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 6
November-December

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, № 6

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2019, V. 54, № 6, pp. 1065-1079
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Reviews

UDC 636.38:636.082:577.21

doi: 10.15389/agrobiology.2019.6.1065eng
doi: 10.15389/agrobiology.2019.6.1065rus

A STUDY OF GENETIC MECHANISMS UNDERLYING THE FAT TAIL PHENOTYPE IN SHEEP: METHODOLOGICAL APPROACHES AND IDENTIFIED CANDIDATE GENES

(review)

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation (project No. 19-16-00070)
Received August 6, 2019

Abstract

Fat-tailed sheep breeds comprise 25 % of the global sheep population and are widely distributed in Africa, Asia (A. Davidson, 1999), the Middle East (S.P. Alves et al., 2013), as well as in Russia (I.M. Dunin et al., 2013). The fat-tailed sheep breeds were valued since their fat was an important ingredient of national cuisine in many ethnic groups (C. Perry, 1995; A. Hajihosseinlo et al., 2015). To-date the customers prefer lean food and cut down the fat intake, and therefore the benefits of large fat tails of sheep have reduced their importance for food production (A. Nejati-Javaremi et al., 2007; M. Moradi et al., 2012). The development of genomic editing technologies (N.A. Zinovieva et al., 2019) makes it relevant to search for genes that determine the "fat tail" phenotype for the subsequent knockout without side effects on other valuable traits of the fat-tailed sheep breeds. This review summarizes the results of studies on identification of candidate genes associated with fat tail trait. Various methods are used to identify candidate genes, including search for selective sweeps (signatures of selection) based on the calculation of differences in allele frequencies (F_{st} values) or haplotypes frequencies between populations (hapFLK method) (M.H. Moradi et al., 2012; M.I. Fariello et al., 2013; C.M. Rochus et al., 2018); genome-wide association studies (GWAS) that require an availability of a phenotypic variability base for the studied traits of economic importance (S.S. Xu et al., 2017); analysis of copy number variation (CNV) that can alter gene expression due to deletion or duplication of genes in the regions of variation (C. Zhu et al., 2016; Q. Ma et al., 2017; V. Bhanuprakash et al., 2018); study of gene expression using RNA-seq technology based on transcriptome analysis using new generation sequencing technology (NGS) (W.A. Hoeijmakers, 2013). Summarizing the research results, the most significant candidate genes associated with the fat deposition of the tail of sheep are *BMP2* and *VRTN* (Z. Yuan et al., 2017; S. Mastrangelo et al., 2018; Z. Pan et al., 2019); *PDGFD* (C. Wei et al., 2015; S. Mastrangelo et al., 2018); genes of the *Homeobox* family (D. Kang et al., 2017; A.A. Yurchenko et al., 2019; A. Ahbara et al., 2019); *SP9* (Z. Yuan et al., 2017; D. Kang et al., 2017); *WDR92* and *ETAA1* (Z. Yuan et al., 2017; L. Ma et al., 2018); *CREB1* (S.S. Xu et al., 2017; L. Ma et al., 2018); *FABP4* (M.R. Bakhtiarizadeh et al., 2013; B. Li et al., 2018); *PPARA*, *RXRA*, *KLF11, ADD1, FASN, PPP1CA* and *PDGFA* (C. Zhu et al., 2016; Q. Ma et al., 2017). To search for candidate genes involved in the formation of a fat tail phenotype in the Russian sheep breeds a QTL mapping resource sheep population was established by crossing the long-fat-tailed Karachaev and the short-thin-tailed Romanov breeds, to perform a genome-wide association study.

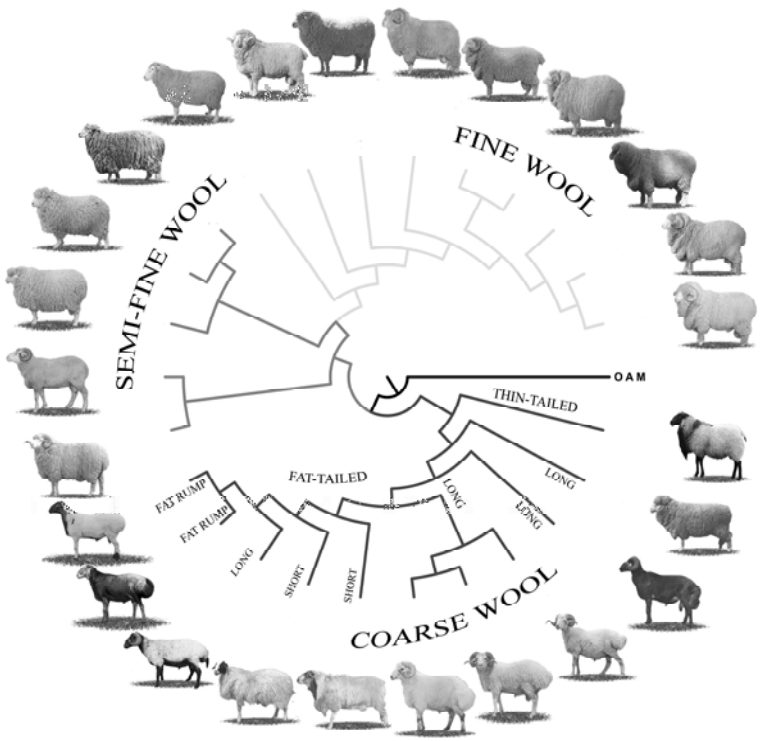
Keywords: domestic sheep, fat tail, fat rump, genetic marker, SNP, DNA chips, RNA-seq, GWAS, CNV.

The identification of genes underlying economically significant phenotypes can indicate potential targets for marker-assisted and genomic selection, as well as genomic editing, making gene identification a necessary basis for the development of genetic technologies in animal husbandry [1].

Significant changes in human dietary requirements, including an increased demand for lean food [2], lead to important changes in the goals of breeding for a few livestock species, including sheep. Fat-tailed and fat-rumped sheep constitute 25% of the global sheep population and are widespread in the countries of North Africa, Asia [3, 4] and the Middle East [5], as well as in Russia [12] of the 15 semicoarse wool and coarse wool sheep breeds in Russia are fat-tailed or fat-rumped [6].

The predominance of these breeds is because, for a long time, fat from the fat rumps of sheep was an important ingredient in national cuisine [7, 8]. Currently, among people worldwide, including ethnic groups in southern Russia, there is a tendency toward a decrease in fat intake. This recent preference has resulted in sheep breeds with high levels of fat deposition, reared over millennia, losing their importance for the production of food products [2, 9].

Sheep (*Ovis aries*) were domesticated approximately 9000-11000 years ago in the "Fertile Crescent" region in the territory of modern Iran [10]. The fat-tailed phenotype appeared much later than the thin tail phenotype.



Phylogenetic tree of Russian sheep breeds (*Ovis aries*) according to the type of wool and the type of tail. The photographs used are taken from the directory of breeds and reflect the types of livestock bred in the Russian Federation [6]. OAM — *Ovis ammon* (argali), a group that was used to root the tree (outgroup).

First, the Asiatic mouflon (*Ovis orientalis*), the presumed ancestor of modern domestic sheep [11-13], has a short, thin tail («wild» phenotype). Thus, it has been hypothesized that the first domesticated sheep were also of the short- and thin-tailed type, and breeding for fat storage in the tail or rump began several millennia after domestication [14]. Moreover, the first archeological evidence indicat-

ing the presence of fat-tailed sheep was found in the form of depictions on a stone bowl from Ur (Mesopotamia) dating to the Uruk III period of 3000 BCE [14, 15].

The formation of a fat tail as a valuable source of energy, an analog of the hump in camels and zebu cattle [9], occurred as a result of adaptation mechanisms in response to harsh natural factors: drought, severe winters, and lack of food and/or water [16].

Hardy and unpretentious sheep with fat tails and fat rumps were indispensable companions of nomadic tribes during long migrations in Eurasia and Africa [17, 18]. In terms of phylogeny (Fig.), the type of tail is the second differentiating factor, as confirmed by the results of genome studies of 25 Russian sheep breeds [19].

The advent of new technologies, including genomic editing, has created the possibility for directed and pointwise changes to the sheep genome [20-22]. Knocking out genes associated with the fat tail might be an effective strategy to eliminate the undesirable phenotype while maintaining other valuable traits of the fat-tailed breeds, such as their adaptive qualities, disease resistance, body size, and meat quality. In this regard, it is important to identify causative mutations that determine the processes of fat deposition in the tail region of sheep.

In this review, we summarize and examine the results of studies on the genetic mechanisms critical for the fat tail phenotype in sheep breeds from different parts of the world and identified candidate genes involved in the phenotype acquisition. A detailed analysis of the applied methodological and bioinformatics approaches for studying the genetics underlying the processes of fat deposition in the tails of the sheep was performed.

Scanning for signatures of selection. Domestication and long-term selection based on economically useful traits (quantity and quality of wool, meat and dairy production, and quality of sheepskins), as well as animal adaptation to new breeding conditions (climate, feed resources, and keeping systems), have significantly shaped the genomes of modern sheep breeds, including the so-called “signatures” of selection [23]. The search for genome regions under selection pressure is one of the most popular approaches for identifying candidate genes and for mapping quantitative trait loci (QTLs). This approach offers a tool for studying the evolutionary history of the populations and analyzing adaptive mutations [23] and also has important and valuable applications.

Therefore, over the past decade, signatures of selection were found in genome regions containing candidate genes that are likely associated with skin pigmentation [24-26], muscle building [27], milk production [28], reproductive traits [25, 29], wool quality [30], parasite resistance [31], and the formation of horned or hornless phenotypes [25, 29] in sheep.

Bioinformatics methods based on differences in allele frequencies (calculated Fst values) [32, 33] or on differences in haplotype frequencies between populations (the hapFLK method) [34] are used to search for selective sweeps.

The first attempts to search for candidate genes associated with fat deposition in sheep tails were made upon the advent of the OvineSNP50K BeadChip DNA assay with medium genome coverage (~ 50 K SNPs). Using this genotyping array, the International Sheep Genomics Consortium (ISGC), within the framework of the Ovine HapMap project, genotyped 2819 sheep from 74 breeds and provided access to the generated SNP profiles [25].

In 2012, M.H. Moradi et al. [9] performed a comparative screening of genome-wide genotyping data on sheep with thin and fat tails from local Iranian breeds and sheep included in the Ovine HapMap project. The research resulted in the identification of selection signatures in three genomic regions on chromosomes five, seven and X. It was reported that a high degree of homozygosity in the re-

gions of chromosomes 5 and X promotes the formation of a fat tail, and a high degree of homozygosity on chromosome 7 leads to the development of a thin tail. B. Moioli et al. studied groups including thin-tailed Italian sheep and only two European fat-tailed breeds (Laticauda and Cypriot fat-tailed) [35]. They found *BMP2* and *VRTN* and suggested that these genes are likely the most critical for the regulation of fat deposition in the tail region of sheep. Specifically, bone morphogenetic protein 2 (*BMP2*) plays an important role in the development of bones and cartilage, and vertin (*VRTN*) is crucial for variations in the number of vertebrae [36]. The *BMP2* gene was reported to be under selection pressure in local Egyptian [37] and Chinese fat-tailed sheep breeds [38].

Using an OvineSNP50K Beadchip assay, C. Wei et al. [4] proposed the *PDGFD* gene as a possible candidate involved in the differentiation of preadipocytes with high expression in adipose tissues. Recent studies have confirmed the role of the *BMP2, VRTN*, and *PDGFD* genes in the formation of the fat tail, as well as in adaptation to desert climates [39, 40]. Studying local Chinese fat-tailed sheep breeds, Z. Yuan et al. [41] found 40 potential candidate genes, the most significant of which were *HOXA11, BMP2, PPP1CC, SP3, SP9, WDR92, PROKR1*, and *ETAA1*. It should be noted that the *PROKR1* and *ETAA1* genes are involved in controlling obesity in humans [42, 43] and are likely associated with the formation of fat tails in sheep.

Genes from the *homeobox* family play important roles in the development and morphology of the skeleton, sacrum, and tail [44]. It is assumed that the *HOXA11-13* genes, regulating the number of coccygeal vertebrae, are likely more critical to the length of the tail than to the deposition of fat around the tail. Interestingly, the *HOXA11* gene was previously identified, but it was not assigned to candidates associated with the length or size of the tail [26].

A study using a high-density DNA chip, Ovine Infinium® HD SNP BeadChip assay (~ 600 K SNP), showed that the genes of the *homeobox* family were under pressure of selection in Russian fat-tailed sheep breeds. Specifically, the *HOXC* gene group on chromosome 3 in the Lezgin, Edilbaev and Karakul breeds and the *HOXA* gene group on chromosome 4 in the Karachaev and Buubei breeds were identified [45].

Using an OvineSNP50K Beadchip assay to address the genetic state of Ethiopian and Libyan local fat-tailed sheep, A. Ahbara et al. [46] found that the *ALX4, HOXB13,* and *BMP4* genes were associated with the growth and development of the limbs and skeleton and with tail formation. Further whole-genome next-generation sequencing with deep coverage revealed a strong selection signal in the representatives of these breeds in the region of the *HOXB13* gene, which confirmed the influence of it on the formation of tail types in sheep [47].

To identify candidate genes important to the short fat-tail phenotype, D. Zhi et al. [48] sequenced the whole genome of a local Chinese sheep breed Hulunbuir. Despite the ambiguous results, it was shown that the c.G334T mutation in the *T* gene, which regulates vertebra development, affects the formation of a short, fat tail in sheep, a finding partially confirmed by previous studies [49].

In addition, studies on the adaptability of fat-tailed sheep breeds to the arid climatic conditions of Egypt revealed 172 potential candidate genes that were involved in some way in the physiological mechanisms of adaptation and the regulation of the morphology of the body and its parts, including the fat tail [37, 50].

Genome-wide association study (GWAS). The genome-wide association study is a powerful bioinformatics tool for identifying genomic variations associated with quantitative traits in livestock species [51], including sheep [52, 53]. In contrast to searching for selective sweeps, to conduct a GWAS, a database on phenotype variants must be available for the trait of interest.

Combining the body measurements and genotyping data obtained using a high-density DNA chip, S. Xu et al. [54] revealed several SNPs associated with the development of a fat tail, and they were localized in the genes critical for lipid metabolism (*CREB1, STEAP4, CTBP1* and *RIP140*).

Nevertheless, no genes previously proposed as functional candidates were identified [35, 41]. It has also been suggested that the genome region between 88 and 89 Mb on the X chromosome contains a number of significant SNPs, presumably indicating that this region is potentially associated with the formation of a fat tail in sheep [54].

Copy number variation (CNV). Analysis of copy number variation is a bioinformatics approach for detecting candidate genes and identifying QTLs, as well as for studying the evolutionary mechanisms in domesticated animals and their adaptability to various environmental conditions [55]. The CNV phenomenon is based on some genome regions ranging in size from one thousand to millions of base pairs being present in several copies, the number of which varies among individuals within the population [55]. Copy number variation is an important source of genetic variation in an individual, since CNVs can alter gene expression and, accordingly, cause an unexpected phenotype due to the deletion or duplication of genes in regions of variation [56-58].

Copy number variation in the sheep genome was studied for the first time in 2011. Only 135 CNV regions were found, likely due to the unsuitability of the applied hybridization method based on cross-amplification with the cattle genome [59].

In 2016, approximately 3488 autosomal CNV regions were revealed in sheep using the specifically developed Roche NimbleGen 2.1M CGH platform (Roche NimbleGen, Inc., USA) and new CNV validation methods [60]. In addition, attempts were made to search for functional genes overlapping in the CNV regions using a medium density DNA chip [61, 62].

Using a high-density DNA chip, Zhu C. et al. [63] revealed candidate genes that fully overlap in the CNV regions and are associated with fat deposition in the tails of local Chinese sheep (the Han fat-tailed, Altay and Tibetan breeds), including the genes *PPARA, RXRA, KLF11, ADD1, FASN, PPP1CA* and *PDGFA*. Further studies provide evidence that the listed genes also overlap in the CNV region in another local Chinese breed, Tan sheep [64].

Transcriptome analysis based on NGS. The development of next-generation sequencing (NGS) methods has led to the creation of RNA-seq technology, which has taken gene expression studies to a new, high level [65]. The popularity of the RNA-seq approach is due to a number of advantages, including the unsurpassed resolution, the lack of need for preliminary knowledge of the sequence under investigation, and the ability to reanalyze the data obtained using RNA-seq, as in the case when a more relevant genome assembly becomes available [65-67].

The RNA-seq method allowed the identification of the *NELL1* and *FMO3* genes, which are likely involved in the regulation of fat metabolism in adipose tissues [68]. Excessive expression of the *NELL1* gene may be a key factor in reducing the fat deposition in the tail region [68].

The extension of studies to local Chinese sheep breeds [69, 70] revealed candidate genes that play significant roles in increased fat deposition in the tail region: *SP9* and genes of the *homeobox* family, *HOXC11, HOXC12,* and *HOXC13* [69], and *CREB1, WDR92* and *ETAA1* [70]. In addition, according to L. Ma et al. [71], the *FMO2, PLIN2, PLIN3, LEPR, PENK, ELOVL3, ELOVL5, PDK4*, and *SLC22A4* genes play key roles in fat deposition, adipogenesis, and the biosynthesis of fatty acids.

The transcriptome analysis of Chinese breeds was continued by B. Li et al. [72], who investigated the genetic regulation of lipid metabolism in fat-tailed sheep. It was established that *FABP4, ADIPOQ, FABP5* and *CD36* are the most highly transcribed genes associated with the deposition of fat in the sheep body.

The role of the *FABP4* and *FABP5* genes in the accumulation of fat in cattle [73] and in influencing meat tenderness in sheep [74] is well known. The *CD36* and *ADIPOQ* genes are transporters and regulators of fatty acids [75-77]. B. Li et al. [72] noted that the *FHC*, *FHC-pseudogene*, and *ZC3H10* genes may also be involved in the regulation of lipid metabolism in sheep [72]. The involvement of the *ZNF395* gene in adipogenesis processes has also been suggested [78].

The role of the *FABP4* gene in the processes of fat deposition in the tail region in some sheep breeds has been discussed previously, but conflicting results have been obtained. For example, M.R. Bakhtiarizadeh et al. [79], studying the expression of candidate genes, including *FABP4, FASN, SCD*, and *LPL*, found significantly higher expression of the *FABP4* gene in the fat tail compared with their expression in visceral adipose tissues ($p < 0.05$). On the other hand, X. Ruixia et al. [80], noting the high levels of expression of the *FABP4* gene in Altay, a fat-rumped breed, did not find a significant difference with *FABP4* gene expression in the control group, which consisted of sheep with thin tails. Nevertheless, any conclusion on the role of the *FABP4* gene in the formation of the fat tail and/or fat rump is premature, since the listed differences in gene expression can be breed-specific.

There is an assumption that long noncoding RNAs (lncRNAs) might be involved in the regulation of the expression of genes associated with the deposition of fat in the tails of sheep [81]. A high correlation between the expression of Lpin2 and Lpin3 mRNA and the size of fat tails was shown [82].

In addition, a difference in the expression of the *CPT1* [83] and *OXCT1* [84] genes was found in fat-tailed sheep compared with thin-tailed sheep; therefore, these genes might be considered likely candidates.

Information on the potential candidate genes identified to date is summarized in the table.

Candidate genes involved in the acquisition of the fat tail phenotype in sheep (*Ovis aries*) and methods for their identification

Gene	Chromosome	Method	Function	Reference
ADIPOQ (adiponectin)	1	RNA-seq	Oxidation of fatty acids and glucose	[72]
LEPR (leptin receptor)	1	RNA-seq	Fat deposition, adipogenesis	[71]
RIP140 (NRIP1) (nuclear receptor-interacting protein 1)	1	GWAS	Regulation of lipid and glucose metabolism	[54]
CREB1 (cAMP responsive element binding protein 1)	2	RNA-seq GWAS	Lipid metabolism, glucose homeostasis and adipocyte differentiation	[54, 70]
LPL (lipoprotein lipase)	2	RNA-seq	The release of fatty acids and glycerol through triglyceride hydrolysis	[79]
PLIN2 (perilipin 2)	2	RNA-seq	Fat deposition	[71]
SP3 (Sp3 transcription factor)	2	SPS	Inhibition of adipocyte differentiation	[41]
SP9 (Sp9 transcription factor)	2	SPS, RNA-seq	Fat deposition by adherence of mesenchymal cells to adipocytes	[41, 69]
ZNF395 (zinc finger protein 395)	2	RNA-seq	Differentiation of preadipocytes, determination of the line of progenitor cells of the mesenchyme	[78]
SLC22A4 (SLC22A family member)	3	RNA-seq	Energy metabolism, fat accumulation	[71]
CPT1 (carnitine palmitoyltransferase 1)	3	RNA-seq	Involved in the metabolism of fatty acids in the liver	[83]
ETAA1 (Ewing tumor-associated antigen 1)	3	SPS, RNA-seq	Distribution and deposition of fat in the body	[41, 70]
HOXC11 (homeobox C11-13)	3	RNA-seq, SPS	Knockout leads to vertebra transformation	[45, 69]
KLF11 (Krüppel-like factor 11)	3	CNV	Brown fat transcription factor	[64]
PPARA (peroxisome proliferator-activated receptor-α)	3	CNV	Coactivator of fatty acid metabolism	[64]
PROKR1 (prokineticin receptor 1)	3	SPS	Suppression of proliferation and differentiation of preadipocytes	[41]

Table continued

RXRA (retinoic X receptor A)	3	CNV	Lipid homeostasis	[64]
WDR92 (WD repeat domain 92)	3	SPS, RNA-seq	Interaction with phospholipids	[41, 70]
ZC3H10 (zinc finger CCCH-type containing 10)	3	RNA-seq	Adipocyte homeostasis; located in QTL associated with internal fat	[72]
HOXA11 (homeobox A11)	4	SPS	Regulation of variation in the number of coccygeal vertebrae	[26, 41, 45]
PDK4 (pyruvate dehydrogenase kinase 4)	4	RNA-seq	Fat accumulation, adipogenesis	[71]
STEAP4 (STEAP4 metalloreductase)	4	GWAS	Encodes metalloreductase involved in adipocyte metabolism in adipose tissue	[54]
PLIN3 (perilipin 3)	5	RNA-seq	Fat accumulation	[71]
ADD1 (adipocyte determination and differentiation factor 1)	6	CNV	Differentiation of adipocytes and cholesterol homeostasis	[64]
CTBP1 (C-terminal-binding protein 1)	6	GWAS	Oxidation of fatty acids; inhibition leads to fatty liver	[54]
BMP4 (bone morphogenetic protein 4)	7	SPS	Growth and development of limbs, skeleton and tail formation	[46]
VRTN (vertnin, vertebrae development associated)	7	SPS	Change in the number of vertebrae	[35, 39, 40]
T(TBXT) (T-box transcription factor T, T/Brachyury)	8	SPS	Development of vertebrae, the formation of a short, fat tail in sheep	[48, 49]
FABP4 (fatty acid-binding protein 4)	9	RNA-seq	Transportation of fatty acids to locations of accumulation or production of energy; high expression increases adipocyte differentiation time	[72, 79, 80]
FABP5 (fatty acid-binding protein 5)	9	RNA-seq	Compensation for FABP4 loss in adipocytes	[72]
PENK (proenkephalin)	9	RNA-seq	Fat accumulation	[71]
FASN (fatty acid synthase)	11	CNV	De novo fatty acid synthesis, fat accumulation and fatty acid anabolism	[64]
HOXB13 (homeobox B13)	11	SPS	Growth and development of limbs, skeleton and tail formation	4[6, 47]
FMO2 (flavin-containing dimethylaniline monoxygenase 2)	12	RNA-seq	Fat accumulation, adipogenesis and fatty acid biosynthesis	[71]
BMP2 (bone morphogenetic protein 2)	13	SPS	Development of bones and cartilage	[35, 37-41]
Lpin3 (lipin 3)	13	RNA-seq	Lipid metabolism	[82]
ALX4 (ALX homeobox 4)	15	SPS	Growth and development of limbs, skeleton and tail formation	[46]
PDGFD (platelet-derived growth factor D)	15	SPS	Inhibition of differentiation of preadipocytes	[4, 39, 40]
OXCT01 (3-oxoacid CoA-transferase 1)	16	RNA-seq	Metabolism of ketone bodies; knockout leads to lipid accumulation in adipocytes	[84]
PPP1CC (protein phosphatase 1 catalytic subunit gamma)	17	SPS	Dephosphorylation and inactivation of glycogen synthase in skeletal muscle	[41]
ELOVL5 (fatty acid elongase 5)	20	RNA-seq	Elongation of polyunsaturated long-chain fatty acids	[71]
FHC (ferritin heavy chain)	21	RNA-seq	Regulation of fat cell activity	[72]
FMO3 (flavin-containing monooxygenase 3)	21	RNA-seq	Inhibition of fatty acid oxidation	[68]
NELL1 (NEL-like 1 neural EGFL-like 1, a protein strongly expressed in neural tissue encoding an epidermal growth factor-like domain)	21	RNA-seq	Enhancement of osteogenic differentiation and weakens the differentiation of adipose tissue	[68]
PPP1CA (phosphoprotein phosphatase 1 catalytic subunit)	21	CNV	Conversion of phosphorylase A to phosphorylase B	[64]
ELOVL3 (ELOVL fatty acid elongase 3)	22	RNA-seq	Energy metabolism, lengthening saturated and monounsaturated fatty acid chains	[71]
Lpin2 (lipin 2)	23	RNA-seq	Involvement in lipid metabolism	[82]
PDGFA (platelet-derived growth factor alpha)	24	CNV	Differentiation of preadipocytes	[64]

N o t e . RNA-seq — transcriptome analysis of gene expression based on next-generation sequencing (NGS), CNV — copy number variation, SPS — scan for signatures of selection, selective sweeps, GWAS — genome-wide association study

To search for candidate genes associated with the formation of fat tails in Russian sheep breeds, at the Ernst Federal Science Center for Animal Husbandry, a resource sheep population was obtained from crossing the long-fat-tailed Karachaev and short-thin-tailed Romanov sheep breeds. The sizes of the tails of the

F_2 hybrids and backcrosses will be measured 6, 42, 90 and 180 days after birth. The animals of the resource population will be genotyped using high-density DNA chip and analyzed using GWAS. The use of resource populations for QTL mapping has several advantages over the use of random populations: in particular, a decrease in the false discovery rates (FDR) and an increase in the accuracy of mapping [85]. In this regard, the identified candidate genes associated with fat deposition in the tails in the Russian breeds might be used as possible targets for editing the genome of fat-tailed sheep to increase the production of lean mutton.

Thus, we summarized and analyzed the results of the investigations of the world scientific community on the identification of candidate genes associated with increased fat deposition in the tail region of sheep. We reviewed the most popular methods for identifying potential candidate genes, including scanning for selection signatures, genome-wide associative studies, copy number variation, and gene expression studies based on transcriptome analysis. The advantages and limitations of the relevant methodological approaches were analyzed. Therefore, genome-wide association studies are high-resolution and effective methods of QTL mapping, but their implementation requires the availability of a database of phenotype variants of the economically relevant trait under investigation. To search for selection signatures and to address copy number variation, information on the phenotypes is optional, but the results obtained using both approaches require validation by quantitative PCR amplification or sequencing of the identified genome regions. Transcriptome analysis is a high-resolution method, but the collection of the organ and tissue specimens must be considered based on strict time and temperature conditions. The most significant candidate genes associated with the deposition of fat in sheep tails and identified in more than one study, including *BMP2* and *VRTN*; *PDGFD*; genes of the *homeobox* family; *SP9*; *CREB1*; *PPARA*, *RXRA*, *KLF11*, *ADD1*, *FASN*, *PPP1CA* and *PDGFA*, are presented. Despite a significant number of studies aimed at identifying the genetic mechanisms underlying the fat tail phenotype in sheep, the only known fact is that more than one gene is involved in the formation of a fat tail. As a method for searching for candidate genes involved in the acquisition fat tails in Russian sheep breeds, a genome-wide association study on sheep from a resource sheep population, obtained by crossing long-fat-tailed Karachaev sheep with short-thin-tailed Romanov sheep, has been proposed.

REFERENCES

1. Belous A.A., Sermyagin A.A., Kostyunina O.V., Brem G., Zinov'eva N.A. study of genetic architecture of feed conversion rate in duroc young boars (*Sus scrofa*) based on the genome-wide SNP analysis. *Agricultural Biology* [*Sel'skokhozyaistvennaya biologiya*], 2019, 54(4): 705-712 (doi: 10.15389/agrobiology.2019.4.705eng).
2. Nejati-Javaremi A., Izadi F., Rahmati G., Moradi M. Selection in fat-tailed sheep based on two traits of fat-tail and body weight versus single-trait total body weight. *Int. J. Agri. Biol.*, 2007, 9(4): 645-648.
3. Davidson A. *The Oxford companion to food.* Oxford University Press, Oxford, UK, 1999.
4. Wei C., Wang H., Liu G., Wu M., Cao J., Liu Z., Liu R., Zhao F., Zhang L., Lu J., Liu C., Du L. Genome-wide analysis reveals population structure and selection in Chinese indigenous sheep breeds. *BMC Genomics*, 2015, 16: 194 (doi: 10.1186/s12864-015-1384-9).
5. Alves S.P., Bessa R.J.B., Quaresma M.A.G., Kilminster T., Scanlon T., Oldham C., Milton J., Greeff J., Almeida A.M. Does the fat tailed Damara ovine breed have a distinct lipid metabolism leading to a high concentration of branched chain fatty acids in tissues? *PLoS ONE*, 2013, 8(10): e77313 (doi: 10.1371/journal.pone.0077313).
6. Dunin I.M., Dankvert A.G. *Spravochnik porod i tipov sel'skokhozyaistvennykh zhivotnykh, razvodimykh v Rossiiskoi Federatsii* [Directory of farm animals breeds and types bred in the Russian Federation]. Moscow, 2013 (in Russ.).
7. Perry C. The fate of the tail. In: *Disappearing foods: studies in food and dishes at risk: Proceedings of the Oxford symposium on food and cookery.* H. Walker (ed.). Pro-spect Books, UK, 1995.

8. Hajihosseinlo A., Jafari S., Ajdary M. The relationship of GH and LEP gene poly-morphisms with fat-tail measurements (fat-tail dimensions) in fat-tailed Makooei breed of Iranian sheep. *Adv. Biomed. Res.*, 2015, 4: 172 (doi: 10.4103/2277-9175.163995).
9. Moradi M.H., Nejati-Javaremi A., Moradi-Shahrbabak M., Dodds K.G., McEwan J.C. Genomic scan of selective sweeps in thin and fat tail sheep breeds for identifying of candidate regions associated with fat deposition. *BMC Genetics*, 2012, 13: 10 (doi: 10.1186/1471-2156-13-10).
10. Zeder M.A. Domestication and early agriculture in the Mediterranean Basin: origins, diffusion, and impact. *Proceedings of the National Academy of Sciences*, 2008, 105(33): 11597-11604 (doi: 10.1073/pnas.0801317105).
11. Hiendleder S., Kaupe B., Wassmuth R., Janke A. Molecular analysis of wild and domestic sheep questions current nomenclature and provides evidence for domestication from two different subspecies. *Proc. R. Soc. Lond. B.*, 2002, 269(1494): 893-904 (doi: 10.1098/rspb.2002.1975).
12. Rezaei H.R., Naderi S., Chintauan-Marquier I.C., Taberlet P., Virk A.T., Naghash H.R., Rioux D., Kaboli M., Luikart G., Pompanon F. Evolution and taxonomy of the wild species of the genus *Ovis* (*Mammalia*, *Artiodactyla*, *Bovidae*). *Molecular Phylogenetics and Evolution*, 2010, 54(2): 315-326 (doi: 10.1016/j.ympev.2009.10.037).
13. Demirci S., Koban Baştanlar E., Dağtaş N.D., Pişkin E., Engin A., Özer F., Yüncü E., Doğan S.A., Togan İ. Mitochondrial DNA diversity of modern, ancient and wild sheep (*Ovis gmelinii anatolica*) from Turkey: new insights on the evolutionary history of sheep. *PLoS ONE*, 2013, 8(12): e81952 (doi: 10.1371/journal.pone.0081952).
14. Ryder M.L. *Sheep and man*. Gerald Duckworth & Co. Ltd., London, 1983.
15. Epstein H. The Awassi sheep with special reference to the improved dairy type. *FAO Animal production and health paper*, 1985, 57: 282.
16. Kashan N.E.J., Azar G.H.M., Afzalzadeh A., Salehi A. Growth performance and carcass quality of fattening lambs from fat-tailed and tailed sheep breeds. *Small Ruminant Research*, 2005, 60(3): 267-271 (doi: 10.1016/j.smallrumres.2005.01.001).
17. Lv F.H., Peng W.F., Yang J., Zhao Y.X., Li W.R., Liu M.J., Ma Y.H., Zhao Q.J., Yang G.L., Wang F., Li J.Q., Liu Y.G., Shen Z.Q., Zhao S.G., Hehua E., Gorkhali N.A., Farhad Vahidi S.M., Muladno M., Naqvi A.N., Tabell J., Iso-Touru T., Bruford M.W., Kantanen J., Han J.L., Li M.H. Mitogenomic meta-analysis identifies two phases of migration in the history of Eastern Eurasian sheep. *Molecular Biology and Evolution*, 2015, 32(10): 2515-2533 (doi: 10.1093/molbev/msv139).
18. Zhao Y.X., Yang J., Lv F.H., Hu X.J., Xie X.L., Zhang M., Li W.R., Liu M.J., Wang Y.T., Li J.Q., Liu Y.G., Ren Y.L., Wang F., Hehua E., Kantanen J., Arjen Lenstra J., Han J.L., Li M.H. Genomic reconstruction of the history of native sheep reveals the peopling patterns of nomads and the expansion of early pastoralism in East Asia. *Molecular Biology and Evolution*, 2017, 34(9): 2380-2395 (doi: 10.1093/molbev/msx181).
19. Deniskova T.E., Dotsev A.V., Selionova M.I., Kunz E., Medugorac I., Reyer H., Wimmers K., Barbato M., Traspov A.A., Brem G., Zinovieva N.A. Population structure and genetic diversity of 25 Russian sheep breeds based on whole-genome genotyping. *Genet. Sel. Evol.*, 2018, 50(1): 29 (doi: 10.1186/s12711-018-0399-5).
20. Proudfoot C., Carlson D.F., Huddart R., Long C.R., Pryor J.H., King T.J., Lillico S.G., Mileham A.J., McLaren D.G., Whitelaw C.B., Fahrenkrug S.C. Genome edited sheep and cattle. *Transgenic Res.*, 2015, 24(1): 147-153 (doi: 10.1007/s11248-014-9832-x).
21. Zhou S., Yu H., Zhao X., Cai B., Ding Q., Huang Y., Li Y., Li Y., Niu Y., Lei A., Kou Q., Huang X., Petersen B., Ma B., Chen Y., Wang X. Generation of gene-edited sheep with a defined Booroola fecundity gene (*FecB*^{B}) mutation in bone morphogenetic protein receptor type 1B (*BMPR1B*) via clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) 9. *Reproduction, Fertility and Development*, 2018, 30(12): 1616-1621 (doi: 10.1071/RD18086).
22. Zinovieva N.A., Volkova N.A., Bagirov V.A. Genome editing: state of the art and application to animal husbandry. *Biotekhnologiya*, 2018, 34(3): 9-22 (doi: 10.21519/0234-2758-2018-34-3-9-22).
23. Rochus C.M., Tortereau F., Plisson-Petit F., Restoux G., Moreno-Romieux C., Tosser-Klopp G., Servin B. Revealing the selection history of adaptive loci using genome-wide scans for selection: an example from domestic sheep. *BMC Genomics*, 2018, 19(1): 71 (doi: 10.1186/s12864-018-4447-x).
24. García-Gámez E., Reverter A., Whan V., McWilliam S.M., Arranz J.J., Consortium ISG, Kijas J. Using regulatory and epistatic networks to extend the findings of a genome scan: identifying the gene drivers of pigmentation in merino sheep. *PLoS ONE*, 2011, 6(6): e21158 (doi: 10.1371/journal.pone.0021158).
25. Kijas J.W., Lenstra J.A., Hayes B., Boitard S., Porto Neto L.R., San Cristobal M., Servin B., McCulloch R., Whan V., Gietzen K., Paiva S., Barendse W., Ciani E., Raadsma H., McEwan J., Dalrymple B., International Sheep Genomics Consortium Members. Genome-wide analysis of the world's sheep breeds reveals high levels of historic mixture and strong recent selection. *PLoS Biology*, 2012, 10(2): e1001258 (doi: 10.1371/journal.pbio.1001258).
26. Fariello M.I., Servin B., Tosser-Klopp G., Rupp R., Moreno C., Consortium ISG, San Cristo-

bal M., Boitard S. Selection signatures in worldwide sheep populations. *PLoS ONE*, 2014, 9(8): e103813 (doi: 10.1371/journal.pone.0103813).
27. Fleming-Waddell J.N., Olbricht G.R., Taxis T.M., White J.D., Vuocolo T., Craig B.A., Tellam R.L., Neary M.K., Cockett N.E., Bidwell C.A. Effect of *DLK1* and *RTL1* but not *MEG3* or *MEG8* on muscle gene expression in Callipyge lambs. *PLoS ONE*, 2009, 4(10): e7399 (doi: 10.1371/journal.pone.0007399).
28. Moioli B., Scatà M.C., Steri R., Napolitano F., Catillo G. Signatures of selection identify loci associated with milk yield in sheep. *BMC Genet.*, 2013, 14: 76 (doi: 10.1186/1471-2156-14-76).
29. Liu Z.H., Ji Z.B., Wang G.Z., Chao T.L., Hou L., Wang J.M. Genome-wide analysis reveals signatures of selection for important traits in domestic sheep from different ecoregions. *BMC Genomics*, 2016, 17(1): 863 (doi: 10.1186/s12864-016-3212-2).
30. Demars J., Cano M., Drouilhet L., Plisson-Petit F., Bardou P., Fabre S., Servin B., Sarry J., Woloszyn F., Mulsant P., Foulquier D., Carrière F., Aletru M., Rodde N., Cauet S., Bouchez O., Pirson M., Tosser-Klopp G., Allain D. Genome-wide identification of the mutation underlying fleece variation and discriminating ancestral hairy species from modern woolly sheep. *Molecular Biology and Evolution*, 2017, 34(7): 1722-1729 (doi: 10.1093/molbev/msx114).
31. Mcrae K.M., McEwan J.C., Dodds K.G., Gemmell N.J. Signatures of selection in sheep bred for resistance or susceptibility to gastrointestinal nematodes. *BMC Genomics*, 2014, 15: 637 (doi: 10.1186/1471-2164-15-637).
32. Weir B.S., Cockerham C.C. Estimating F-statistics for the analysis of population-structure. *Evolution*, 1984, 38(6): 1358-1370 (doi: 10.2307/2408641).
33. Porto-Neto L.R., Lee S.H., Lee H.K., Gondro C. Detection of signatures of selection using F_{ST}. In: *Methods in molecular biology (methods and protocols)*. C. Gondro, J. van der Werf, B. Hayes (eds.). Humana Press, Totowa, NJ, 2013, vol. 1019: 423-436 (doi: 10.1007/978-1-62703-447-0_19).
34. Fariello M.I., Boitard S., Naya H., SanCristobal M., Servin B. Detecting signatures of selection through haplotype differentiation among hierarchically structured populations. *Genetics*, 2013, 193(3): 929-941 (doi: 10.1534/genetics.112.147231).
35. Moioli B., Pilla F., Ciani E. Signatures of selection identify loci associated with fat tail in sheep. *Journal of Animal Science*, 2015, 93(10): 4660-4669 (doi: 10.2527/jas.2015-9389).
36. Mikawa S., Sato S., Nii M., Morozumi T., Yoshioka G., Imaeda N., Yamaguchi T., Hayashi T., Awata T. Identification of a second gene associated with variation in vertebral number in domestic pigs. *BMC Genet.*, 2011, 12: 5 (doi: 10.1186/1471-2156-12-5).
37. Kim E.S., Elbeltagy A.R., Aboul-Naga A.M., Rischkowsky B., Sayre B., Mwacharo J.M., Rothschild M.F. Multiple genomic signatures of selection in goats and sheep indigenous to a hot arid environment. *Heredity*, 2016, 116: 255-264 (doi: 10.1038/hdy.2015.94).
38. Pan Z., Li S., Liu Q., Wang Z., Zhou Z., Di R., An X., Miao B., Wang X., Hu W., Guo X., Lv S., Li F., Ding G., Chu M., Li Y. Rapid evolution of a retro-transposable hotspot of ovine genome underlies the alteration of *BMP2* expression and development of fat tails. *BMC Genomics*, 2019, 20(1): 261 (doi: 10.1186/s12864-019-5620-6).
39. Mastrangelo S., Moioli B., Ahbara A., Latairish S., Portolano B., Pilla F, Ciani E. Genome-wide scan of fat-tail sheep identifies signals of selection for fat deposition and adaptation. *Animal Production Science*, 2018, 59(5): 835-848 (doi: 10.1071/AN17753).
40. Mastrangelo S., Bahbahani H., Moioli B., Ahbara A., Al Abri M., Almathen F., da Silva A., Belabdi I., Portolano B., Mwacharo J.M., Hanotte O., Pilla F., Ciani E. Novel and known signals of selection for fat deposition in domestic sheep breeds from Africa and Eurasia. *PLoS ONE*, 2019, 14(6): e0209632 (doi: 10.1371/journal.pone.0209632).
41. Yuan Z., Liu E., Liu Z., Kijas J.W., Zhu C., Hu S., Ma X., Zhang L., Du L., Wang H., Wei C. Selection signature analysis reveals genes associated with tail type in Chinese indigenous sheep. *Anim. Genet.*, 2017, 48(1): 55-66 (doi: 10.1111/age.12477).
42. Szatkowski C., Vallet J., Dormishian M., Messaddeq N., Valet P., Boulberdaa M., Metzger D., Chambon P., Nebigil C.G. Prokineticin receptor 1 as a novel suppressor of preadipocyte proliferation and differentiation to control obesity. *PLoS ONE*, 2013, 8(12): e81175 (doi: 10.1371/journal.pone.0081175).
43. Liu C.T., Monda K.L., Taylor K.C., Lange L., Demerath E.W., Palmas W., Wojczynski M.K., Ellis J.C., Vitolins M.Z., Liu S., Papanicolaou G.J., Irvin M.R., Xue L., Griffin P.J., Nalls M.A., Adeyemo A., Liu J., Li G., Ruiz-Narvaez E.A., Chen W.M., Chen F., Henderson B.E., Millikan R.C., Ambrosone C.B., Strom S.S., Guo X., Andrews J.S., Sun Y.V., Mosley T.H., Yanek L.R., Shriner D., Haritunians T., Rotter J.I., Speliotes E.K., Smith M., Rosenberg L., Mychaleckyj J., Nayak U., Spruill I., Garvey W.T., Pettaway C., Nyante S., Bandera E.V., Britton A.F., Zonderman A.B., Rasmussen-Torvik L.J., Chen Y.D., Ding J., Lohman K., Kritchevsky S.B., Zhao W., Peyser P.A., Kardia S.L., Kabagambe E., Broeckel U., Chen G., Zhou J., Wassertheil-Smoller S., Neuhouser M.L., Rampersaud E., Psaty B., Kooperberg C., Manson J.E., Kuller L.H., Ochs-Balcom H.M., Johnson K.C., Sucheston L,. Ordovas J.M., Palmer J.R., Haiman C.A., McKnight B., Howard B.V., Becker D.M., Bielak L.F., Liu Y., Allison M.A., Grant S.F., Burke G.L., Patel S.R., Schreiner P.J., Borecki I.B., Evans M.K., Tay-

lor H., Sale M.M., Howard V., Carlson C.S., Rotimi C.N., Cushman M., Harris T.B., Reiner A.P., Cupples L.A., North K.E., Fox C.S. Genome-wide association of body fat distribution in African ancestry populations suggests new loci. *PLoS Genetics*, 2013, 9(8): e1003681 (doi: 10.1371/journal.pgen.1003681).
44. Mallo M., Wellik D.M., Deschamps J. Hox genes and regional patterning of the vertebrate body plan. *Developmental Biology*, 2010, 344(1): 7-15 (doi: 10.1016/j.ydbio.2010.04.024).
45. Yurchenko A.A., Deniskova T.E., Yudin N.S., Dotsev A.V., Khamiruev T.N., Selionova M.I., Egorov S.V., Reyer H., Wimmers K., Brem G., Zinovieva N.A., Larkin D.M. High-density genotyping reveals signatures of selection related to acclimation and economically important traits in 15 local sheep breeds from Russia. *BMC Genomics*, 2019, 20(Suppl 3): 294 (doi: 10.1186/s12864-019-5537-0).
46. Ahbara A., Bahbahani H., Almathen F., Al Abri M., Agoub M.O., Abeba A., Kebede A., Musa H.H., Mastrangelo S., Pilla F., Ciani E., Hanotte O., Mwacharo J.M. Genome-wide variation and putative candidate regions and genes associated with fat deposition and tail morphology in Ethiopian indigenous sheep. *Front. Genet.*, 2019, 9: 699 (doi: 10.3389/fgene.2018.00699).
47. Ahbara A., Musa H., Clark E., Robert C., Watson M., Abeba A., Latairish S., Hanotte O., Mwacharo J. OP196 indigenous African sheep genomes reveal insights on fat-tail deposition and morphology. *Proc. 37th International Conference on Animal Genetics*. Lleida, Spain, 2019: 56.
48. Zhi D., Da L., Liu M., Cheng C., Zhang Y., Wang X., Li X., Tian Z., Yang Y., He T., Long X., Wei W., Cao G. Whole genome sequencing of Hulunbuir short-tailed sheep for identifying candidate genes related to the short-tail phenotype. *G3: Genes, Genomes, Genetics*, 2018, 8(2): 377-383 (doi: 10.1534/g3.117.300307).
49. Liu Z., Wang H., Liu R., Mingming W.U., Zhang S., Zhang L., Zhao F., Du L., Wei C. Genome-wide detection of selection signatures of distinct tail types in sheep popula-tions. *Acta Veterinaria et Zootechnica Sinica*, 2015, 46(10): 1721-1732 (doi: 10.11843/j.issn.0366-6964.2015.10.004).
50. Mwacharo J.M., Kim E.S., Elbeltagy A.R., Aboul-Naga A.M., Rischkowsky B.A., Rothschild M.F. Genomic footprints of dryland stress adaptation in Egyptian fat-tail sheep and their divergence from East African and western Asia cohorts. *Sci. Rep.*, 2017, 7(1): 17647 (doi: 10.1038/s41598-017-17775-3).
51. Sermyagin A.A., Gladyr' E.A., Kharitonov S.N., Ermilov A.N., Strekozov N.I., Brem G., Zinov'eva N.A. Genome-wide association study for milk production and reproduction traits in Russian Holstein cattle population. *Agricultural Biology* [*Sel'skokhozyaistvennaya biologiya*], 2015, 51(2): 182-193 (doi: 10.15389/agrobiology.2016.2.182eng).
52. Jonas E., Thomson P.C., Raadsma H.W. Genome-wide association study and fine mapping of QTL on OAR 21 for body weight in sheep. *Proc. 9th World Congress on Genetics Applied to Livestock Production*. Leipzig, 2010.
53. Kominakis A., Hager-Theodorides A.L., Zoidis E., Saridaki A., Antonakos G., Tsiamis G. Combined GWAS and 'guilt by association'-based prioritization analysis identifies functional candidate genes for body size in sheep. *Genet. Sel. Evol.*, 2017, 49: 41 (doi: 10.1186/s12711-017-0316-3).
54. Xu S.S., Ren X., Yang G.L., Xie X.L., Zhao Y.X., Zhang M., Shen Z.Q., Ren Y.L., Gao L., Shen M., Kantanen J., Li M.H. Genome-wide association analysis identifies the genetic basis of fat deposition in the tails of sheep (*Ovis aries*). *Anim. Genet.*, 2017, 48(5): 560-569 (doi: 10.1111/age.12572).
55. Bhanuprakash V., Chhotaray S., Pruthviraj D.R., Rawat C., Karthikeyan A., Panigrahi M. Copy number variation in livestock: a mini review. *Veterinary World*, 2018, 11(4): 535-541 (doi: 10.14202/vetworld.2018.535-541).
56. Stranger B.E., Forrest M.S., Dunning M., Ingle C.E., Beazley C., Thorne N., Redon R., Bird C.P., de Grassi A., Lee C., Tyler-Smith C., Carter N., Scherer S.W., Tavaré S., Deloukas P., Hurles M.E., Dermitzakis E.T. Relative impact of nucleotide and copy number variation on gene expression phenotypes. *Science*, 2007, 315(5813): 848-853 (doi: 10.1126/science.1136678).
57. Liu G.E., Ventura M., Cellamare A., Chen L., Cheng Z., Zhu B., Li C., Song J., Eichler E.E. Analysis of recent segmental duplications in the bovine genome. *BMC Genomics*, 2009, 10: 571 (doi: 10.1186/1471-2164-10-571).
58. Zhang F., Gu W., Hurles M.E., Lupski J.R. Copy number variation in human health, disease, and evolution. *Annual Review of Genomics and Human Genetics*, 2009, 10: 451-481 (doi: 10.1146/annurev.genom.9.081307.164217).
59. Fontanesi L., Beretti F., Martelli P.L., Colombo M., Dall'Olio S., Occidente M., Portolano B., Casadio R., Matassino D., Russo V. A first comparative map of copy number variations in the sheep genome. *Genomics*, 2011, 97(3): 158-165 (doi: 10.1016/j.ygeno.2010.11.005).
60. Jenkins G.M., Goddard M.E., Black M.A., Brauning R., Auvray B., Dodds K.G., Kijas J.W., Cockett N., McEwan J.C. Copy number variants in the sheep genome detected using multiple approaches. *BMC Genomics*, 2016, 17: 441 (doi: 10.1186/s12864-016-2754-7).
61. Liu J., Zhang L., Xu L., Ren H., Lu J., Zhang X., Zhang S., Zhou X., Wei C., Zhao F., Du L. Analysis of copy number variations in the sheep genome using 50K SNP BeadChip array. *BMC*

Genomics, 2013, 14: 229 (doi: 10.1186/1471-2164-14-229).
62. Ma Y., Zhang Q., Lu Z., Zhao X., Zhang Y. Analysis of copy number variations by SNP50 BeadChip array in Chinese sheep. *Genomics*, 2015, 106(5): 295-300 (doi: 10.1016/j.ygeno.2015.08.001).
63. Zhu C., Fan H., Yuan Z., Hu S., Ma X., Xuan J., Wang H., Zhang L., Wei C., Zhang Q., Zhao F., Du L. Genome wide detection of CNVs in Chinese indigenous sheep with different types of tails using ovine high-density 600K SNP arrays. *Scientific Reports*, 2016, 6: 27822 (doi: 10.1038/srep27822).
64. Ma Q., Liu X., Pan J., Ma L., Ma Y., He X., Zhao Q., Pu Y., Li Y., Jiang L. Genome-wide detection of copy number variation in Chinese indigenous sheep using an ovine high-density 600 K SNP array. *Scientific Reports*, 2017, 7: 912 (doi: 10.1038/s41598-017-00847-9).
65. Hoeijmakers W.A., Bártfai R., Stunnenberg H.G. Transcriptome analysis using RNA-Seq. In: *Malaria. Methods in molecular biology (methods and protocols)*. R. Ménard (ed.). Humana Press, Totowa, NJ, 2012, vol. 923: 221-239 (doi: 10.1007/978-1-62703-026-7_15).
66. Shendure J. The beginning of the end for microarrays? *Nature Methods*, 2008, 5(7): 585-587 (doi: 10.1038/nmeth0708-585).
67. Oshlack A., Robinson M.D., Young M.D. From RNA-seq reads to differential expression results. *Genome Biol.*, 2010, 11(12): 220 (doi: 10.1186/gb-2010-11-12-220).
68. Wang X., Zhou G., Xu X., Geng R., Zhou J., Yang Y., Yang Z., Chen Y. Transcriptome profile analysis of adipose tissues from fat and short-tailed sheep. *Gene*, 2014, 549(2): 252-257 (doi: 10.1016/j.gene.2014.07.072).
69. Kang D., Zhou G., Zhou S., Zeng J., Wang X., Jiang Y., Yang Y., Chen Y. Comparative transcriptome analysis reveals potentially novel roles of Homeobox genes in adipose deposition in fat-tailed sheep. *Sci. Rep.*, 2017, 7(1): 14491 (doi: 10.1038/s41598-017-14967-9).
70. Ma L., Li Z., Cai Y., Xu H., Yang R., Lan X. Genetic variants in fat- and short-tailed sheep from high-throughput RNA-sequencing data. *Anim. Genet.*, 2018, 49(5): 483-487 (doi: 10.1111/age.12699).
71. Ma L., Zhang M., Jin Y., Erdenee S., Hu L., Chen H., Cai Y., Lan X. Comparative transcriptome profiling of mRNA and lncRNA related to tail adipose tissues of sheep. *Front. Genet.*, 2018, 9: 365 (doi: 10.3389/fgene.2018.00365).
72. Li B., Qiao L., An L., Wang W., Liu J., Ren Y., Pan Y., Jing J., Liu W. Transcriptome analysis of adipose tissues from two fat-tailed sheep breeds reveals key genes involved in fat deposition. *BMC Genomics*, 2018, 19(1): 338 (doi: 10.1186/s12864-018-4747-1).
73. Avilés C., Polvillo O., Peña F., Juárez M., Martínez A.L., Molina A. Associations between *DGAT1*, *FABP4*, *LEP*, *RORC*, and *SCD1* gene polymorphisms and fat deposition in Spanish commercial beef. *Journal of Animal Science*, 2013, 91(10): 4571-4577 (doi: 10.2527/jas.2013-6402).
74. Xu Q.L., Tang G.W., Zhang Q.L., Huang Y.K., Liu Y.X., Quan K., Zhu K.Y., Zhang C.X. The *FABP4* gene polymorphism is associated with meat tenderness in three Chinese native sheep breeds. *Czech Journal of Animal Science*, 2011, 56(1): 1-6.
75. Bokor S., Legry V., Meirhaeghe A., Ruiz J.R., Mauro B., Widhalm K., Manios Y., Amouyel P., Moreno L.A., Molnàr D., Dallongeville J., HELENA Study group. Single-nucleotide polymorphism of CD36 locus and obesity in European adolescents. *Obesity*, 2010, 18(7): 1398-1403 (doi: 10.1038/oby.2009.412).
76. Kern P.A., Di Gregorio G.B., Lu T., Rassouli N., Ranganathan G. Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. *Diabetes*, 2003, 52(7): 1779-1785 (doi: 10.2337/diabetes.52.7.1779).
77. Zhang L., Yang M., Li C., Xu Y., Sun J., Lei C., Lan X., Zhang C., Chen H. Identification and genetic effect of a variable duplication in the promoter region of the cattle *ADIPOQ* gene. *Anim. Genet.*, 2014, 45(2): 171-179 (doi: 10.1111/age.12112).
78. Erdenee S., Li J., Kang Z., Xu H., Zang R., Cao X., Yang J., Cai Y., Lan X. Sheep zinc finger proteins 395 (*ZNF395*): insertion/deletion variations, associations with growth traits, and mRNA expression. *Animal Biotechnology*, 2019, 19: 1-8 (doi: 10.1080/10495398.2019.1585865).
79. Bakhtiarizadeh M.R., Moradi-Shahrbabak M., Ebrahimie E. Underlying functional genomics of fat deposition in adipose tissue. *Gene*, 2013, 521(1): 122-128 (doi: 10.1016/j.gene.2013.03.045).
80. Ruixia X., Lei G., Weili Z., Wei Z., Guangchao S., Shangquan G., Guoqing S. [Analysis of FABP4 expression pattern in rump fat deposition and metabolism of Altay sheep]. *Yi Chuan*, 2015, 37(2): 174-182 (doi: 10.16288/j.yczz.14-369).
81. Bakhtiarizadeh M.R., Salami S.A. Identification and expression analysis of long noncoding RNAs in fat-tail of sheep breeds. *G3: Genes, Genomes, Genetics*, 2019, 9(4): 1263-1276 (doi: 10.1534/g3.118.201014).
82. Jiao X.L., Jing J.J., Qiao L.Y., Liu J.H., Li L.A., Zhang J., Jia X.L., Liu W.Z. Ontogenetic expression of *Lpin2* and *Lpin3* genes and their associations with traits in two breeds of Chinese fat-tailed sheep. *Asian-Australasian Journal of Animal Sciences*, 2016, 29(3): 333-342 (doi: 10.5713/ajas.15.0467).
83. Zakariapour Bahnamiri H., Ganjkhanlou M., Zali A., Sadeghi M., Moradi Shahrbabak H., Nehzati Paghaleh G.A. Expression of genes related to liver fatty acid metabolism in fat-tailed

and thin-tailed lambs during negative and positive energy balances. *J. Anim. Physiol. Anim. Nutr.*, 2019, 103(2): 427-435 (doi: 10.1111/jpn.13036).
84. Zeng J., Zhou S.W., Zhao J., Jin M.H., Kang D.J., Yang Y.X., Wang X.L., Chen Y.L. Role of OXCT1 in ovine adipose and preadipocyte differentiation. *Biochemical and Biophysical Research Communications*, 2019, 512(4): 779-785 (doi: 10.1016/j.bbrc.2019.03.128).
85. Ledur M.C., Navarro N., Pérez-Enciso M. Large-scale SNP genotyping in crosses between outbred lines: how useful is it? *Heredity*, 2009, 105(2): 173-182 (doi: 10.1038/hdy.2009.149).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1080-1094
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.5:57.08:577.21

doi: 10.15389/agrobiology.2019.6.1080eng
doi: 10.15389/agrobiology.2019.6.1080rus

TRANSGENIC POULTRY: DERIVATION AND AREAS OF APPLICATION
(review)

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The authors declare no conflict of interests
Received October 7, 2019
doi: 10.15389/agrobiology.2019.6.1080eng

Abstract

Transgenic poultry is a powerful instrument for the biotechnologic research in agriculture and medicine as well as a useful biological model (H. Sang, 2004). The technologies of transgenesis can be also aimed at the improvement of qualitative and quantitative characteristics of poultry products (L.G. Korshunova, 2011); development of poultry crosses genetically resistible to infectious diseases (L.G. Korshunova et al., 2014); derivation of poultry that can produce recombinant proteins of different usage areas within the eggs (D. Cao et al., 2015). The most popular way to induce transgenicity is the microinjection of foreign DNA into the ovicell in the proper moment when the organism consists of a single cell (zygote). Certain peculiarities of avian reproduction, however, constrain the induction of the transgenesis. A hen produces daily a single fertilized ovicell which is large in size and extremely sensitive to any manipulation like those to be performed on the mammal ovicells at the injection of foreign DNA. Furthermore, normal embryonic development in avian eggs requires the integrity of tertiary coats — albumen, inner shell membrane, and eggshell itself. The cleavage of chicken ovicell starts as early as in the magnum while freshly laid egg contains ca. 50,000-60,000 cells. As a consequence, first transgenic bird was produced via retroviral vectors. Retroviruses were the first contenders for the role of vectors in the gene transfer since they normally can enter the genomic DNA of the host with subsequent replication. At present the induction of retroviral (D.W. Salter et al., 1986; D.W. et al. Salter, 1987; D.W. et al. Salter, 1989; R.A. Bosselman et al., 1989; L.B. Crittenden, 1991; L.B. Crittenden et al., 1992) and lentiviral (H.A. Kaleri et al., 2011; A.H. Seidl et al., 2013; N.A. Volkova et al., 2015) transgenesis in chicken and quails was reported. The technologies of genomic modification in chicken and quails are continuing their development: e.g. the methods with the use of zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) (T.S. Park et al., 2014), CRISPR/Cas9 (I. Oishi et al., 2016; Q. Zuo et al., 2016) were developed. The CRISPR/Cas9 technology allows for the further progress in the genetic manipulations to produce genome-edited lines of poultry (N. Veron et al., 2015). Avian embryos, primarily chicken (*Gallus gallus domesticus*) and quail (*Coturnix coturnix japonica*), served as a model for the embryologic studies in vertebrates for more than a century. Modern targeted genetic manipulations in chicken embryo as an in vivo model became possible via the CRISPR/Cas9 editing system (V. Morin et al., 2017). The alternative non-viral methods of the induction of transgenesis in avian species can be used; one of these methods involves the transfer of foreign embryonic cells (as foreign DNA vector) into the recipient embryo resulting in the chimeric birds (J.N. Petitte et al., 1990; J.Y. Han et al., 2017; N.A. Volkova et al., 2017). Another attractive technology for the transfer of foreign genetic material into avian embryos involves the use of spermatozoa as the vectors since artificial insemination is the traditional and common technique for poultry (E. Harel-Markowitz et al., 2009; A.V. Samoylov et al., 2013). The combination of spermatozoa vectors and CRISPR/Cas9 technology can result in transgenesis in the first generation and hence allows for the substantial savings in time and resources (C.A. Cooper et al., 2017). The microinjection of DNA into the zygote is still a classic technology of non-viral transgenesis. The method involves direct injection of gene construct into the cytoplasm of freshly fertilized ovicell and subsequent incubation of injected eggs. The ovicell for injection should be taken immediately after its fertilization which means that its movement down the oviduct should be interrupted; further development of the injected embryo require special cultivation system (C. Mather, 1994). Another microinjection technique for avian ovicells involves natural formation of tertiary coats in the oviduct. The method is based on the surgical operation to get the access to the ovicell, its microinjection with foreign DNA, and implantation of injected ovicell back

to the maternal oviduct for the formation of normal egg suitable for incubation (R.V. Karapetyan, 1995). The populations of transgenic chicken and quails with different foreign gene constructs were produced with the use of this method (R.V. Karapetyan, 1996; L.G. Korshunova et al., 2013).

Keywords: transgenesis, poultry, retroviruses, microinjection, primordial cells, sperm cells, genome editing

In the age of the developed poultry industry, the number of poultry species used in production has decreased significantly. Genotypes selected for certain conditions have become the most profitable but when market requirements change, the economic necessity for species and lines with new properties appears.

Genetic engineering provides powerful tools for changing living organisms. Since there is no species barrier for the integration of foreign genes, transgenic individuals of a specified type that cannot be obtained by classical breeding methods can be created. Transgenic organisms differ from natural ones in that they are obtained by genetic engineering methods and contain foreign genetic engineering material in their genome. They are capable of reproducing and transferring artificially altered genetic material to offspring. Transgenesis technologies in poultry farming can be aimed at improving the qualitative and quantitative characteristics of poultry products; developing poultry crosses genetically resistible to infectious diseases; deriving poultry that can produce recombinant proteins of different usage areas within the eggs. For practical use, a transgenic bird must have a phenotype that exceeds the level already achieved in poultry farming. Such signs may include an increase in growth rate, improved feed conversion, increased egg production, reduced body fat, increased disease resistance, etc. In comparison with traditional breeding methods, when the transfer of the desired gene from one bird line to another requires subsequent multiple backcrosses to remove unnecessary genes inherited during natural sexual hybridization, transgenesis gives a long-term advantage in time. In contrast to traditional breeding, transgenesis provides a unique opportunity to obtain a bird that produces and accumulates proteins useful for medical and industrial purposes in the egg, which can also have a wide practical application [1-4].

The purpose of this review is to summarize data on bioengineering methods for the creation and possible applications of transgenic poultry.

Research to develop practical methods of transgenesis in poultry has been ongoing since the creation of a transgenic mouse in the 1980s. The first manipulations with the chicken zygote initiated the development of more successful technologies [5]. Currently, there are several methods of genetic modification of the body. Most often, the microinjection of foreign DNA into the ovicells is used. At the same time, it is important to ensure that the transgene is contained in all cells of the body and obligatorily in the germ cells (for transfer to offspring). Genes are transferred at the earliest stages of the body's development, including the zygote stage. However, the peculiarities of birds breeding create serious problems here. A hen produces daily a single fertilized ovicell which is large in size and extremely sensitive to any manipulations. Normal embryonic development in avian eggs requires tertiary coats – albumen, inner shell membrane, and eggshell. The cleavage of chicken ovicell starts as early as in the magnum while freshly laid egg contains ca. 50,000-60,000 cells.

The first transgenic bird was produced via retroviral vectors. Retroviruses have become the first contenders for the role of vectors in the gene transfer since they normally can enter the genomic DNA of the host with subsequent replication. Many researchers have tried to introduce foreign genes into the germ line, infecting embryos with both replication-capable and non-replicating retroviral vectors [6, 7]. The frequency of successful embedding of the transgene in these exper-

iments ranged from 0.8 to 5%. A positive result on the transfer of genetic material to the germ line was obtained by introducing a recombinant vector of avian leukemia into the blastoderm of incubated chicken embryos [8, 9]. Complete transgenes were created with the help of the recombinant virus [10-12].

In the work of Lee *et al.* [3], the possibility of using chickens as a bioreactor to produce a human urokinase-type plasminogen activator (*huPA*) was studied. The recombinant *huPA* gene under the control of the Rous sarcoma virus promoting agent was injected into freshly laid eggs at stage X using retroviral vectors based on the mouse leukemia virus. In general, 38 chickens from 573 eggs injected with the virus hatched and contained the *huPA* gene in different parts of the body. The mRNA transcript of the *huPA* gene was present in various organs, including blood and ovicell, and was a germ line passed on to the next generation. The content of the active *huPA* protein in the blood of transgenic poultry was 16 times higher than that of non-transgenic poultry ($p < 0.05$). *HuPA* protein expression in eggs increased from 7.82 IU/egg in generation G_0 to 17.02 IU/egg in generation G_1. However, embryos expressing *huPA* had reduced survival and hatchability on Days 18 and 21 of incubation. In transgenic chickens, blood clotted much more slowly than in non-transgenic counterparts ($p < 0.05$). In addition, adult transgenic roosters had reduced fertility ($p < 0.05$): ejaculate volume, spermatozoa concentration and viability decreased. As a whole, the obtained data suggest that transgenic *huPA* chickens can be successfully obtained using a retroviral vector system. Such chickens can be used as a bioreactor in the production of the pharmacological *huPA* drug for the treatment of vascular diseases, as well as in the study of *HuPA*-induced bleeding and other disorders. Urokinase-type plasminogen activator (uPA; Swiss-Prot: P00749) – inducible serine protease (EC 3.4.21.73) found in blood and extracellular matrix plays an important role in fibrinolysis, extracellular proteolysis, and tissue remodeling.

In chicken transgenesis, the advantages of using a viral vector that carries its integrase were identified, which can be applied to embryos in newly laid eggs. This formed the basis for a more effective method using lentivirus vectors [13-16]. Byun *et al.* [4] used lentiviral vectors to create transgenic chickens that express the human extracellular superoxide dismutase (hEC-SOD) gene. Recombinant lentiviruses were injected into the sub-germ cavity of freshly laid eggs. The embryos were then incubated before hatching using phases II and III of the ex vivo surrogate shell culture system. Sixteen chickens (G_0) out of 158 such embryos hatched. In one of them, the polymerase chain reaction (PCR) method was used to identify a transgene in the germ line cells that form germ cells. This transgenic bird (G_0) was crossed with a normal one and, as a result, two transgenic chickens (G_1) were obtained. In G_2 transgenic chickens, the hEC-SOD protein was contained in egg white and showed antioxidant activity.

The ability to manipulate gene expression when creating transgenic chicken embryos using viral vectors has proven useful for analyzing gene functions in tissues. Modern transgenesis technologies using lentiviral vectors have significant potential for genetic research on embryos and adult birds. One of the main problems in vertebrate embryogenesis is to understand how differentiation and complete anatomical development are coordinated in organs consisting of many cell types, and how differences in gene expression affect the phenotype. These and many other issues can be solved by using transgenic bird lines [2, 17].

Currently, retroviral and lentiviral transgenesis is used in poultry. Lentiviral vectors have been successfully used to create several transgenic lines of chickens and quails [5, 18-21]. The development of this technology continues [22, 23]. However, despite the effectiveness of viral transduction, there are many

obstacles to its application due to the relatively low and variable frequencies of transgene transfer along the germ line, as well as safety problems when using viral vectors. Therefore, obtaining transgenic poultry using non-viral technologies remains a necessary condition for applying transgenesis in poultry farming practice [24, 25].

Effective genome modification technologies are currently available and continue to be developed: zinc finger nucleases method (ZFNs – "zinc fingers"), TALENs (transcription activator-like effector nucleases) [26], CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats – short palindromic repeats arranged in groups regularly) [27, 28]. All techniques of genomic engineering are available for poultry.

The possibilities and consequences of using CRISPR/Cas9 are being studied actively [29]. CRISPR/Cas9 is a revolutionary genome-editing system that provides a powerful tool for studying the molecular mechanisms that regulate development [30-32]. Recent achievements using programmable nucleases have increased the efficiency of making precise changes to the genomes of eukaryotic cells significantly. Genome editing technologies allow expanding knowledge about genetic diseases and create more accurate models of pathological processes [33]. The prospect of direct correction of genetic mutations in affected tissues and cells for the treatment of diseases that do not respond to traditional therapy is particularly tempting [34]. CRISPR/Cas9 can be used to accelerate progress in the genetic manipulation of poultry and create lines with the edited genome [35].

Let us note that due to the similarity in general ontogenesis and gene expression patterns, the bird can serve as an excellent model for sequestering genetic, molecular, and biochemical processes in mammals, including humans. For more than a century, bird embryos, mainly chicken (*Gallus gallus domesticus*) and quail (*Coturnix japonica*) have been the basis for studying vertebrate embryology, cell movement in morphogenesis, mechanisms of induction and differentiation, the pathogenesis of embryonic diseases, and toxicology. Transgenic birds remain one of the most powerful research tools in biotechnology.

Targeted genetic manipulations on a chicken embryo as an in vivo model were made possible by the CRISPR/Cas9 system [36-39]. Gandhi *et al.* [40] optimized CRISPR/Cas9 using a three-step strategy applied to early embryos. First, the authors used Cas9 with two signal sequences to improve nuclear localization. Second, due to the modified RNA (gRNA), there was no premature termination of transcription, and Cas9-gRNA interactions were stable. Third, the involved chicken-specific U6 promoting agent provided 4 times higher gRNA expression than human U6. For rapid in vivo gRNAs screening, a cell line of chicken fibroblasts expressing Cas9 was also created. To prove the principle, the authors conducted electroporation studies of loss of function in an early chicken embryo during protein knockout of Pax7 and Sox10, key transcription factors with a known role in the development of the neural crest. CRISPR/Cas9-mediated deletion has been shown to cause loss of the corresponding proteins and transcripts. The results confirmed the usefulness of the optimized CRISPR/Cas9 method for targeted gene knockout in chicken embryos in a reproducible, reliable, and specific way [40]. CRISPR/Cas9 can be combined with other methods of studying gene function in chicken and quail embryos, for example, with electroporation (in particular, for targeted editing of genes of the genitourinary birds' system) [41, 42].

Among other methodological approaches for virus-free transgenesis in birds, it is possible to note the production of chimeras when transplanting foreign cells into embryos. Early blastoderm cells or primordial cells are isolated,

foreign DNA is injected into their nuclei, then the cells are implanted in embryos, where they survive and divide [43-47]. Thus, Petitte *et al.* [43] obtained blastodermal cells from chicken embryos of the Barred Plymouth Rock line (it has a black pigment in the feathers due to a recessive allele at the I locus) and introduced them into the sub-germ cavity of embryos from the inbred Dwarf White Leghorns line with white feathers due to the dominant allele at the I locus. Six (11.3%) of 53 such embryos of the Dwarf White Leghorn line turned out to be phenotypic chimeras in feather color, one of them (cockerel) survived to hatch. In recipients, the distribution of black feathers varied and was not limited to any specific area, although in one case they prevailed on the head. This somatically chimeric rooster was cross-bred with several Barred Plymouth Rock hens to assess the extent to which donor cells were included in the testes. Two of received 719 chicks were Barred Plymouth Rocks by phenotype. It means that cells capable of turning into germ line cells were passed on to recipients. The DNA fingerprint technique from the blood and sperm of germ chimeras showed that both of these tissues differed from those of the inbred Dwarf White Leghorns line. Somatic chimeras and germ-line chimeras were obtained in the same way [43, 48, 49]. Chimeric individuals were raised and their offspring were removed from them. Some of the offspring were transgenic.

These and similar studies [20, 50-54] show that the isolation, transfer, and introduction of primordial germ cells can be used to produce transgenes in poultry farming. However, the described technology [43] is quite complex and expensive. Modern progress in isolation of primordial germ cells and cultivation opens up new opportunities for reproductive biotechnologies in poultry farming [55-57]. Methodological techniques for transferring foreign DNA with embryonic cells (including using CRISPR/Cas9) continue to be developed. For example, Nakamura *et al.* [58] showed that X-ray irradiation reduced the number of endogenous primordial germ cells and increased the transmission of transferred primordial germ cells in chimeric chickens. Reducing the number of endogenous primordial germ cells is also possible when using busulfan [59, 60]. Genetically modified primordial germ cells were transplanted directly into the testicles of sterilized adult roosters. Their ability to mature into functional spermatozoa and restore spermiogenesis was shown [61].

Transgenesis with spermatozoa as vectors for foreign genetic material to the zygote looks very tempting since artificial insemination is widely used in poultry farming. Studies have been carried out on the use of sperms as a vector for the delivery of foreign DNA to the zygotes of chickens. It has been shown that lipofectin interacts with DNA, forming lipid-DNA complexes that can connect to the plasma membrane of the cell, contributing to the ingress of DNA into the cell. In the case of lipofectin application, 51.6% of the sperm of the rooster showed the presence of exogenous DNA. When artificial insemination of chickens with sperms transfected using lipofectin was carried out, exogenous DNA was observed in the blastoderm of 67% of eggs. However, the presence of foreign DNA in the genome of hatched chickens was not detected, although its episomal integration was shown. To increase the efficiency of sperm-mediated gene transfer (SMGT), the method was supplemented with the use of restriction enzyme-mediated integration. Linear DNA, together with restriction, penetrates the target cell by lipofection or electroporation. Restrictase is expected to specifically cut genomic DNA to facilitate the integration of exogenous DNA with complementary sticky ends. Lipofection of plasmid DNA with a restriction enzyme is considered a highly effective method for obtaining transfected sperm for artificial insemination [62]. Using liposomes, chickens with the human granulocyte colony-stimulating factor gene were created by artificial insemination with

transfected sperm. The share of transgenic chickens was 33.3%, and the frequency of a foreign gene inheritance was 37.5% [63].

To use rooster sperms as vectors, the permeability of sperm membranes under various electroporation regimes was studied. The range of physical and chemical effects is determined, within which the formation of pores in the sperm shell is observed, and the sperms themselves retain motility. An increase in the amount of trypan blue dye deposited with rooster sperms after electroporation is shown, with an increase in the number of electrical pulses and a decrease in the osmolarity of the electroporation medium. Parameters that can be changed to increase the efficiency of the electroporation are determined [64]. However, the passive transfer of non-viral constructs via rooster sperm does not always lead to the appearance of transgenic individuals [65]. Although the successful transfer of an exogenous gene using spermatozoa has been shown in birds, there are few studies in which the expression of a foreign gene has been observed. It is assumed that if DNA is not internalized in the sperm nucleus, but only delivered in the ovicell cytoplasm by it, the DNA copies in the form of plasmids are found in early embryos, but not detected in chickens.

The inability to obtain stable and diverse modifications through SMGT has led to the need to look for other technologies. Wang *et al.* [66] successfully isolated haploid embryonic stem cells (haESCs) with the genome of highly specialized gametes. They can consistently maintain haploidy (through periodic cell sorting based on the amount of DNA), self-renewal, and pluripotency in the transplanted cell culture. In particular, haESCs derived from androgenetic haploid blastocysts (AG-haESCs) that carry only the sperm genome can provide generation of SC (semi-cloned) animals by injection into oocytes. It is noteworthy that after removing the imprinted control regions of H19-DMR (a differentially methylated section of DNA) and IG-DMR by double knockout (DKO) when using DKO-AG-haESCs blastocysts, it is possible to obtain SC-animals with high efficiency, i.e., DKO-AG-haESCs serves as the equivalent of sperm. It is important that DKO-AG-haESCs can be used for multiple rounds of in vitro gene modification to create generations of fertile animals with specified genetic characteristics. Thus, DKO-AG-haESCs ("artificial spermatozoa") modified using CRISPR/Cas technology can become a convenient fertilization agent for effectively obtaining genetically modified offspring and serve as a universal tool for analyzing the function of genes in vivo.

However, using sperms to deliver foreign DNA to ovicells in combination with CRISPR/Cas9 allows the creation of a first-generation transgenic bird, which significantly saves time and resources. In addition, the developed methods can be easily adapted to different types of birds [67].

The classic technology of virus-free transgenesis is the microinjection of DNA into the zygote. Its use in poultry is complicated due to the too large size of the oocyte. The possibility of incubating in vitro fertilized chicken ovicells extracted from the upper part of the protein section of the oviduct was studied [68]. Since the embryo is single-celled at this stage, it is theoretically possible to micro-inject foreign DNA into such an embryo. A fertilized chicken ovicell can be cultured in a shell with the protein of another egg [68]. The method includes three stages (taking into account the different needs of the embryo during development) and involves using the eggshell as a "vessel" for cultivation. The initial version involved culturing in glass vessels for the first 24 hours, followed by moving the embryos from vessel to vessel (from shell to shell) for cultivation between stages I and II, II and III. Further on, the method was simplified by culturing the embryo at the first stage of development in the shell, which eliminated the need to transfer it from the glass vessel to the shell. As a result, the number of

viable hatched chickens was 20%. The described cultivation or its separate stages can be used for various experimental purposes since this technique gives access to the developing embryo at all stages of its development [69].

As a method of creating a transgenic bird, the possibility of injecting DNA with further embryo cultivation was considered. The gene construct with the reporter gene β-galactosidase was injected. The expression of the gene construct in the first 7 days of development was studied histochemically by the manifestation of β-galactosidase activity in embryonic tissues. Cells with the β-galactosidase activity were observed at the stage of 250-500 cells in the center of the blastodisc. On Day 2, they were visible in large segments of the blastoderm, at later stages – in the corresponding smaller segments of extra-germ shells. β-galactosidase-positive cells were in most cases scattered around the primitive blastula band, but after gastrulation, they were observed in embryonic tissue in only 7% of embryos. This indicates transcriptional activity at the crushing stages and confirms data on the loss of foreign DNA during the early development of the chicken. The results of exogenous DNA injection at the stage of a single cell with further embryo cultivation showed that the inclusion of injected DNA in chicken chromosomes was a fairly rare event. The lack of integration into the genome can be hidden by the presence of extra-chromosomal copies of injected plasmids [68].

The approach successfully used in creating a transgenic bird [70] is an injection of a gene construct into the cytoplasm of a freshly fertilized chicken ovicell with its incubation before hatching. Ovicells for injection were extracted from the upper part of the protein section of the oviduct already covered with a thin layer of protein, but not the shell. The culture system has been improved to optimize the survival of the injected ovicell. The system is still three-stage, the eggshell is used as a vessel for cultivation, and the liquid protein diluted with a salt solution is used as a nutrient medium. At the first stage of cultivation, which lasts 24 hours, the injected ovicell is placed in a shell containing such an amount of nutrient medium that the germ disk is not immersed in it. At this stage, the embryo develops from one cell to the blastoderm (60 thousand cells), which is typical for laid fertilized eggs. Then, the shell is filled with diluted egg white until the embryo is completely submerged, and the window in the shell is sealed with tape. At this second stage, the embryo should develop a heart and extra-fetal blood circulation within 65 hours. At the third, the longest stage, which lasts until hatching, the contents of the shell are moved to another, larger shell. This is done to create the air space needed during the paraphytic period when the embryo begins to breathe with the lungs and prepares for hatching. For 1-2 days before hatching, with the beginning of pulmonary ventilation, the sealing film is perforated for free access of air to the shell. Further on, the film is loosened so that the chicken can come out of the shell. Thus, incubation at the first stage takes place in the surrogate oviduct – the incubator, where the bird's body temperature (42 °C), high relative humidity and high concentration of carbon dioxide are maintained. At the end of the first stage, there is a short period at room temperature to imitate egg-laying. The incubation conditions during stages II and III, equivalent to the incubation period of normal chicken eggs, are changed in comparison with traditionally used in poultry farming. The relative humidity is increased to about 75% (up to 18 days of incubation), then it is reduced to about 65% (before the hatching stage). During stage II, the frequency of rotations is increased (4 times per hour). At stage III, the angle of rotation is reduced so that the embryo does not touch tape that covers the window in the shell.

Creating transgenic chickens using the described method is quite time-consuming. Only 50% of injected eggs reach stage III, and the percentage of

hatched chickens is about 15%. However, the results of the analysis of embryos and chickens for transgenicity are encouraging. When using PCR, it was shown that the DNA of almost 50% of embryos and chickens that reached the last Day 12 of cultivation contained a transgene. Thus, two of the hatched chickens were found to have a transgene in the chorioallantoic membrane, rib pulp, and blood. In one of the roosters, the transgene was preserved until puberty. Its sperm contained a transgene (reporter gene) that was passed on to 3.4% of the offspring, i.e. 14 of the 412 chickens [70]. Thus, transgenic chickens were created by this method and the inheritance of the transgene was shown. All primary transgenic individuals were mosaic. In subsequent generations, the inheritance of the transgene corresponded to Mendel's laws. According to the authors' reports, about 60% of embryos [70] survived the hatching stage. However, when using this method in other laboratories, the same indicator did not exceed 3-10% [71].

Another variant of DNA microinjections in bird ovicells involves the formation of tertiary ovicell coats (protein, subshell, and shell) naturally in the bird's oviduct. To access the ovulated chicken ovicell, it is necessary to define the time when the abdominal cavity should be opened. In the bird used in the experiment (the work was carried out on White Leghorn chickens at the age of 180-300 days), ovulation occurred 20-25 minutes after egg-laying. Approximately 5 minutes after ovulation, the ovicell is located in the funnel of the oviduct completely, where it is fertilized. The time until the ovicell is completely moved to the protein section is a few minutes. An ovicell extracted from the abdominal cavity or the oviduct was injected and placed through the funnel into the protein section of the oviduct with or without a specially designed expander [72].

In the ovulatory process, the oviduct funnel is active in the chicken immediately before ovulation. In this state, it is sufficiently elastic and strong to place the transplanted ovicell in it. The active state of the funnel is observed for about 5 minutes, after which it becomes extremely loose and damaged when stretched. In such cases, either the ovicell was lost due to mechanical damage when trying to return it to the compressed oviduct funnel, or transplantation became impossible due to ruptures of the funnel itself. Only half of the implanted ovicells produced morphologically normal eggs.

To exclude the stages of ovulating ovicell extraction and its reimplantation into the oviduct, it was proposed to inject DNA into the blastodisc of the ovicells through a sufficiently transparent shell of the oviduct funnel. As a result, ovicell losses were reduced to almost zero, and the number of morphologically normal eggs was close to 100%. Thus, the effectiveness of the method has increased significantly [73, 74].

The techniques described above make it possible to obtain no more than one viable ovicell injected with foreign DNA from a single chicken in a single operation. An option that allowed a sharp increase in the number of such ovicells for each operation was an injection into the ovicells located in the ovary, in other words, into the follicles [75]. The chicken ovary has 4-5 large follicles, which are quite suitable for microinjections. However, this approach increases the technical difficulties. Surgical access to the ovary is somewhat more difficult than to the oviduct, and the follicle membranes are tougher and thicker than those of the oviduct funnel. Nevertheless, the positive aspects of the proposed option prevail. In addition, there is no need to set ovulatory cycles of poultry, which simplifies the preparation and conduct of the experiment, and the complexity of visual control during microinjections is easily overcome due to the use of modern ophthalmological and otolaryngological optics and microtechnics. Thus, hypothetically, it is possible to process and obtain 4-5 injected foreign DNA ovicells from each chicken for each operation, and without possible prob-

lems associated with non-fertilization of ovicells or abnormal formation of tertiary coats.

To assess the possibility of DNA microinjections into the follicles, it was necessary to make sure that an ovicell was formed from the injected follicle, and that a full-fledged egg was formed from it. To do it, a dye – ink or methylene blue was injected into the yolk of the follicles. Eggs were collected and opened from operated chickens for 12 days. The obtained results showed that eggs were formed from injected follicles. After 7 days, the dye in the eggs was never observed. It should be noted that surgery and injections can disrupt the order in the follicle hierarchy, according to which the largest follicle should ovulate first. Quite often, the dye was found in eggs laid not among the first, but later, although it was always injected into the three largest follicles. Autopsy of the chickens showed that the part of follicles has been reabsorbed. Quite often, only traces of the dye were observed in the eggs, which means that it is removed from the follicle during maturation. It is possible that some eggs marked as not containing dye came from injected follicles. Although the obtained results are not unambiguous enough for strict conclusions about the hierarchy of follicles, the order of their ovulation, etc., one can conclude that on average, at least 50% of eggs laid by chickens in the first 7 days after surgery come from injected follicles.

The developed surgical operations and methods of manipulating the eggs of chickens and quails allow making microinjections of DNA into the ovicell and ensure its further normal embryonic development. Using these technologies, transgenic chickens [76-78] with different gene structures and quail with the bovine somatotropin gene were created. At the same time, transgenic quails had obvious phenotypic differences from native quails. Integration of the bovine growth hormone gene into the quail genome increased their immune status, resulting in accelerated antibody production. The LaSota vaccine strain of the Newcastle disease virus was used as an antigen [79]. Another most notable phenotypic feature was large eggs laid by quails. The average weight of eggs in the experimental quail population was 20-50% higher than normal. The live weight of transgenic quails was 5-15% higher than that of both typical for the breed and the control group of native individuals. These phenotypic features were preserved in all 33 studied generations of transgenic quail offspring [80].

Thus, some progress has already been made in creating a transgenic bird based on several methodological approaches (retroviral and lentiviral vector systems, virus-free transgenosis using sperms, "artificial spermatozoa", microinjections of foreign DNA into a zygote, a freshly fertilized egg or follicles). However, the development of transgenesis in poultry farming is mostly still at an early stage, although all known genetic engineering technologies can be used in poultry, including actively developed methods of genomic editing based on CRISPR/Cas9, which are close to the natural mechanisms of action of these systems in cells. Modern leaders in successful genome editing developments are the United States, China, and the United Kingdom; such studies are also being conducted in Russia. This modern technology can be useful in solving a range of scientific and practical problems, including the creation of new breeds of poultry. However, it is not fully clear yet what are the consequences of bioengineering manipulations with the genome, including genomic editing to correct an existing feature or introduce a new one.

REFERENCES

1. Cao D., Wu H., Li Q., Sun Y., Liu T., Fei J., Zhao Y., Wu S., Hu X., Li N. Expression of recombinant human lysozyme in egg whites of transgenic hens. *PLoS ONE*, 2015, 10(2): e0118626 (doi: 10.1371/journal.pone.0118626).

2. Lin S.L., Chang S.E., Ying S.Y. Transgene-like animal models using intronic microRNAs In: *MicroRNA protocols. Methods in molecular biology. V. 1733*. S.Y. Ying (ed.). Humana Press, NY, 2018: 239-254 (doi: 10.1007/978-1-4939-7601-0_20).
3. Lee S.H., Gupta M.K., Ho Y.T., Kim T., Lee H.T. Transgenic chickens expressing human urokinase-type plasminogen activator. *Poultry Science*, 2013, 92(9): 2396-2403 (doi: 10.3382/ps.2013-03223).
4. Byun S.J., Ji M.R., Jang Y.J., Hwang A.I., Chung H.K., Kim J.S., Kim K.W., Chung H.J., Yang B.C., Jeon I., Park J.K., Yoo J.G., Kim T.Y. Human extracellular superoxide dismutase (EC-SOD) expression in transgenic chicken. *BMB Reports*, 2013, 46(8): 404-409 (doi: 10.5483/bmbrep.2013.46.8.251).
5. Sang H. Prospects for transgenesis in the chick. *Mechanisms of Development*, 2004, 121(9): 1179-1186 (doi: 10.1016/j.mod.2004.05.012).
6. Bosselman R.A., Hsu R.-Y., Boggs T., Hu S., Bruszewski J., Ou S., Kozar L., Martin F., Green C., Jacobsen F., Nicholson M., Schultz J.A., Semon K.M., Rishell W., Stewart, R.G. Germline transmission of exogenous genes in the chicken. *Science*, 1989, 243(4890): 533-535 (doi: 10.1126/science.2536194).
7. Crittenden L.B. Retroviral elements in the genome of the chicken: implications for poultry genetics and breeding. *Critical Reviews of Poultry Biology*, 1991, 3(2): 73-109.
8. Salter D.W., Smith E.J., Hughes S.H., Wright S.E., Crittenden L.B. Transgenic chickens: insertion of retroviral genes into the chicken germ line. *Virology*, 1987, 157(1): 236-240 (doi: 10.1016/0042-6822(87)90334-5).
9. Salter D.W., Smith E.J., Hughes S.H., Wright S.E., Fadly A.M., Witter R.L., Crittenden L.B. Gene insertion into the chicken germ line by retroviruses. *Poultry Science*, 1986, 65(8): 1445-1458 (doi: 10.3382/ps.0651445).
10. Salter D.W., Crittenden L.B. Artificial insertion of a dominant gene for resistance to avian leukosis virus into the germ line of the chicken. *Theoretical and Applied Genetics*, 1989, 77(4): 457-461 (doi: 10.1007/BF00274263).
11. Crittenden L.B., Salter D.W. A transgene, *alv6*, that expresses the envelope of subgroup A avian leukosis virus reduces the rate of congenital transmission of a field strain of avian leukosis virus. *Poultry Science*, 1992, 71(5): 799-806 (doi: 10.3382/ps.0710799).
12. Petropoulos C.J., Payne W., Salter D.W., Hughes S.H. Appropriate in vivo expression of a muscle-specific promoter by using avian retroviral vectors for gene transfer [corrected]. *Journal of Virology*, 1992, 66(6): 3391-3397.
13. Poynter G., Huss D., Lansford R. Japanese quail: an efficient animal model for the production of transgenic avians. *Cold Spring Harbor Protocols*, 2009, 2009(1): pdb emo112 (doi: 10.1101/pdb.emo112).
14. McGrew M.J., Sherman A., Lillico S.G., Taylor L., Sang H. Functional conservation between rodents and chicken of regulatory sequences driving skeletal muscle gene expression in transgenic chickens. *BMC Developmental Biology*, 2010, 10: 26 (doi: 10.1186/1471-213X-10-26).
15. Kwon M.S., Koo B.C., Choi B.R., Park Y.Y., Lee Y.M., Suh H.S., Park Y.S., Lee H.T., Kim J.H., Roh J.Y., Kim N.H., Kim T. Generation of transgenic chickens that produce bioactive human granulocyte-colony stimulating factor. *Molecular Reproduction and Development*, 2008, 75(7): 1120-1126 (doi: 10.1002/mrd.20860).
16. Scott B.B., Lois C. Generation of tissue-specific transgenic birds with lentiviral vectors. *Proceedings of the National Academy of Sciences*, 2005, 102(45): 16443-16447 (doi: 10.1073/pnas.0508437102).
17. Ayers K.L., Smith C.A., Lambeth L.S. The molecular genetics of avian sex determination and its manipulation. *Genesis*, 2013, 51(5): 325-336 (doi: 10.1002/dvg.22382).
18. Seidl A.H., Sanchez J.T., Schecterson L., Tabor K.M., Wang Y., Kashima D.T., Poynter G., Huss D., Fraser S.E., Lansford R., Rubel E.W. Transgenic quail as a model for research in the avian nervous system: a comparative study of the auditory brainstem. *The Journal of Comparative Neurology*, 2013, 521(1): 5-23 (doi: 10.1002/cne.23187).
19. Park F. Lentiviral vectors: are they the future of animal transgenesis? *Physiological Genomics*, 2007, 31(2): 159-173 (doi: 10.1152/physiolgenomics.00069.2007).
20. Intarapat S., Stern C.D. Chick stem cells: current progress and future prospects. *Stem Cell Research*, 2013, 11(3): 1378-1392 (doi: 10.1016/j.scr.2013.09.005).
21. Byun J.S., Yuk S.S., Jang Y.J., Choi H., Jeon M.H., Erdene-Ochir T.O., Kwon J.H., Noh J.Y., Kim J.S., Yoo J.G., Song C.S. Transgenic chickens expressing the 3D8 single chain variable fragment protein suppress avian influenza transmission. *Scientific Reports*, 2017, 7(1): 5938 (doi: 10.1038/s41598-017-05270-8).
22. Kaleri H.A., Xu S.Y., Lin H.L. Generation of transgenic chicks using an oviduct-specific expression system. *Genetics and Molecular Research*, 2011, 10(4): 3046-3055 (doi: 10.4238/2011.December.8.1).
23. Volkova N.A., Fomin I.K., Tomgorova E.K., Vetokh A.N., Mennibaeva E.R., Brem G., Zinov'eva N.A. The study of factors affected the gene transfer efficiency in chicken embryonic cells by application of lentiviral vectors. *Agricultural Biology* [*Sel'skokhozyaistvennaya biologiya*], 2015, 50(4): 458-466 (doi: 10.15389/agrobiology.2015.4.458eng).

24. Park S.H., Kim J.N., Park T.S., Lee S.D., Kim T.H., Han B.K., Han J.Y. CpG methylation modulates tissue-specific expression of a transgene in chickens. *Theriogenology*, 2010, 74(5): 805-816.e1 (doi: 10.1016/j.theriogenology.2010.04.005).
25. Park T.S., Lee H.G., Moon J.K., Lee H.J., Yoon J.W., Yun B.N., Kang S.C., Kim J., Kim H., Han J.Y., Han B.K. Deposition of bioactive human epidermal growth factor in the egg white of transgenic hens using an oviduct-specific minisynthetic promoter. *The FASEB Journal*, 2015, 29(6): 2386-2396 (doi: 10.1096/fj.14-264739).
26. Park T.S., Lee H.J., Kim K.H., Kim J.S., Han J.Y. Targeted gene knockout in chickens mediated by TALENs. *Proceedings of the National Academy of Sciences*, 2014, 111(35): 12716-12721 (doi: 10.1073/pnas.1410555111).
27. Oishi I., Yoshii K., Miyahara D., Kagami H., Tagami T. Targeted mutagenesis in chicken using CRISPR/Cas9 system. *Scientific Reports*, 2016, 6: 23980 (doi: 10.1038/srep23980).
28. Zuo Q., Wang Y., Cheng S., Lian C., Tang B., Wang F., Lu Z., Ji Y., Zhao R., Zhang W., Jin K., Song J., Zhang Y., Li B. Site-directed genome knockout in chicken cell line and embryos can use CRISPR/Cas gene editing technology. *G3: Genes, Genomes, Genetics*, 2016, 6(6): 1787-1792 (doi: 10.1534/g3.116.028803).
29. Davey M.G., Balic A., Rainger J., Sang H.M., McGrew M.J. Illuminating the chicken model through genetic modification. *Int. J. Dev. Biol.*, 2018, 62: 257-264 (doi: 10.1387/ijdb.170323mm).
30. Fineran P.C., Charpentier E. Memory of viral infections by CRISPR-Cas adaptive immune systems: acquisition of new information. *Virology*, 2012, 434(2): 202-209 (doi: 10.1016/j.virol.2012.10.003).
31. Jinek M., Chylinski K., Fonfara I., Hauer M., Doudna J.A., Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, 2012, 337(6096): 816-821 (doi: 10.1126/science.1225829).
32. Wiedenheft B., Sternberg S.H., Doudna J.A. RNA-guided genetic silencing systems in bacteria and archaea. *Nature*, 2012, 482(7385): 331-338 (doi: 10.1038/nature10886).
33. Cox D.B.T., Platt R.J., Zhang F. Therapeutic genome editing: prospects and challenges. *Nature Medicine*, 2015, 21(2): 121-131 (doi: 10.1038/nm.3793).
34. Ramanan V., Shlomai A., Cox D.B.T., Schwartz R.E., Michailidis E., Bhatta A., Scott D.A., Zhang F., Rice C.M., Bhatia S.N. CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. *Scientific Reports*, 2015, 5: 10833 (doi: 10.1038/srep10833).
35. Véron N., Qu Z., Kipen P.A.S., Hirst C.E., Marcelle C. CRISPR mediated somatic cell genome engineering in the chicken. *Developmental Biology*, 2015, 407(1): 68-74 (doi: 10.1016/j.ydbio.2015.08.007).
36. Abu-Bonsrah K.D., Zhang D., Newgreen D.F. CRISPR/Cas9 targets chicken embryonic somatic cells in vitro and in vivo and generates phenotypic abnormalities. *Scientific Reports*, 2016, 6: 34524 (doi: 10.1038/srep34524).
37. Cheng Y., Lun M., Liu Y., Wang H., Yan Y., Sun J. CRISPR/Cas9-mediated chicken TBK1 gene knockout and its essential role in STING-mediated IFN-beta induction in chicken cells. *Frontiers in Immunology*, 2018, 9: 3010 (doi: 10.3389/fimmu.2018.03010).
38. Tang N., Zhang Y., Pedrera M., Chang P., Baigent S., Moffat K., Shen Z., Nair V., Yao Y. A simple and rapid approach to develop recombinant avian herpesvirus vectored vaccines using CRISPR/Cas9 system. *Vaccine*, 2018, 36(5): 716-722 (doi: 10.1016/j.vaccine.2017.12.025).
39. Williams R.M., Senanayake U., Artibani M., Taylor G., Wells D., Ahmed A.A., Sauka-Spengler T. Genome and epigenome engineering CRISPR toolkit for in vivo modulation of cis-regulatory interactions and gene expression in the chicken embryo. *Development*, 2018, 145(4): dev160333 (doi: 10.1242/dev.160333).
40. Gandhi S., Piacentino M.L., Vieceli F.M., Bronner M.E. Optimization of CRISPR/Cas9 genome editing for loss-of-function in the early chick embryo. *Developmental Biology*, 2017, 432(1): 86-97 (doi: 10.1016/j.ydbio.2017.08.036).
41. Morin V., Véron N., Marcelle C. CRISPR/Cas9 in the chicken embryo. In: *Avian and reptilian developmental biology. Methods in molecular biology, vol. 1650.* G. Sheng (ed.). Humana Press, NY, 2017: 113-123 (doi: 10.1007/978-1-4939-7216-6_7).
42. Hirst C.E., Serralbo O., Ayers K.L., Roeszler K.N., Smith C.A. Genetic manipulation of the avian urogenital system using in ovo electroporation. In: *Avian and reptilian developmental biology. Methods in molecular biology, vol. 1650.* G. Sheng (ed.). Humana Press, NY, 2017: 177-190 (doi: 10.1007/978-1-4939-7216-6_11).
43. Petitte J.N., Clark M.E., Liu G., Verrinder Gibbins A.M., Etches R.J. Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. *Development*, 1990, 108(1): 185-189.
44. Han J.Y., Lee B.R. Isolation and characterization of chicken primordial germ cells and their application in transgenesis. In: *Avian and reptilian developmental biology. Methods in molecular biology, vol. 1650.* G. Sheng (ed.). Humana Press, NY, 2017: 229-242 (doi: 10.1007/978-1-4939-7216-6_15).
45. Volkova N.A., Bagirov V.A., Tomgorova E.K., Vetokh A.N., Volkova L.A., Zinov'eva N.A. Isolation, cultivation and characterization of quail primordial germ cells. *Agricultural Biology* [*Sel'skokhozyaistvennaya biologiya*], 2017, 52(2): 261-267 (doi: 10.15389/agrobiology.2017.2.261eng).

46. Choi H.J., Lee H.C., Kang K.S., Lee H.G., Ono T., Nagai H., Sheng G., Han J.Y. Production of interspecific germline chimeras via embryo replacement. *Biology of Reproduction*, 2015, 93(2): Article 36 (doi: 10.1095/biolreprod.114.127365).
47. Haraguchi S., Matsubara Y., Hosoe M. Chick embryos can form teratomas from microinjected mouse embryonic stem cells. *Develop. Growth Differ.*, 2016, 58(2): 194-204 (doi: 10.1111/dgd.12260).
48. Brazolot C.L., Petitte J.N., Etches R.J., Verrinder Gibbins A.M. Efficient transfection of chicken cells by lipofection, and introduction of transfected blastodermal cells into the embryo. *Molecular Reproduction and Development*, 1991, 30(4): 304-312 (doi: 10.1002/mrd.1080300404).
49. Petitte J.N., Karagenc L., Ginsburg M. The origin of the avian germ line and transgenesis in birds. *Poultry Science*, 1997, 76(8): 1084-1092 (doi: 10.1093/ps/76.8.1084).
50. Etches R.J. Transgenic chickens. *Proc. 10th European poultry conference* «*The poultry industry towards the 21st century*». Jerusalem, Israel, 1998: 3-6.
51. Savva D., Page N., Vick L., Simkiss K. Detection of foreign DNA in transgenic chicken embryos using the polymerase chain reaction. *Research in Veterinary Science*, 1991, 50(2): 131-133 (doi: 10.1016/0034-5288(91)90094-5).
52. Petitte J.N., Liu G., Yang Z. Avian pluripotent stem cells. *Mechanisms of Development*, 2004, 121(9): 1159-1168 (doi: 10.1016/j.mod.2004.05.003).
53. Song Y., Duraisamy S., Ali J., Kizhakkayil J., Jacob V.D., Mohammed M.A., Eltigani M.A., Amisetty S., Shukla M.K., Etches R.J., de Lavoir M.C. Characteristics of long-term cultures of avian primordial germ cells and gonocytes. *Biology of Reproduction*, 2014, 90(1): Article 15 (doi: 10.1095/biolreprod.113.113381).
54. Leighton P.A., van de Lavoir M.C., Diamond J.H., Xia C., Etches R.J. Genetic modification of primordial germ cells by gene trapping, gene targeting, and phiC31 integrase. *Molecular Reproduction and Development*, 2008, 75(7): 1163-1175 (doi: 10.1002/mrd.20859).
55. Motono M., Yamada Y., Hattori Y., Nakagawa R., Nishijima K., Iijima S. Production of transgenic chickens from purified primordial germ cells infected with a lentiviral vector. *Journal of Bioscience and Bioengineering*, 2010, 109(4): 315-321 (doi: 10.1016/j.jbiosc.2009.10.007).
56. Nakamura Y., Usui F., Ono T., Takeda K., Nirasawa K., Kagami H., Tagami T. Germline replacement by transfer of primordial germ cells into partially sterilized embryos in the chicken. *Biology of Reproduction*, 2010, 83(1): 130-137 (doi: 10.1095/biolreprod.110.083923).
57. Park K.J., Kang S.J., Kim T.M., Lee Y.M., Lee H.C., Song G., Han J.Y. Gamma-irradiation depletes endogenous germ cells and increases donor cell distribution in chimeric chickens. *In Vitro Cell. Dev. Biol.-Animal*, 2010, 46(10): 828-833 (doi: 10.1007/s11626-010-9361-8).
58. Nakamura Y., Usui F., Miyahara D., Mori T., Ono T., Kagami H., Takeda K., Nirasawa K., Tagami T. X-irradiation removes endogenous primordial germ cells (PGCs) and increases germline transmission of donor PGCs in chimeric chickens. *Journal of Reproduction and Development*, 2012, 58(4): 432-437 (doi: 10.1262/jrd.2012-045).
59. Song Y., D'Costa S., Pardue S.L., Petitte J.N. Production of germline chimeric chickens following the administration of a busulfan emulsion. *Molecular Reproduction and Development*, 2005, 70(4): 438-444 (doi: 10.1002/mrd.20218).
60. Nakamura Y., Yamamoto Y., Usui F., Atsumi Y., Ito Y., Ono T., Takeda K., Nirasawa K., Kagami H., Tagami T. Increased proportion of donor primordial germ cells in chimeric gonads by sterilisation of recipient embryos using busulfan sustained-release emulsion in chickens. *Reproduction, Fertility, and Development*, 2008, 20(8): 900-907 (doi: 10.1071/rd08138).
61. Mucksová J., Reinišová M., Kalina J., Lejčková B., Hejnar J., Trefil P. Conservation of chicken male germline by orthotopic transplantation of primordial germ cells from genetically distant donorsdagger. *Biology of Reproduction*, 2019, 101(1): 200-207 (doi: 10.1093/biolre/ioz064).
62. Harel-Markowitz E., Gurevich M., Shore L.S., Katz A., Stram Y., Shemesh M. Use of sperm plasmid DNA lipofection combined with REMI (restriction enzyme-mediated insertion) for production of transgenic chickens expressing eGFP (enhanced green fluorescent protein) or human follicle-stimulating hormone. *Biology of Reproduction*, 2009, 80(5): 1046-1052 (doi: 10.1095/biolreprod.108.070375).
63. Samoilov A.V., Kesyan A.Z., Suraeva N.M. *Izvestiya Rossiiskoi akademii nauk. Seriya biologicheskaya*, 2013, 5: 517-521 (doi: 10.7868/S0002332913040140) (in Russ.).
64. Korshunova L.G., Karapetyan R.V., Ziadinova O.F. *Ptitsevodstvo*, 2017, 4: 22-25 (in Russ.).
65. Chaparian S., Abdulahnejad A., Rashidi F., Toghyani M., Gheisari A., Eghbalsaied S. Is passive transmission of non-viral vectors through artificial insemination of sperm-DNA mixtures sufficient for chicken transgenesis? *Journal of Reproduction and Development*, 2016, 62(3): 265-270 (doi: 10.1262/jrd.2015-176).
66. Wang L., Li J. “Artificial spermatid”-mediated genome editing. *Biology of Reproduction*, 2019, 101(3): 538-548 (doi: 10.1093/biolre/ioz087).
67. Cooper C.A., Challagulla A., Jenkins K.A., Wise T.G., O'Neil T.E., Morris K.R., Tizard M.L., Doran T.J. Generation of gene edited birds in one generation using sperm transfection assisted gene editing (STAGE). *Transgenic Research*, 2017, 26(3): 331-347 (doi: 10.1007/s11248-016-0003-0).

68. Sang H.M., Perry M.M., Gribbin C., Mather C., Morrice D., Love J. Chick embryo culture and gene transfer. In: *AFRC Institute of Animal Physiology and Genetics Research. Report for 1990-1991.* R.B. Heap, H.D. Griffin, G. Leng et al. (eds.). Agricultural and Food Research Council, Cambridge, 1992: 52-53.
69. Andacht T., Hu W., Ivarie R. Rapid and improved method for windowing eggs accessing the stage X chicken embryo. *Molecular Reproduction and Development*, 2004, 69(1): 31-34 (doi: 10.1002/mrd.20155).
70. Mather C. Transgenic chicken by DNA microinjection. *Poultry International*, 1994, 33(6): 16-18.
71. Ellendorff F., Gulyas N., Muhlbauer E., Klein S. Potential use of molecular sex recognition in layer birds. *Proc. XX World's Poultry Congress.* New Delhi, India, 1996: 3-4.
72. Ernst L.K., Fisinin V.I., Zhuravlev I.V., Karapetyan R.V., Matveenko N.P., Ziadinova O.F., Kudryavtsev I.V., Kuzin B.A., Enikolopov G.N. *Sposob transplantatsii kurinoi yaitsekletki. Pat. 1565025 SU. № 4396482. Zayavl. 23.03.88. Opubl. 15.01.90. Byul. № 1* [Method for chicken egg transplantation. Patent 1565025 SU. № 4396482. Claims 23.03.88. Publ. 15.01.90. Bul. № 1] (in Russ.).
73. Karapetyan R.V. *Doklady RASKHN*, 1995, 4: 27-29 (in Russ.).
74. Karapetyan R.V. *Doklady RASKHN*, 1996, 2: 19-20 (in Russ.).
75. Fisinin V.I., Ernst L.K., Karapetyan R.V., Matveenko N.P., Zazykina T.V., Ziadinova O.F., Zhadanov A.B. *Sposob povysheniya yaichnoi produktivnosti ptitsy. Pat. 2061366 RU. № 93019970/15. Zayavl. 19.04.93. Opubl. 10.06.96. Byul. № 16* [Method for increase egg productivity of poultry. Patent 2061366 RU. № 93019970/15. Claims 19.04.93. Publ. 10.06.96. Bul. № 16] (in Russ.).
76. Korshunova L.G., Karapetyan R.V., Fisinin V.I. Methods for genetic modification in poultry (review). *Agricultural Biology* [*Sel'skokhozyaistvennaya biologiya*], 2013, 6: 3-15 (in Russ.).
77. Korshunova L.G., Fisinin V.I., Karapetyan R.V. *Ptitsa i ptitseprodukty*, 2015, 2: 47-49 (in Russ.).
78. Korshunova L.G., Karapetyan R.V., Ziadinova O.F. Modification of chicken genome by interferon gene. *Russian Agricultural Sciences*, 2014, 40(5): 379-381 (doi: 10.3103/s1068367414050140).
79. Korshunova L.G., Karapetyan R.V. *Veterinariya*, 2009, 5: 15-16 (in Russ.).
80. Korshunova L.G. Biological and productive properties of quails transgenic on bovine somatotropin gene. *Agricultural Biology* [*Sel'skokhozyaistvennaya biologiya*], 2011, 2: 46-50 (in Engl.).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1095-1109
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.52/.58:637.4:636.083:57.04

doi: 10.15389/agrobiology.2019.6.1095eng
doi: 10.15389/agrobiology.2019.6.1095rus

THE EFFECTS OF LIGHTING REGIMES ON THE OVIPOSITION TIME AND EGG QUALITY IN LAYING HENS

(review)

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The authors declare no conflict of interests
Received October 8, 2019

Abstract

Oviposition is a complex process where oviposition time (OT), clutch length (CL), and interval length (IL) are interrelated (B.G. Roy et al., 2014). The review presented highlights the effects of lighting regimes on the oviposition time in laying hens (*Gallus gallus domsticus* L.) in the relation with egg productivity and quality. OT is directly related to the ovulation time which, in turn, depends on the time of peak circulatory concentration of the luteinizing hormone (LG) released by the anterior pituitary (S.C. Wilson et al., 1984). In standard 24-hour light-dark cycles with single dark-to-light switch oviposition occurs predominantly during the light phase (F. Noddegaard, 1998; G.A. Kirdyashkina et al., 2009) while in conditions of intermittent asymmetric lighting regimes it occurs during the «subjective day» period (P.D. Lewis et al., 1990; A.Sh. Kavtarashvili et al., 2002; A.Sh. Kavtarashvili, 2007). The most of daily laid eggs in a flock are laid during ca. 5-6 hours after the switch-on (A.H. Zakaria, 2005), corresponding to average OT 13-15 hours after the switch-off (K. Lillpers, 1991; P.H. Patterson, 1997; R.J. Etches, 1990; A.Sh. Kavtarashvili et al., 2019). Average OT is determined by complex interaction of «dawn» and «dusk» signals, the latter being the most influential in this case (S.S. Liou et al., 1987; B.M. Bhati et al., 1988). In 24-30-hour light-dark cycles every 1 hour of the cycle length with the same length of the light phase decreases average OT by 1.89-1.90 hours. The increase in the length of the light phase within given light-dark cycle by 1 hour shifts average OT by 0.26-0.27 hours toward the «dusk» point. OT is known to affect egg quality. E.g. the weight of eggs laid in the morning is higher in compare to the eggs laid later (E. Tůmová et al., 2010; M. Akif Boz et al., 2014; S. Samiullah et al., 2016; A.J. Kryeziu et al., 2011). Some researchers (R.H. Harms, 1991; E. Tůmová et al., 2009) reported that egg weight, eggshell thickness and strength were higher in the eggs laid in the morning while in other studies (E. Tůmová et al., 2005, 2007; A.J. Kryeziu et al., 2011; C. Hrnčár et al., 2013) these parameters of egg quality were better in the eggs laid in the midday. The eggs laid in the morning were reported to have more intense brown eggshell pigmentation as compared to eggs laid in the midday (S. Samiullah et al., 2016; A.J. Kryeziu et al., 2011) and higher calcium content in the eggshell (E. Tůmová et al., 2014), 22.8 % lower content of cholesterol (E. Tůmová et al., 2008; M.A. Abdalla et al., 2018), as well as lower phosphorus and magnesium levels (E. Tůmová et al., 2014). Hens that laid eggs preferably in the morning were reported to have longer CL and higher egg production in compare to hens which preferably lay eggs later. The heritability coefficient (h^2) of OT varies from 0.38 to 0.78 (K. Lillpers, 1991). The positive correlation ($r = 0.54$) between the CL and egg production was also reported (M. Bednarczyk et al., 2000; P. Miandmients et al., 1993). In view of the above mentioned average OT and CL can be reasonably included as the criteria into the selection programs for laying hens and broiler parental hens. In addition, the optimization of OT can improve egg quality and facilitate the rational organization of egg collection in the farms. The studies on the effects of lighting regimes on OT and egg quality were performed primarily in conditions of constant lighting schemes. The further research is necessary for the intermittent lighting regimes, on individually caged hens (with the recording of egg position within the clutches) and on hens kept in group cages.

Keywords: *Gallus gallus domesticus*, laying hens, lighting regime, oviposition time, egg quality

The introduction of windowless poultry houses in intensive poultry practice has significantly increased the role of lighting in the egg production process and expanded the possibilities of developing and applying lighting modes to regulate poultry puberty, the daily rhythm of egg laying and egg quality, and increase hens' productivity. This review is focused on assessing the effect of lighting on egg oviposition time (OT) and its relation to egg quality and hens' performance.

The mechanism of egg growth, development and ovulation, egg formation and laying. Hens of modern egg crosses can produce more than 330-340 eggs per year [1, 2]. In the ovary of a 1-day-old chick, 3,500-12,000 follicles are present, that is, significantly more than a hen lays during its lifetime [2-4]. In terms of structure, the follicle is an ovum with a shell without a yolk. They gradually increase with the onset of puberty [5]. Ovum growth, maturation and ovulation, as well as egg formation and oviposition, are determined by the chicken genome and depend on the coordinated action of the hormones of the hypothalamus, pituitary and ovary in interaction with environmental conditions [2, 6]. Follicular development in the reproductive process of laying hens includes two main groups of follicles. The first group is pre-hierarchical, which includes small white (less than 2 mm), large white (2-4 mm) and small yellow follicles (4-8 mm). The second group is hierarchical, formed by 5-7 large yellow rapidly growing follicles (9-35 mm), which reach the preovulatory size in 7-10 days [7]. The fewer large follicles in the hierarchical group, the faster the yolk accumulates in them and the egg matures [2]. Each of the follicles belonging to the hierarchical group is identified by a decreasing figure depending on its size (the largest follicle is F1, the second is F2, the third is F3, etc.). Once a follicle falls into a hierarchical category, it cannot suffer from atresia [8]. Each time the largest follicle ovulates and a new one is recruited from pre-hierarchical small yellow follicles to enter the hierarchy [6].

After ovulation, the ovum-yolk, secreted from the largest follicle, is captured by the funnel of the oviduct and, with further advancement through it, is subjected to successive deposits of the remaining components (egg white and shell) of the egg [9]. The duration of egg formation in the oviduct is usually 22.5-26.2 hours, including in the funnel — 20-30 minutes, the magnum — 2-3.2 hours, the isthmus — 1-1.3 hours, the uterus — 16-21 hours (average — 18-19 hours) [2, 4, 10-12]. Egg white is formed during 3.25-3.5 hours [3, 13]. The longest stage of egg formation is the formation of an egg shell, which takes up to 19-20 hours. It begins 4.5-5 hours after ovulation and ends 1.5 hours before the egg is laid. The end result is a complete egg [3, 13]. After the egg is laid, another ovulation occurs after 15-45 minutes. In highly productive hens, the ovulation cycle (the period between two consecutive ovulations) lasts almost 24 hours, while in low-productivity poultry, it can take 24-27 hours [9, 14]. The shorter the average interval, the longer the oviposition cycle (OC) and the higher the egg production capacity of the hen (15).

Four-six hours before ovulation, the content of luteinizing hormone (LH, the hormone of the anterior pituitary gland) increases sharply in the blood, which, in turn, stimulates the secretion of progesterone (the hormone of the preovulatory follicle). LH regulates the breakdown of connective tissues in the stigma of the follicle, the rupture of the follicle wall and the output of the ovum [13]. Croze et al. [16] suggested that the preovulatory release of testosterone exerts a preparatory effect on the hypothalamic-pituitary-ovarian system and thereby facilitates the preovulatory release of LH. Later studies showed that blocking the action of testosterone by its specific antagonist flutamide led to a stop of the preovulatory burst of plasma testosterone, progesterone, estradiol, LH and, therefore, the predicted ovum in laying hens [17].

Oviposition is a complex process in which OT (the flock average time of

the day when chickens lay eggs), the OC (the period during which the birds lay eggs daily) and the oviposition interval (OI, the period between two consecutive OCs) are interconnected [18]. It is known that OT depends on the genotype, keeping system, age [18], and the time of feeding the hen [19, 20], the lighting mode [21], and the temperature in the poultry house [22]. According to the literature, the most significant effect on the OT in hens is provided by lighting.

OT is directly related to ovulation time, which, in turn, depends on the release of LH by the anterior pituitary gland [23]. The pre-ovulatory LH release in laying hens lasts about 6 hours [17, 24, 25]. It was previously believed that LH secretion was subordinate to the circadian rhythm, starting from the moment the light is switched off ("sunset point"), and lasts 8-10 hours ("open period") [25-28]. This concept was revised in 2007 when Nakao et al. [29] showed follicles in quails close to ovulation had "clock genes" associated with the expression of the steroidogenic acute regulatory protein StAR, which is critical for the transport of cholesterol to the inner mitochondrial membrane and is a limiting factor for the start of progesterone synthesis (P_4) [30]. That is, the expression of *StAR* in the F_1 follicle changes with a frequency of 24 hours along with the expression of the clock gene *Per2*. In addition, sections for binding to the *CLOCK/BMAL1* genes that initiate transcription were found in the *StAR* gene. Consequently, the time of follicles ovulation in the ovary of birds is controlled by a circadian rhythm regulated by clock genes. The latter enhance the genetic expression of *StAR* in the F_1 follicle and provide an increase in the concentration of progesterone in the blood plasma sufficient to trigger pre-ovulatory LH release [31]. In addition, LH is involved in the regulation of *CLOCK/BMAL1* gene activity and stimulates increased expression of the *StAR* gene, which increases the secretion of progesterone and accelerates the process of pre-ovulatory LH release even more. The synthesis of progesterone is a key event that determines the time of LH secretion and, as a result, the duration of the open period due to the positive feedback regulatory relationship between these two hormones [32].

The main function of LH release is to initiate ovulation [14, 23]; therefore, the absence of a peak in the hormone concentration blocks ovulation in laying hens [17]. In addition, LH is involved in the process of follicular maturation [33] and steroidogenesis in small and large follicles [34, 35]. It is shown that the injection of exogenous LH stimulates the secretion of P_4 by a mature follicle before ovulation and the secretion of estradiol (E_2) by the F_3 follicle [36]. LH receptors (LH-R) are present in granular follicular cells in the hierarchy (from 9 mm in diameter), and LH-R mRNA expression increases as the follicle matures [37, 38].

In the 16L:8D mode (16 hours of light, 8 hours of darkness), the beginning of the increase in the preovulatory LH concentration in blood in hens occurs at a time approximately corresponding to the "sunset point", whereas in laying hens kept under the 8L:16D mode, it starts 3-4 hours later [23, 39]. The interval between the achievement of a peak concentration of LH in the blood and ovulation is usually about 5 hours and does not depend on the egg production capacity, the position of the egg in the OC or the mode of lighting of the laying hens [40-42].

The egg formation time in the oviduct varies more. It depends on the intensity of egg laying by the hen and the position of the egg in the OC. It was reported that the egg formation time increases by about 1 h when the laying hens are kept under light-dark cycles 27 hours or more in length [43]. The total interval between the achievement of a peak concentration of LH in the blood and the laying of a fully formed egg can vary from 29 to 31 hours [44].

The effect of lighting conditions on the OT. With the usual duration (24 h) of light-dark cycles, egg laying and hence ovulation occur

during the 8-hour “open period” [14, 28, 45, 46]. Observations showed that in standard light-dark cycles with a single alternation of light (L) and darkness (D) (for example, 16L:8D, 14L:10D), egg laying occurred mainly during the light period [47-49], and under intermittent asymmetric lighting modes (for example, 2L:4D:8L:10D, 1L:5D:3L:4D:3L:8D, 1L:4D:4L:1D:4L:10D) – during the “subjective day” (the period that the bird perceives as daylight hours) [50-53]. In 28-hour light-dark cycles (for example, 12L:16D), 84.5% of the eggs from the daily output of hens are laid during the last 9 hours of the dark period [48].

According to Gumulka et al. [54], the highly productive meat chickens of the Arbor Acres cross from 168 to 448 days old under 16L:8D mode (photoperiod from 5^{00} to 21^{00}) laid the first egg in the OC approximately 3.5 hours after the light was switched on. With an increase in the duration of the OC, the first egg was laid earlier, and the OI decreased. In the study of laying hens of the high and low line of the White Leghorn breed under 12L:12D lighting conditions (photoperiod from 6^{00} to 18^{00}) from 270 to 360 days old, the first egg in a cycle of 3, 4, 7, and 10 eggs was laid, respectively after 1 h 35 min and 1 h 51 min; 1 h 25 min and 56 min; 1 h 06 min and 30 min; 34 and 30 min after switching on the light [55].

It was found that the time of laying the first egg depended on the duration of the OC if it consisted of 2-5 eggs: the longer the cycle, the earlier the laying of the first egg occurred after the light was turned on. With a longer cycle, this pattern is violated [14].

With group keeping of hens against intermittent lighting, the time of the beginning of oviposition depends on the length of the “subjective day” [56]. So, with its duration of 16, 15, 14 and 13 hours/day (the beginning of the “subjective day” is at 2^{00} in all groups), egg laying in the herd began at night: at 4^{00}, 3^{00}, 2^{00} and 1^{00}, respectively.

There is evidence that the majority of eggs from the daily output are laid approximately 5-6 hours after the light is switched on [57], which corresponds to an average OT of 13-14 hours after dark [45, 58]. Similar results were obtained by Roy *et al.* [18], who report that under the 16L:8D mode (photoperiod from 6^{00} to 22^{00}), the average OT was ~ 10^{59}, i.e., it occurred 5 hours after the light was switched on (or 13 hours after the beginning of the dark period).

With the hens' age, the average OT increases. Thus, under the same lighting mode, young (33 weeks) hens of egg crosses laid 50% of the eggs from daily output 13 hours after switching off the light, and laying hens of older age (76 weeks) – 30-60 minutes later [59]. Similar results were obtained on hens of meat cross: the young herd (34 weeks) laid the majority of eggs between 7^{00} and 13^{00}, while the older herd (59 weeks) – between 7^{00} and 15^{00} [57].

When keeping hens in conditions of round-the-clock lighting or round-the-clock darkness [60], as well as intermittent lighting of a symmetrical type with short cycles, for example 3L:3D and 4L:4D [61, 62], oviposition continued for 24 hours a day. However, in the 2L:10D:2L:10D mode, the main number of eggs was laid in the first 10-hour dark phase, more precisely, in the first hour of this phase [63].

According to Patterson [58], the oviposition of hens of high-yielding crosses with 24-hour light-dark cycles rarely occurs in the dark. As a rule, under standard lighting conditions (for example, 14L:10D), eggs are laid between 7^{30} and 8^{15} and between 15^{30} and 16^{00} [64]. According to other sources, the largest proportion of eggs is laid between 10^{00} and 12^{00} [65], 10^{00} and 14^{00} [66] or in the early morning hours of the light phase under the 14L:10D and 17L:7D modes [67]. It is also known that under many non-standard lighting conditions, laying hens also lay eggs in the dark. It was shown that under the 14L:7D mode,

chickens laid many eggs immediately after switching off the light, and in the 14L:14D mode, hens laid during the dark phase [68].

Lewis *et al.* [69] studied the OT of hybrid laying hens with brown and white shells with a photoperiod of 8, 10, 13, and 18 hours. It was found that the average OT in both crosses increased relative to the "sunset point" by approximately 0.5 hours for every 1 hour of photoperiod extension. Moreover, the average OT in brown cross was 1.2-1.4 hours less than in white under each lighting mode. Similar results were obtained in Backhouse [28]. An increase in the duration of daylight hours from 10 to 14 hours in 24-hour light-dark cycles led to a shift in the average time for laying an egg to "sunset" by 0.5 hours for every 1 hour of increase in photoperiod, while with daylight hours of 14 and 16 hours it was almost the same. The herd OT of 50% of the eggs from the daily output was also shifted to "sunset" by 0.5 hours for each additional hour of the photoperiod, and this indicator continued to shift after 14-hour daylight. With daylight shorter than 12.25 hours, the number of eggs laid before "dawn" (switching on the light) increased by 4.5% for every 1-hour reduction in the duration of the photoperiod.

Lewis et al. [70] studied lighting modes with additional periods of dim light before and after the usual 8-hour photoperiod in comparison with the 16-hour photoperiod. With an 8-hour photoperiod and additional dim light, the OT was the same, but with a 16-hour photoperiod, it decreased by 3 hours.

Tůmová *et al.* [71] found that under floor-standing conditions, the time for laying the bulk of the eggs shifted closer to the middle of the day (by 10^{00}) compared with that for the cage keeping system (when the bird laid most of the eggs at about 6^{00}) under the same lighting conditions.

In the authors' studies [56], with the duration of the "subjective day" of 16, 15, 14, and 13 hours/day and the simultaneous switching on of light at 2^{00} a.m. against intermittent lighting (1L:6D:4L:2D:3L:8D, 1L:5D:4L:2D:3L:9D, 1L:4D:4L:2D:3L:10D and 1L:3D:4L:2D:3L:11D) the average OT was 8^{40}, 7^{22}, 5^{22} and 5^{16} (or 14 hours 40 minutes, 14 hours 22 minutes, 13 hours 22 minutes and 14 hours 16 minutes after the onset of the LONGEST dark period – "subjective night", that is, "sunset point").

The role of the light switching on and off time ("sunrise" and "sunset") in the rhythm of oviposition has been described in many papers. Naito *et al.* [72] note that "sunrise" and "sunset" have approximately the same effect on setting the OT. According to many authors [26, 44, 73-75], the sunset time is more important, regardless of the length of the light period. So, when keeping laying hens of the White Leghorn breed under continuous lighting 16L:8D or 20L:4D, the shift in the time for switching off the light had a stronger (response – 74%) effect on the OT than the shift in time for switching on the light (response – 35-38%) [73]. With a simultaneous shift in the time for switching the light on and off, the OT response was 94%. Oviposition time shifted in the same direction in which the dark phase was shifted.

With a longer dark period, the average OT shifted backward, counting from the "sunset point": under the 20L:4D mode, it was 9^{19}-9^{35}, under the 16L:8D – 10^{30}-10^{59}. These data indicate that during 24-hour light-dark cycles, the OT is influenced not only by the time of "sunrise" and "sunset" but also by the length and position of the dark phase. It should be noted that if the "sunset" time is shifted forward or backward at a constant time of switching on the light, then the time shift of oviposition will not be equal to the "sunset" time shift [76]. The paper of Wilson *et al.* [23] shows that when the hens were switched from the 16L:8D light mode ("sunrise" at 24^{00}) to 20L:4D ("sunrise" at 20^{00}), the backward shift of the average OT was only 30 minutes (5^{00} against 5^{30}). However, with the same 20L:4D scheme ("sunset" at 20^{00}), but with a forward shift of "sunset"

time by the same 4 hours, the average egg-laying time was 8^{38}, that is, it moved forward by 3 hours 8 minutes.

According to some authors [73, 77], the average OT under standard light-dark cycles is determined primarily by the "sunset" time, although "sunrise" also has a certain effect. It can be assumed that the OT in hens is based on the complex interaction between these two signals. According to Etches [68], with a standard 14L:10D cycle, the average OT is about 15 hours after "sunset". However, the extension of the dark phase to 18 hours or the reduction to 6 hours led to a shift in the average OT by 4 hours forward or 3 hours back.

The experiment of Tůmová et al. [78] showed that when the light was switched on at 3^{00}, the majority of the eggs of the hen were laid at 6^{00}, and then the number of laid eggs was evenly reduced during the rest of the day. Switching on the light at 6^{00} under the floor-standing keeping of laying hens led to the same uniform decrease in the percentage of eggs laid. These results indicate that the uniformity of egg laying during the day depends on the time the light is switched on, and also, most probably, on the keeping system.

Bhatti et al. [44] after analyzing the results of numerous studies with the duration of light-dark cycles from 24 to 30 hours and the actual dark phase from 5 to 23 hours, proposed an equation that allowed estimating the average laying time (ALT, h) of eggs from the "sunset point" at these cycles:

$$ATL = 16.619 - 2(C - 24) - 0.161C + 0.268D,$$

where C is the duration of the cycle, h; D is the duration of the dark phase, h, It is noted that in cycles longer than 24 h, a change in the duration of the dark phase has a more pronounced effect on the egg-laying time than in 24-hour cycles [79].

Average egg laying time (ALT, h) **by hens (*Gallus gallus domesticus*) depending with light-dark cycles**

Cycle time, h	Lighting mode	ALT from the "sunset" point	ALT reduction with an 1 h increase in cycle due to the dark period	ALT shift to the "sunset" point with an increase in the dark phase by 1 h inside the cycle
24	16L:8D	14.90		
	15L:9D	15.17		0.27
	14L:10D	15.44		0.27
	13L:11D	15.70		0.26
25	16L:9D	13.00	1.90	
	15L:10D	13.27	1.90	0.27
	14L:11D	13.54	1.90	0.27
	13L:12D	13.81	1.89	0.27
26	16L:10D	11.11	1.89	
	15L:11D	11.38	1.89	0.27
	14L:12D	11.65	1.89	0.27
	13L:13D	11.92	1.89	0.27
27	16L:11D	9.22	1.89	
	15L:12D	9.49	1.89	0.27
	14L:13D	9.76	1.89	0.27
	13L:14D	10.02	1.90	0.26
28	16L:12D	7.33	1.89	-
	15L:13D	7.59	1.90	0.27
	14L:14D	7.86	1.90	0.27
	13L:15D	8.13	1.89	0.27
29	16L:13D	5.43	1.90	
	15L:14D	5.70	1.89	0.27
	14L:15D	5.97	1.89	0.27
	13L:16D	6.24	1.73	0.27
30	16L:14D	3.54	1.89	
	15L:15D	3.81	1.89	0.27
	14L:16D	4.08	1.89	0.27
	13L:17D	4.35	1.89	0.27

N o t e. L is light, D is darkness. The cycles were compared at the same photoperiod duration.

However, the authors' calculations (Table) performed using this equation showed that an increase in the duration of the cycle by 1 h for the same dura-

tion of the photoperiod reduced the average egg-laying time by 1.89-1.90 hours. An increase in the duration of the dark period by 1 h inside each cycle shifts the ALT of the eggs by 0.26-0.27 h towards the "sunset".

The aging of the hens' reproductive system is expressed in the lengthening of the intervals between ovulation and the eggs laying within one cycle of oviposition, as well as in a greater number of pauses between OCs, that is, the days when the chicken does not lay eggs at all. According to the assumption of Emmans et al. [80], the duration of egg formation increases with the hens' age, which leads to a decrease in the frequency of ovulation and egg laying. A decrease in the frequency of ovulation and a decrease in the duration of oviposition can also be associated with changes in the circadian rhythm, the process of maturation of the follicles, or with both of these factors. With age, hens not only narrow the amplitude of the circadian rhythm but also change the response of some circadian processes in the body to light stimulation. In general, in older laying hens, the OC is always shorter than in young ones [81].

Delays in egg laying can occur due to stresses caused by conditions of detention (especially with alternative containment systems), transplants, and contact with unfamiliar individuals, and removal of egg-laying nests usual for laying hens [82, 83]. The study of Mills et al. [84] shows that anxiety increases the interval between laying eggs in laying hens.

It has been established that the duration of illumination in the photosensitive phase has a significant effect on the circadian rhythm of oviposition (it begins 11 hours after the first switching on of the light and lasts for 5 hours) [53]. So, when illuminated during the photosensitive phase for 1, 3 and 5 hours under the conditions of 1L:5D:5L:4D:1L:8D, 1L:5D:3L:4D:3L:8D and 1L:5D:1L:4D:5L:8D modes oviposition began 2 hours after the first switching on of the light. In this case, 90.9; 85.7 and 80.5% of the daily eggs output were laid until 13^{00}, the egg mass was 61.9; 61.2 and 60.1 g, and the elastic deformation of the shell — 22.4; 22.6 and 22.5 microns, respectively.

Effect of OT on egg quality and hens' productivity. Numerous studies have established that OT affects egg quality and hens' productivity [85-87]. Thus, the mass of eggs laid early in the morning was higher than that of eggs laid during the day [82, 88-92]. The egg-laying time plays an important role in the formation of the quality of the shell, since the mass of the laid shell linearly depends on the time spent by the egg in the uterus of the oviduct [64, 93].

It is known that the egg-laying time has a significant effect on the shell mass: it is higher for eggs laid before 7^{45} than for eggs laid between 7^{45} and 11^{45} [94]. Then, the mass of the shell increases significantly until the laying time 12^{45} and remains high throughout the rest of the daylight hours, excluding the period between 14^{45} and 16^{45}. Pavlovski et al. [88] showed that eggs laid in a later period of the day had better shell quality characteristics than in the morning. According to Tůmová et al. [95], the shell mass decreased during daylight hours, and especially strongly in the Isa Brown cross (ISA-Brown, Hendrix Genetics, Netherlands): in this genotype, the average egg shell mass was 6.38 g at 6^{00} and 6.23 g at 14^{00}. In other studies [78, 96], shell mass was maximum in the middle of daylight hours (at 14^{00}). Based on this, the authors suggested that the mass of the shell tended to increase to the last egg in the clutch.

OT also affects the thickness of the shell. There is an opinion [97] that the best quality of the shell of eggs laid in the middle of the day is due to its greater thickness. This assumption is consistent with the data that the shell thickness in morning eggs is less than in midday eggs [78, 96]. At the same time, it has been reported that shell thickness in the morning hours (6^{00}) is significantly larger with a subsequent decrease in this indicator, which is probably due to

the genotype of the hens used in different experiments [71].

Hrnčár et al. [98] studied the influence of OT on egg quality indicators using laying hens of three breeds (Brown Leghorn, Oravka, Brahma) from 20 to 64 weeks of age. The hens were kept on a deep litter. Eggs were collected at 6^{00}, 10^{00} and 14^{00} – at the beginning, middle and end of the productive period. In chickens of the Brahma breed, the maximum egg weight (59.96 g) was noted at 6^{00}, and the relative weight (10.53%), strength (29.88 n/cm^2) and shell thickness (381.88 μm) were higher ($p \leq 0.05$) at 14^{00}. In brown leghorns, OT did not significantly affect the egg weight, specific weight, and yolk index. The largest ($p \leq 0.05$) shell thickness (398.84 μm) was noted at 6^{00}, and the maximum values of the egg white index (7.39%) and the number of Howe units (72.21) – at 14^{00}. In Oravka chickens, OT did not significantly affect the egg mass, the relative mass of egg white, yolk and shell, the white and yolk index, and Howe units. At the same time, a higher shell thickness (381.88 μm, $p \leq 0.05$) occurred at 14^{00}. In another experiment [92], when studying the effect of OT on the quality of eggs, the mass of eggs collected at 10^{00} and 14^{00} was 65.25 and 63.94 g; absolute and specific shell weight 7.78 and 7.64 g (11.93 and 11.98%); the egg white weight 40.91 and 39.94 g (62.65 and 62.42%); the yolk weight 16.56 and 16.35 g (25.42 and 25.60%); egg shape index 76.72 and 76.70%; Howe units 76.60 and 76.70; shell thickness 0.51 and 0.54 mm; shell color 12.18 and 12.16 points.

Tůmová et al. [99] report a significant effect of egg-laying time on the accumulation of mineral substances in the shell. So, in eggs laid at 7^{30}, the maximum calcium content was 352 g/kg, while at 15^{30} it was 342 g/kg. The content of phosphorus and magnesium in the shell increased with a shift in the time of egg laying to a later time: at 7^{30} these values were 1.20 and 3.56 g/kg and at 15^{30} 1.43 and 3.88 g/kg. Kebreab et al. [100] suggest that a higher calcium content in the shell of morning eggs is associated with increased deposition of calcium in the medullary bone in the dark phase of the day.

According to a number of authors [90, 92], OT affects the color of the brown shell. So, dark-colored eggs are laid in the morning, and lighter – later during daylight hours (after 10^{00}). The brown color intensity decreased with the age of the herd, and the position of the egg in the clutch had relatively little effect on the brown color of the eggshell [90]. In addition to the fact that the brown color of the eggshell plays a role in consumer preferences, the presence of the pigment responsible for this coloring positively correlates with the strength and thickness of the shell, as well as the hatchability of eggs [101-104]. Japanese scientists have shown that brown pigment has photodynamically independent antibacterial properties against certain gram-positive microorganisms, such as *Staphylococcus aureus* and *Bacillus cereus* [105].

It was established that the time of oviposition affected the accumulation of cholesterol in the egg. So, the content in the morning eggs is 22.8% less than in daytime ones, 12.53 against 16.23 mg/g of yolk, respectively. In addition, the content of total cholesterol in the yolk of the morning eggs is significantly lower (176.63 mg/egg) than in the daytime eggs (221.14 mg/egg) [93, 106].

Lillpers [45] reports that chickens characterized by early egg laying are more productive than those which lay at a later time. According to these data, the heritability (h^2) of the time of laying is in the range from 0.38 to 0.78. The genetic and phenotypic relationships between this and the classic features (egg production capacity, egg mass, etc.) are mostly positive. Later studies [11, 107] establish a positive relationship between the duration of the OC and egg production capacity: the correlation coefficient (r) between these indicators is 0.54. That is, a genetically determined increase in the average length of the OC will lead to a significant increase in the overall egg production capacity of hens.

Thus, lighting is one of the main exogenous factors that have a significant effect on the OT of hens. The average egg laying time is directly related to the ovulation period, which occurs 5 hours after the peak concentration of LH in the blood is reached and does not depend on the serial number of the egg in the laying cycle and egg production capacity of hens. In the absence of a peak concentration of this hormone, ovulation is blocked. At standard 24-hour light-dark cycles under the conditions of constant illumination (for example, 16L:8D, 14L:10D), eggs are laid mainly during the light period, and under the conditions of asymmetric intermittent lighting modes (for example, 2L:4D:8L:10D, 1L:4D:4L:2D:3L:10D) during the "subjective day". In the conditions of a round-the-clock photoperiod or constant darkness, as well as intermittent lighting of a symmetrical type (for example, 3L:3D, 4L:4D), hens lay eggs for 24 hours a day. The shorter the daylight hours or "subjective" day, the earlier the laying of eggs begins and the shorter the average egg-laying time in the herd. Laying of the bulk of eggs from daily output occurs within 5-6 hours after the start of daylight or "subjective" day, while the average egg-laying time is within 13-15 hours after dark. At 24-hour light-dark cycles, the average laying time is the result of a complex interaction of the times for switching the light on and off, the "sunrise" and "sunset" with the prevailing influence of the "sunset point". Under conditions of a single alternation of light and dark in cycles of 24-30 hours, an increase in the duration of the cycle by 1 hour with the same photoperiod reduces the average egg-laying time by 1.89-1.90 hours. An increase in the duration of the dark period by 1 h inside each cycle shifts the average OT by 0.26-0.27 h towards the "sunset point". The shorter the average egg-laying time, the longer the OC and the higher the productivity of the hens. The heritability of OT is 0.38-0.78, the correlation coefficient between the duration of the OC and egg production capacity is 0.54. Data on the effect of OT on egg quality are contradictory. Some authors note better indicators (weight, shell thickness, and strength) of eggs laid in the morning, others, on the contrary, in the middle of the day. In morning eggs, the color of the shell is more intense and the content of calcium in the shell is higher, while the content of phosphorus, magnesium and cholesterol in the yolk is lower (by 22.8%) than in eggs laid in the afternoon (after 12^{00}). These facts indicate the feasibility and prospect of including indicators of average OT and its cycle duration (OC) in the selection program for improving egg and meat crosses. In addition, the optimization of OT will streamline the collection of eggs and improve their quality in poultry farms. It should be noted that the study of the effect of lighting on the OT and the quality of eggs was carried out mainly under continuous lighting. In-depth studies under the conditions of intermittent daylight hours both with an individual (taking into account the serial number of eggs in the cycle) and group keeping of hens are necessary.

REFERENCES

1. Lukić M., Pavlovski Z., Škrbić Z. Adequate calcium nutrition and quality of egg shell and bones in layers: innovative approach. *Biotechnology in Animal Husbandry*, 2011, 27(3): 485-497 (doi: 10.2298/BAH1103485L).
2. Shtelle A.L. *Ptitsa i ptitseprodukty*, 2011, 6: 19-23 (in Russ.).
3. Nys Y., Guyot N. Egg formation and chemistry. In: *Improving the safety and quality of eggs and egg products. Food Science, Technology and Nutrition*. Y. Nys, M. Bain, F. Van Immerseel (eds.). Woodhead Publishing Limited, 2011: 83-132 (doi: 10.1533/9780857093912.2.83).
4. Sidorenko L.I., Shcherbatov V.I. *Biologiya kur* [Chick biology]. Krasnodar, 2016: 244 (in Russ.).
5. Johnson A.L., Woods D.C. Ovarian dynamics and follicle development. In: *Reproductive biology and phylogeny of birds*. B.G.M. Jamieson (ed.). Science Publishers, Enfield, USA, 2007, 6A: 243-277.
6. Rangel P.L., Gutierrez C.G. Reproduction in hens: is testosterone necessary for the ovulatory process? *General and Comparative Endocrinology*, 2014, 203: 250-261 (doi: 10.1016/j.ygcen.2014.03.040).

7. Johnson A.L. Regulation of follicle differentiation by gonadotropins and growth factor. *Poultry Science*, 1993, 72(5): 867-873 (doi: 10.3382/ps.0720867).
8. Johnson A.L. The avian ovarian hierarchy: a balance between follicle differentiation and atresia. *Avian and Poultry Biology Reviews*, 1996, 7(2-3): 99-110.
9. Sauveur B., de Reviers M. Qualite d'œufs. In: *Reproduction des volailles et production d'œufs*. INRA, Paris, 1988: 377-436.
10. Sheldon B.L., Yoo B.H., Podger R.N. Increasing egg yield under normal light cycles by selecting for short interval between eggs under continuous light. *Annales Agriculturae Fenniae*, 1984, 23(4): 216-225.
11. Bednarczyk M., Kiecłzewski K., Szwaczkowski, T. Genetic parameters of the traditional selection traits and some clutch traits in a commercial line of laying hens. *Archiv für Geflügelkunde*, 2000, 64(3): 129-133.
12. Chen C.F., Tixier-Boichard M. Correlated responses to long-term selection for clutch length in dwarf brown-egg layers carrying or not carrying the naked neck gene. *Poultry Science*, 2003, 82(5): 709-720 (doi: 10.1093/ps/82.5.709).
13. Bain M.M., Nys Y., Dunn I.C. Increasing persistency in lay and stabilising egg quality in longer laying cycles. What are the challenges? *British Poultry Science*, 2016, 57(3): 330-338 (doi: 10.1080/00071668.2016.1161727).
14. Etches R.J. *Reproduction in poultry*. CAB International, Wallingford, UK, 1996.
15. Icken W., Cavero D., Schmutz M., Thurner S., Wendl G., Preisinger R. Analysis of the time interval within laying sequences in a transponder nest. *World's Poultry Science Journal*, 2008, 64: 231-234.
16. Croze F. Etches R.J. The physiological significance of androgen-induced ovulation in the hen. *Journal of Endocrinology*, 1980, 84(1): 163-171 (doi: 10.1677/joe.0.0840163).
17. Rangel P.L., Sharp P.J., Gutierrez C.G. Testosterone antagonist (flutamide) blocks ovulation and preovulatory surges of progesterone, luteinizing hormone and oestradiol in laying hens. *Reproduction*, 2006, 131(6): 1109-1114 (doi: 10.1530/rep.1.01067).
18. Roy B.G., Kataria M.C., Roy U. Study of oviposition pattern and clutch traits in a White leghorn (WL) layer population. *IOSR Journal of Agriculture and Veterinary Science*, 2014, 7(1): 59-67 (doi: 10.9790/2380-07115967).
19. Backhouse D., Gous R.M. The effect of feeding time on shell quality and oviposition time in broiler breeders. *British Poultry Science*, 2005, 46(2): 255-259 (doi: 10.1080/00071660500066258).
20. Backhouse D., Gous R.M. Responses of adult broiler breeders to feeding time. *World's Poultry Science Journal*, 2006, 62(2): 269-281 (doi: 10.1079/WPS200596).
21. Lewis Dr P.D., Ciacciariello M., Ciccone N.A., Sharp P.J., Gous R.M. Lighting regimens and plasma LH and FSH in broiler breeders. *British Poultry Science*, 2005, 46(3): 349-353 (doi: 10.1080/00071660500098509).
22. Tůmová E., Gous R.M. Interaction between oviposition time, age, and environmental temperature and egg quality traits in laying hens and broiler breeders. *Czech Journal of Animal Science*, 2012, 57(12): 541-549 (doi: 10.17221/6411-CJAS).
23. Wilson S.C., Cunningham F.J. Endocrine control of ovulation cycle. In: *Reproductive biology of poultry*. F.J. Cunningham, P.E. Lake, D. Hewitt (eds.). British Poultry Science Ltd., Edinburgh, UK, 1984: 29-51.
24. Johnson A.L., van Tienhoven A. Hypothalamo-hypophyseal sensitivity to hormones in the hen. I. Plasma concentrations of LH, progesterone and testosterone in response to central injections of progesterone and R5020. *Biology of Reproduction*, 1980, 23(5): 910-917 (doi: 10.1095/biolreprod23.5.910).
25. Wilson S.C., Jennings R.C., Cunningham F.J. An investigation of diurnal and cyclic changes in the secretion of luteinizing hormone in the domestic hen. *Journal of Endocrinology*, 1983, 98(1): 137-145 (doi: 10.1677/joe.0.0980137).
26. Morris T.R. The effects of ahemeral light and dark cycles on egg production in the fowl. *Poultry Science*, 1973, 52(2): 423-445 (doi: 10.3382/ps.0520423).
27. Johnson P.A., van Tienhoven A. Investigations of the significance of the crepuscular LH peak in the ovulatory cycle of the hen (*Gallus domesticus*). *Journal of Endocrinology*, 1984, 100(3): 307-313 (doi: 10.1677/joe.0.1000307).
28. Backhouse D. *The effect of photoperiod and feeding time on broiler breeder eggshell quality and oviposition time*. University of KwaZulu-Natal, Pietermaritzburg, 2004: 74.
29. Nakao N., Yasuo S., Nishimura A., Yamamura T., Watanabe T., Anraku T., Okano T., Fukada Y., Sharp P.J., Ebihara S., Yoshimura T. Circadian clock gene regulation of steroidogenic acute regulatory protein gene expression in preovulatory ovarian follicles. *Endocrinology*, 2007, 148(7): 3031-3038 (doi: 10.1210/en.2007-0044).
30. Stocco D.M. StAR protein and the regulation of steroid hormone biosynthesis. *Annual Review of Physiology*, 2001, 63: 193-213 (doi: 10.1146/annurev.physiol.63.1.193).
31. Johnson A.L., Solovieva E.V., Bridgham J.T. Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development. *Biology of Reproduction*, 2002, 67(4): 1313-1320 (doi: 10.1095/biolreprod67.4.1313).

32. Wilson S.C., Sharp P.J. Effects of androgens, oestrogens and deoxycorticosterone acetate on plasma concentrations of luteinizing hormone in laying hens. *Journal of Endocrinology*, 1976, 69(1): 93-102 (doi: 10.1677/joe.0.0690093).
33. Cunningham F.J., Wilson S.C., Knight P.G., Gladwell R.T. Chicken ovulation cycle. *Journal of Experimental Zoology*, 1984, 232(3): 485-494 (doi: 10.1002/jez.1402320315).
34. Robinson F.E., Etches R.J. Ovarian steroidogenesis during follicular maturation in the domestic fowl (*Gallus domesticus*). *Biology of Reproduction*, 1986, 35(5): 1096-1105 (doi: 10.1095/biolreprod35.5.1096).
35. Robinson F.E., Etches R.J., Anderson-Langmuir C.E., Burke W.H., Cheng K.W., Cunningham F.J., Ishii S., Sharp P.J., Talbot R.T. Steroidogenic relationships of gonadotrophin hormones in the ovary of the hen (*Gallus domesticus*). *General and Comparative Endocrinology*, 1988, 69(3): 455-466 (doi: 10.1016/0016-6480(88)90038-X).
36. Imai K., Nalbandov A.V. Plasma and follicular steroid levels of laying hens after the administration of gonadotropins. *Biology of Reproduction*, 1978, 19(4): 779-784 (doi: 10.1095/biolreprod19.4.779).
37. Johnson A.L., Bridgham J.T., Wagner B. Characterization of a chicken luteinizing hormone receptor (cLH-R) complementary deoxyribonucleic acid, and expression of cLH-R messenger ribonucleic acid in the ovary. *Biology of Reproduction*, 1996, 55(2): 304-309 (doi: 10.1095/biolreprod55.2.304).
38. Yamamura N., Takeishi M., Goto H., Tagami M., Mizutani T., Miyamoto K., Doi O., Kamiyoshi M. Expression of messenger RNA for gonadotropin receptor in the granulosa layer during the ovulatory cycle of hens. *Comparative Biochemistry and Physiology Part A: Molecular & integrative physiology*, 2001, 129(2-3): 327-337 (doi: 10.1016/S1095-6433(00)00350-0).
39. Wilson S.C., Cunningham F.J. Effect of photoperiod on the concentrations of corticosterone and luteinizing hormone in the plasma of the domestic hen. *Journal of Endocrinology*, 1981, 91(1): 135-143 (doi: 10.1677/joe.0.0910135).
40. Etches R.J. Maturation of ovarian follicles. In: *Reproductive biology of poultry*. F.J. Cunningham, P.E. Lake, D. Hewitt (eds.). British Poultry Science Ltd., Edinburgh, UK, 1984: 51-73.
41. Gow C.B., Sharp P.J., Carter N.B., Scaramuzzi R.J., Sheldon B.L., Yoo B.H., Talbot R.T. Effects of selection for reduced oviposition interval on plasma concentrations of luteinizing hormone during the ovulatory cycle in hens on a 24-hr lighting cycle. *British Poultry Science*, 1985, 26(4): 441-451 (doi: 10.1080/00071668508416834).
42. Morris T.R., Melek O., Cunningham F.J. Luteinizing hormone concentrations in the plasma of laying hens exposed to a 27-hr cycle of light and darkness. *Journal of Reproduction and Fertility*, 1975, 42(2): 381-384 (doi: 10.1530/jrf.0.0420381).
43. Melek O., Morris T.R., Jennings R.C. The time factor in egg formation for hens exposed to ahemeral light-dark cycles. *British Poultry Science*, 1973, 14(5): 493-498 (doi: 10.1080/00071667308416056).
44. Bhatti B.M., Morris T.R. Model for the prediction of mean time of oviposition for hens kept in different light and dark cycles. *British Poultry Science*, 1988, 29(2): 205-213 (doi: 10.1080/00071668808417045).
45. Lillpers K. Genetic variation in the time of oviposition in the laying hen. *British Poultry Science*, 1991, 32(2): 303-312 (doi: 10.1080/00071669108417354).
46. Etches R.J. Physiology of reproduction: the female. In: *Poultry production*. P. Hunton (ed.). Elsevier, Amsterdam, the Netherlands, 1995: 221-241.
47. Cain J.R., Wilson W.O. The influence of specific environmental parameters on the circadian rhythm of chickens. *Poultry Science*, 1974, 53(4): 1438-1447 (doi: 10.3382/ps.0531438).
48. Noddegaard F. Oviposition patterns and plasma melatonin rhythms in response to manipulations of the light:dark cycle. *British Poultry Science*, 1998, 39(5): 653-661 (doi: 10.1080/00071669888539).
49. Kirdyashkina G.A., Mal'tsev A.B., Kavtarashvili A.Sh. *Materialy X Ukrainskoi konferentsii po ptitsevodstvu s mezhdunarodnym uchastiem «Aktual'nye problemy sovremennogo ptitsevodstva»* [Proc. X Ukrainian conference on poultry farming with international participation «Actual problems of modern poultry farming»]. Khar'kov, 2009: 222-225 (in Russ.).
50. Lewis P.D., Perry G.C. Response of laying hens to asymmetrical interrupted lighting regimens: physiological aspects. *British Poultry Science*, 1990, 31(1): 45-52 (doi: 10.1080/00071669008417229).
51. Naito M., Ueno T., Komiyama T. The effect of intermittent lighting on the time of oviposition in the domestic fowl. *Japanese Poultry Science*, 1982, 19(4): 234-237 (doi: 10.2141/jpsa.19.234).
52. Kavtarashvili A.Sh. *Ptitsa i ptitseprodukty*, 2007, 5: 45-47 (in Russ.).
53. Kavtarashvili A.Sh., Marchev S.V., Ridzhal S.P. V knige: *Sbornik nauchnykh trudov VNITIP* [In: Collection of scientific works of VNITIP. Vol. 77]. Sergiev Posad, 2002, tom 77: 21-25 (in Russ.).
54. Gumulka M., Kapkowska E., Maj D. Laying pattern parameters in broiler breeder hens and intrasequence changes in egg composition. *Czech Journal of Animal Science*, 2010, 55(10): 428-435 (doi: 10.17221/1698-CJAS).
55. Miyoshi S., Inoue K., Minh Luc K., Kuchida K., Mitsumoto T. Intra-clutch changes in egg composition and shell quality in laying hens. *Japanese Poultry Science*, 1997, 34(4): 273-281 (doi: 10.2141/jpsa.34.273).

56. Kavtarashvili A.Sh., Novotorov E.N., Gusev V.A., Prisyazhnaya L.M. *Ptitsa i ptitseprodukty*, 2019, 3: 38-41 (in Russ.).
57. Zakaria A.H., Plumstead P.W., Romero-Sanchez H., Leksrisompong N., Osborne J., Braket J. Oviposition pattern, egg weight, fertility, and hatchability of young and old broiler breeders. *Poultry Science*, 2005, 84(9): 1505-1509 (doi: 10.1093/ps/84.9.1505).
58. Patterson P.H. The relationship of oviposition time and egg characteristics to the daily light:dark cycle. *The Journal of Applied Poultry Research*, 1997, 6(4): 381-390 (doi: 10.1093/japr/6.4.381).
59. Lewis Dr P.D., Backhouse D., Gous R.M. Photoperiod and oviposition time in broiler breeders. *British Poultry Science*, 2004, 45(4): 561-564 (doi: 10.1080/00071660412331286244).
60. Bhatti B.M. Distribution of oviposition times of hens in continuous darkness or continuous illumination. *British Poultry Science*, 1987, 28(2): 295-306 (doi: 10.1080/00071668708416962).
61. Duplaix M., Williams J., Mongin, P. Effects of an intermittent lighting schedule on the time of egg-laying and the levels of luteinizing hormone, progesterone and corticosterone in the plasma of the domestic hen. *Journal of Endocrinology*, 1981, 91(3): 375-383 (doi: 10.1677/joe.0.0910375).
62. Sauveur B., Mongin P. Performance of layers reared and/or kept under different 6-hour light-dark cycles. *British Poultry Science*, 1983, 24(3): 405-416 (doi: 10.1080/00071668308416755).
63. Mongin P. Food intake and oviposition by domestic fowl under symmetric skeleton photoperiods. *British Poultry Science*, 1980, 21(5): 389-394 (doi: 10.1080/00071668008416686).
64. Campo J.L., Gil M.G., Dávila S.G. Differences among white-, tinted-, and brown-egg laying hens for incidence of eggs laid on the floor and for oviposition time. *Archiv für Geflügelkunde,* 2007, 71(3): 105-109.
65. Washburn K.W., Potts P.L. Effect of strain and age on the relationship of oviposition time to shell strength. *British Poultry Science*, 1975, 16(6): 599-606 (doi: 10.1080/00071667508416235).
66. Halaj M. Study of dynamics of egg laying and properties during a day. *Acta Zootechnica*, 1974, 28: 162-171.
67. Etches R.J., Petitte J.N., Anderson-Langmuir C.E. Interrelationship between the hypothalamus, pituitary gland, ovary, adrenal gland and the open period for LH release in the hen (*Gallus domesticus*). *Journal of Experimental Zoology*, 1984, 232(3): 501-511 (doi: 10.1002/jez.1402320317).
68. Etches R.J. The ovulatory cycle of the hen. *Critical Reviews in Poultry Biology*, 1990, 2(4): 293-318.
69. Lewis P.D., Perry G.C., Morris T.R. Effect of photoperiod on the mean oviposition time of two breeds of laying hen. *British Poultry Science*, 1995, 36(1): 33-37 (doi: 10.1080/00071669508417750).
70. Lewis P.D., Perry G.C., Morris T.R., English J. Supplementary dim light differentially influences sexual maturity, oviposition time, and melatonin rhythms in pullets. *Poultry Science*, 2001, 80(12): 1723-1728 (doi: 10.1093/ps/80.12.1723).
71. Tůmová E., Ledvinka Z. The effect of time of oviposition and age on egg weight, egg components weight and eggshell quality. *Archiv für Geflügelkunde*, 2009, 73(2): 110-115.
72. Naito M., Ueno T., Komiyama T., Miyazono Y. Oviposition times under various light-dark cycles in the domestic fowl. *Japanese Poultry Science*, 1980, 17(3): 146-150 (doi: 10.2141/jpsa.17.146).
73. Liou S.S., Biellier H.V. Light-dark cues entrain oviposition time in the chicken hen. *International Journal of Biometeorology*, 1987, 31(4): 285-292 (doi: 10.1007/bf02188939).
74. Wilson W.O. Photocontrol of oviposition in gallinaceous birds. *Annals of the New York Academy of Science*, 1964, 117(1): 194-202 (doi: 10.1111/j.1749-6632.1964.tb48173.x).
75. Bhatti B.M., Morris T.R. The relative importance of light and temperature as phase setting signals for oviposition in the fowl. *British Poultry Science*, 1977, 18(4): 391-395 (doi: 10.1080/00071667708416378).
76. Bhatti B.M., Morris T.R The relative importance of sunrise and sunset for entrainment of oviposition in the fowl. *British Poultry Science*, 1978, 19(3): 365-371 (doi: 10.1080/00071667808416488).
77. Bhatti B.M. Exogenous regulation of the time of oviposition in the domestic fowl. *World's Poultry Science Journal*, 1987, 43(2): 116-131 (doi: 10.1079/WPS19870009).
78. Tůmová E., Ebeid T. Effect of time of oviposition on egg quality characteristics in cages and in a litter housing system. *Czech Journal of Animal Science*, 2005, 50(3): 129-134 (doi: 10.17221/4006-CJAS).
79. Bhatti B.M., Morris T.R. Entrainment of oviposition in the fowl using light-dark cycles. *British Poultry Science*, 1978, 19(3): 333-340 (doi: 10.1080/00071667808416485).Emmans G.C., Fisher C. Problems in nutritional theory. In: *Nutrient requirements of poultry and nutritional research*. I. Fisher, K.N. Boorman (eds.). Butterworths, London, 1986: 9-39.
81. Johnston S.A., Gous R.M. An improved mathematical model of the ovulatory cycle of the laying hen. *British Poultry Science*, 2003, 44(5): 752-760 (doi: 10.1080/00071660310001643723).
82. Charvátová V., Tůmová E. Time of oviposition and egg composition: a review. *Scientia Agriculturae Bohemica*, 2010, 41(3): 190-195.
83. Hughes B.O., Gilbert A.B., Brown M.F. Categorisation and causes of abnormal egg shells: relationship with stress. *British Poultry Science*, 1986, 27(2): 325-337 (doi: 10.1080/00071668608416885).
84. Mills A.D., Nys Y., Gautron J., Zawadski J. Whitening of brown shelled eggs: individual variation and relationships with age, fearfulness, oviposition interval and stress. *British Poultry Science*, 1991, 32(1): 117-129 (doi: 10.1080/00071669108417333).

85. Brake J. Relationship of egg weight, specific gravity, and shell weight to time of oviposition and feeding in broiler breeders. *Poultry Science*, 1985, 64(11): 2037-2040 (doi: 10.3382/ps.0642037).
86. Lee K.D., Choi J.H. Interrelationships among time of oviposition, egg weight, shell weight, and rate of production of laying hens. *Poultry Science*, 1985, 64(12): 2256-2258 (doi: 10.3382/ps.0642256).
87. Hashiguchi M. Relationship of position of egg in sequence to eggshell quality in laying hen. *Japanese Poultry Science*, 1996, 33: 230-234 (doi: 10.2141/jpsa.33.230).
88. Pavlovski Z., Vitorović D., Škrbić Z., Vračar S. Influence of limestone particle size in diets for hens and oviposition time on eggshell quality. *Acta Veterinaria, Belgrade*, 2000, 50(1): 37-42 (doi: 123456789/3).
89. Akif Boz M., Sarıca M., Yamak U.S. The effect of oviposition time on hatching traits of different chicken genotypes. *European Poultry Science*, 2014, 78 (doi: 10.1399/eps.2014.11).
90. Samiullah S., Roberts J., Chousalkar K. Oviposition time, flock age, and egg position in clutch in relation to brown eggshell color in laying hens. *Poultry Science, 2016,* 95(9)*:* 2052-2057 (doi: 10.3382/ps/pew197).
91. Zakaria A.H., Plumstead P.W., Romero-Sanchez H., Leksrisompong N., Brake J. The effects of oviposition time on egg weight loss during storage and incubation, fertility, and hatchability of broiler hatching eggs. *Poultry Science*, 2009, 88(12): 2712-2717 (doi: 10.3382/ps.2009-00069).
92. Kryeziu A.J., Mestani N., Kamberi M., Berisha H. Effect of hen age and oviposition time on egg quality parameters. *Proc. XIV European Symposium on the Quality of Eggs and Egg Products and XX European Symposium on the Quality of Poultry Meat*. Leipzig, Germany, 2011: b-047 (doi: 10.13140/RG.2.1.1675.2808).
93. Tůmová E, Ledvinka Z., Skřivan M., Englmaierová M., Zita L. Effect of time of oviposition on egg quality in egg and meat type hens. *Scientia Agriculturae Bohemica*, 2008, 39(3): 269-272.
94. Harms R.H. Specific gravity of eggs and eggshell weight from commercial layers and broiler breeders in relation to time of oviposition. *Poultry Science*, 1991, 70(5): 1099-1104 (doi: 10.3382/ps.0701099).
95. Tůmová E., Skřivan M., Englmaierová M., Zita L. The effect of genotype, housing system and egg collection time on egg quality in egg type hens. *Czech Journal of Animal Science*, 2009, 54(1): 17-23 (doi: 10.17221/1736-CJAS).
96. Tůmová E., Zita L., Hubený M., Skřivan M., Ledvinka Z. The effect of oviposition time and genotype on egg quality characteristics in egg type hens. *Czech Journal of Animal Science*, 2007, 52(1): 26-30 (doi: 10.17221/2326-CJAS).
97. Yannakopoulos A.L., Tserveni-Gousi A.S., Nikokyris P.N. Egg composition as influenced by time of oviposition, egg weight, and age of hens. *Archiv für Geflügelkunde*, 1994, 58(5): 206-213.
98. Hrnčár C., Hässlerová M., Bujko J. The effect of oviposition time on egg quality parameters in Brown Leghorn, Oravka and Brahma hens. *Scientific Papers: Animal Science and Biotechnologies*, 2013, 46(1): 53-57.
99. Tůmová E., Gous R.M., Tyler N. Effect of hen age, environmental temperature, and oviposition time on egg shell quality and eggshell and serum mineral contents in laying and broiler breeder hens. *Czech Journal of Animal Science*, 2014, 59(9): 435-443 (doi: 10.17221/7655-CJAS).
100. Kebreab E., France J., Kwakkel R.P., Lesson S., Darmani Kuhi H., Dijkstra J. Development and evaluation of a dynamic model of calcium and phosphorus flows in layers. *Poultry Science*, 2009, 88(3): 680-689 (doi: 10.3382/ps.2008-00157).
101. Campo J.L., Escudero J. Relationship between egg-shell colour and two measurements of shell strength in the Vasca breed. *British Poultry Science*, 1984, 25(4): 467-476 (doi: 10.1080/00071668408454888).
102. Curtis P.A., Gardner F.A., Mellor D.B. A comparison of selected quality and compositional characteristics of brown and white shell eggs. II. Interior quality. *Poultry Science*, 1985, 64(2): 302-306 (doi: 10.3382/ps.0640302).
103. Jones D.R., Musgrove M.T., Anderson K.E., Thesmar H.S. Physical quality and composition of retail shell eggs. *Poultry Science*, 2010, 89(3): 582-587 (doi: 10.3382/ps.2009-00315).
104. Mertens K., Vaesen I., Loffel J., Kemps B., Kamers B., Perianu C., Zoons J., Darius P., Decuypere E., De Baerdemaeker J., De Ketelaere B. The transmission color value: A novel egg quality measure for recording shell color used for monitoring the stress and health status of a brown layer flock. *Poultry Science*, 2010, 89(3): 609-617 (doi: 10.3382/ps.2009-00261).
105. Ishikawa S., Suzuki K., Fukuda E., Arihara K., Yamamoto Y., Mukai T., Itoh M. Photodynamic antimicrobial activity of avian eggshell pigments. *FEBS Letters,* 2010, 584(4): 770-774 (doi: 10.1016/j.febslet.2009.12.041).
106. Abdalla M.A., Ochi E.B. Effect of laying hen's age and oviposition time on egg cholesterol contents. *Science Letters*, 2018, 6(1): 42-46.
107. Myandmets R., Khyammaya Ya., Rummel' K. *Ptitsevodstvo*, 1993, 8: 14-15 (in Russ.).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1110-1121
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

DNA technologies

UDC 636.2:577.21:577.088

doi: 10.15389/agrobiology.2019.6.1110eng
doi: 10.15389/agrobiology.2019.6.1110rus

COMPARATIVE STUDY OF DIFFERENT METHODS OF DNA EXTRACTION FROM CATTLE BONES SPECIMENS MAINTAINED IN A CRANIOLOGICAL COLLECTION

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The authors declare no conflict of interests
Acknowledgements:
The equipment of the Sharing Center for Farm Animal Bioresources and Bioengineering (FSC for Animal Husbandry) was used.
Supported financially by Russian Science Foundation, project No. 19-76-20012
Received September 3, 2019

Abstract

The development of molecular-genetic methods allows elucidating the origin and demographic history of breeds of farm animals. Samples of bones and teeth maintained in craniological collections can serve as a source of DNA for such studies. The work with historical samples is complicated by the presence of a very low quantity of DNA, the high degree of its degradation and by the contamination of samples by PCR inhibitors. The aim of this work was the comparison of the efficiency of various methods of DNA extraction from historical cattle skulls, suitable for molecular genetic studies. The material was teeth extracted from historical skulls of cattle of the Yaroslavl and Kholmogor breeds stored in the craniological collection of the Liskun Museum of Livestock (Timiryazev Russian State Agrarian University—Moscow Agrarian Academy). At the first stage, we compared various DNA isolation methods implemented in the form of commercial kits, i.e. Prep Filer™ BTA Forensic DNA Extraction Kit («Thermo Fisher Scientific Inc.», USA), COrDIS Extract decalcine («GORDIZ» LLC, Russia), M-sorb-bone («Syntol» LLC, Russia), QIAamp DNA Investigator Kit («Qiagen», USA), with the modification of the amount of bone material and conditions of lylis. Based on preliminary research results, we selected for more detailed studies two kits, the QIAamp DNA Investigator Kit («Qiagen», USA) which implements the technology of column with silica gel membrane, and Prep Filer™ BTA Forensic DNA Extraction Kit («Thermo Fisher Scientific Inc.», USA) which is based on using magnetic particles. The quantitative and qualitative characteristics of the obtained DNA were evaluated by measuring the concentration of double-stranded DNA using a Qubit™ fluorimeter («Invitrogen, Life Technologies», USA) and determining the ratio of the absorption at 260 nm and 280 nm ($OD_{260/280}$) on a NanoDrop 8000 instrument («Thermo Fisher Scientific, Inc.», USA). The suitability of the obtained DNA extracts for molecular genetic studies was assessed based on the multiplex analysis of 11 microsatellite loci (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA122, INRA23, TGLA126, BM1818, ETH225, BM1824) as well as genome-wide genotyping on high-density DNA chips containing 777 thousand SNPs (Bovine HD BeadChip, «Illumina, Inc.», USA). Concentrations of double-stranded DNA (dsDNA) obtained using QIAamp DNA Investigator Kit and Prep Filer™ BTA Forensic DNA Extraction Kit ranged from 0.146 ng/μl

to 2.060 ng/μl and from 0.110 ng/μl to 13,600 ng/μl, respectively, and averaged 0.83±0.23 ng/μl and 2.75±1.33 ng/μl. The correlation coefficient (*r*) between the concentrations of dsDNA in isolations DNA obtained by two different methods was 0.84. Analysis of microsatellites showed that each of the samples has its own unique genotype which differs from other historical and modern samples of individuals. Efficiency of SNP genotyping (Call Rate) of the historical samples was 0.533-0.878 and 0.958-0.977 for DNA preparations produced using QIAamp DNA Investigator Kit и Prep Filer™ BTA Forensic DNA Extraction Kit, respectively. The results of microsatellite analysis and SNP genotyping, on the one hand, indicate the suitability of the obtained DNA for polymorphism research, on the other hand, confirm the compliance of the laboratory in which this analysis was performed with the authenticity criteria for working with ancient DNA. Conducting large-scale studies of historical samples using different types of DNA markers will clarify the origin and demographic history of domestic cattle breeds and develop effective programs for their conservation.

Keywords: historical DNA, craniological collection, DNA extraction, microsatellite analysis, SNP genotyping, cattle, local breeds

Involving historical and fossil specimens in researches is a way of studying evolution and demographic history of farm animal breeds [1]. Although after the death of an organism, DNA is destroyed in the process of enzymatic reactions, the molecules can persist for hundreds and thousands of years under favorable conditions [2, 3].

The main problem in the study of fossil DNA is the contamination of extracts or reagents with modern DNA molecules. Almost from the very beginning of work with fossil samples, the question arose as to whether the resulting DNA belonged to historical samples. As a result of numerous studies, the following so-called authenticity criteria have been formulated: conducting a reaction in each batch of samples with a "pure extract", i.e. without using fossil DNA; examining each sample in duplicates with subsequent comparison of the results; controlling the length of amplified fragments; the presence of polymerase chain reaction (PCR) products longer than 500-700 bps may cause suspicion [4-6]. Compliance with the above criteria is a prerequisite when working with fossil samples. The minimum requirements for working with ancient DNA are considered to be the presence of a physically isolated work area in which all studies are carried out before the amplification stage [7]; the use of negative controls during amplification and avoiding (if possible) positive control, since it carries a risk of contamination [8, 9]; the reproducibility of results for different DNA extracts of the same sample [10].

Since soft tissues are very poorly to preserve, bones of historical and fossil specimens are mainly used to extract DNA [11, 12]. The inner part of the temporal bone and the cement layer in the roots of the teeth are the best to extract DNA while no systematic difference in the DNA content between the two substrates was found [13]. However, bone tissue is extremely difficult to grind and dissolve. In addition, ancient bones and teeth often contain a large number of PCR inhibitors that are co-extracted with DNA [12, 14, 15]. Ancient DNA is quite severely damaged [16-18], so overly aggressive sample treatments, such as high temperatures or strong detergents, should be avoided [19]. There are several ways to extract ancient DNA, including sedimentation in water-ethanol or water-isopropanol solutions [12, 15], DNA concentration in membranes and separation by DNA molecules binding to silicon dioxide [20-22].

Regardless of the DNA extraction method, sample preparation involves several steps. First, the bones (teeth) are washed with detergent and distilled water, and then 2-3 mm of tissue is ground off to remove surface contamination and modern DNA. Purified bone fragments are treated with UV light (λ = 254 nm) for 30 minutes and mechanically ground into fine flour. It is followed by the steps of dissolving the bone powder in a lysing solution, washing away impurities

that inhibit PCR, and obtaining a purified DNA extract. Lysing solutions may differ in composition, but usually contain proteinase K, ethylenediaminetetraacetic acid (EDTA), and the sodium salt of N-lauroyl sarcosinate. A distinctive feature of the bone powder lysis process is the duration of incubation. For effective cell dissolution, lysis is recommended at a given temperature and continuous stirring for at least 24 hours. Increasing the lysis time to 48 hours or more is only appropriate for large sample weight portions and solution volumes. Otherwise, it does not lead to a significant improvement in the quality and does not increase the yield of the final product. It is followed by purification of DNA from lysates, for which various methods are used.

The simplest method is to sediment DNA with ethanol or isopropanol, wash the sediment to remove impurities from the solution, and dissolve the DNA in bidistilled water or buffer. The main advantages of this method are its rapidity and low cost. Disadvantages include high labor intensity, increased probability of sample loss or cross-contamination if several samples are processed simultaneously [23, 24].

To obtain pure DNA preparations, the use of columns with a silica gel membrane is effective. Nucleic acids are selectively bound to the membrane, and impurities are removed by the successive addition of washing buffers and centrifugation. At the final stage, an eluting buffer is used, which washes out the nucleic acids from the membrane. The advantages of the method include low labor intensity and low probability of errors of the researcher, good quality of the resulting DNA. It is also not necessary to use reagents that are dangerous to humans and the environment, such as phenol and chloroform. The method has been implemented in several commercial kits: Pure Link® Genomic DNA Mini Kit (Thermo Fisher Scientific, Inc., USA); Nucleo Spin® Tissue (Macherey-Nagel GmbH & Co. KG, Germany); QIAamp DNA Investigator Kit (Qiagen, USA) [25], which allows standardizing the DNA extraction process. The disadvantage of using commercial kits is their relatively high cost.

A promising method of nucleic acid purification is the use of magnetic particles as sorbents made from various synthetic polymers, biopolymers, porous glass or based on inorganic magnetic materials such as iron oxide [26]. DNA reversibly binds to the surface of magnetic particles and after a series of washings and removal of impurities is easily removed from the sorbent using an eluting buffer. This method is convenient, technological, and suitable for preparing samples for PCR amplification. However, product losses are possible due to irreversible sorption on the carrier, as well as during numerous washings, which is critical when working with small amounts of DNA in the sample [27]. The method has been successfully used in commercial Prep Filer™ BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific, Inc., USA); Quick-DNA/RNA MagBead (Zymo Research, USA).

When studying fossil samples of human bones, the advantage of the DNA extraction method using silica gel columns is shown concerning both the amount of DNA obtained [28, 29] and the size of fragments [29]. Thus, when using silica gel columns, a higher concentration of DNA was obtained in 68.4% of samples, while using QIAquick PCR Purification Kit® (Qiagen, USA) – in 21.05% of samples [28].

Historical skulls and their parts preserved in craniological collections are of interest as material for research on the demographic history of domestic animal breeds. The craniological method proposed in 1865 by Rütimeyer was the main method in the study of domestic animals origin in the late 19th to first half of the 20th century [30]. It led to the creation of well-documented craniological

collections, but only a few of them have survived to the present day. One of these collections, collected by Prof. Adametz, which contains about 1,300 skulls of old breeds of large domestic animals in Central Europe, is kept in the Natural History Museum Vienna (Naturhistorisches Museum Wien); https://www.nhm-wien.ac.at/en/research/1_zoology_vertebra-tes/archaeo-zoological_collection).

A unique collection of skulls of domestic and imported to old Russia foreign breeds of domestic animals (cattle, pigs, horses) collected by Prof. Liskun at Russian State Agrarian University—Moscow Timiryazev Agricultural Academy. The collection contains more than 700 animal skulls, including 350 cattle of 41 breeds and breed groups [31].

However, the complications in using material of craniological collections for DNA extraction is due to the fact that the technique of a skull accession preparation includes simmering for a few hours, in some cases bleaching in ammonia solution with hydrogen peroxide, and treating with phosphoric acid or chloramine to give a natural shade. Such treatment leads to significant degradation of nucleic acids, so when using historical specimens as a source of DNA, it is necessary to optimize the technique that allows obtaining DNA preparations that are suitable for quantitative and qualitative characterization of various types of polymorphisms. It is especially critical to obtain a sufficient amount of nuclear DNA, represented by only two copies per cell, in contrast to mitochondrial DNA the copy number of which is from 100 to 10,000 per cell [32].

In this paper, we obtained for the first time DNA preparations suitable for molecular genetic research using cattle skull accessions dated to the first half of the 20th century and subjected to thermal and chemical treatment prior to depositing in collections.

The work objective was to compare the effectiveness of various methods of DNA extraction from historical cattle skulls.

Techniques. The material was teeth from historical skulls of cattle of the Yaroslavl and Kholmogor breeds stored in the craniological collection of the State Museum of Stock-Breeding of Russian State Agrarian University—Timiryazev Moscow Agricultural Academy. All the standards set by the criteria for the authenticity of ancient DNA were met during the research [33].

Teeth were cleaned of mechanical impurities, washed with detergent and distilled water to remove surface contamination and modern DNA. Then, they were irradiated with ultraviolet light (λ = 254 nm) for 30 minutes. Using a table-top MBS240/E electric saw (Proxxon, Germany) equipped with a bimetallic blade, the teeth were cut longitudinally into two parts. After that, fine powder was drilled from the inside of the teeth using a Dremel 3000-15 mini drill (Dremel, USA) with diamond boron. Drilling was performed at minimum speeds (~ 8,000 rpm) with breaks to prevent excessive heating of the dentin and destruction of DNA under the influence of high temperatures. The resulting bone powder was poured into pre-irradiated Eppendorf-type sterile tubes with a Safe-Lock system (1.5 ml).

At the first stage, various methods of DNA isolation implemented in the form of commercial kits were compared with modifications of the amount of bone material used and lysis conditions: Prep Filer™ BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific, Inc., USA), COrDIS Extract decalcin (LLC (OOO) GORDIZ, Russia), M-Sorb-kost (LLC (OOO) Syntol, Russia), QIAamp DNA Investigator Kit (Qiagen, USA).

For more detailed research, QIAamp DNA Investigator Kit, which implements the technology of selective DNA binding on a silicon membrane, and Prep Filer™ BTA Forensic DNA Extraction Kit, based on the use of magnetic

particles, were selected. To obtain comparable results, the extraction protocols recommended by the manufacturers for each set were modified. Sample weight portions resulted in a single mass (100±3 mg), the lysis time was increased to 24 h at a temperature of 56 °C and the rotation speed was 1100 rpm. A further increase in lysis time to 48 hours did not have a significant positive effect on the quality and quantity of the resulting DNA. The elution time for QIAamp DNA Investigator Kit extraction was increased to 30 minutes.

The quality of the obtained DNA preparations was evaluated as follows: the total DNA concentration and the drug absorption ratio were measured at λ = 260 nm and λ = 280 nm using a NanoDrop 8000 device (Thermo Fisher Scientific, Inc., USA); the concentration of double-stranded DNA was determined on the Qubit™ (1.0) fluorimeter (Invitrogen, Life Technologies, USA).

To assess the suitability of the obtained DNA preparations for molecular genetic research, a multiplex analysis was performed on 11 microsatellites (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA122, INRA23, TGLA126, BM1818, ETH225, BM1824) using a panel developed at the Federal Science Center for Animal Husbandry named after Academy Member L.K. Ernst, as well as full-genomic genotyping for about 777 thousand SNPs (single nucleotide polymorphisms) on high-density DNA chips (Bovine HD BeadChip, Illumina, Inc., USA). PCR amplification was performed on a SimpliAmp Thermal Cycler (Life Technologies, USA). Polymorphism of microsatellite markers was studied on the capillary genetic analyzer ABI3500 (Applied Biosystems, USA) using the software Gene Mapper v. 5 (Applied Biosystems, USA). Microsatellite profiles of the representatives of the modern breeds were taken from the database of the Ernst Federal Science Center for Animal Husbandry.

SNP was examined using the iScan® system scanner (Illumina, Inc., USA) and the software supplied with the device.

A phylogenetic tree for modern and historical bovine samples was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method based on genetic distances of Nei (Ds) [34] calculated from microsatellites in the Populations program, 1.2.32 [35], and visualized in the SplitsTree program, 4.13.1 [36].

Results. Kits based on magnetic particles (Prep Filer™ BTA Forensic DNA Extraction Kit, COrDIS Extract decalcin and M-Sorb-kost), in general, allowed obtaining DNA preparations with a higher concentration (2.94-9.80 ng/μl of double-stranded DNA, the value of $OD_{260}/_{280}$ = 1.00-1.64). However, the purity of extracts obtained using spin columns with a silicon membrane (QIAamp DNA Investigator Kit) was higher at a lower concentration of the extracted DNA (0.56-6.42 ng/μl) ($OD_{260}/_{280}$ = 1.47-1.90). QIAamp DNA Investigator Kit and Prep Filer™ BTA Forensic DNA Extraction Kit were selected for a more detailed study.

Significant differences in DNA concentration were found between samples and between preparations obtained from the same sample using different methods (Table 1). Concentrations of double-stranded DNA isolated using QIAamp DNA Investigator Kit and Prep Filer™ BTA Forensic DNA Extraction Kit ranged from 0.146 to 2.060 ng/μl and 0.110 to 13.600 ng/μl, respectively, averaging 0.83±0.23 and 2.75±1.33 ng/μl. In 8 of the 10 samples, the concentration of double-stranded DNA was 1.43-6.60 times higher when using the Prep Filer™ BTA Forensic DNA Extraction Kit compared to the QIAamp DNA Investigator Kit, while in the two remaining samples it was 1.76-2.80 times lower. The correlation coefficient (r) between the concentrations of double-stranded DNA in preparations obtained by two different methods was 0.84.

1. Concentration and purity of DNA preparations obtained from historical samples of Yaroslavl and Kholmogor cattle teeth using various methods

Sample No.	QIAamp DNA Investigator Kit			Prep Filer™ BTA Forensic DNA Extraction Kit		
	Qubit, ng/μl	NanoDrop, ng/μl	$OD_{260}/_{280}$	Qubit, ng/μl	NanoDrop, ng/μl	$OD_{260}/_{280}$
1	0.434	141.50	0.82	0.246	5.60	1.70
2	2.060	23.02	1.71	13.600	79.40	1.87
3	0.308	9.12	1.46	0.110	70.68	1.96
4	0.316	33.80	1.83	1.260	26.51	1.67
5	0.318	14.70	1.58	0.454	17.38	0.67
6	0.334	16.44	1.54	0.540	18.75	1.59
7	1.590	19.13	1.73	2.400	40.09	1.74
8	0.884	15.19	1.72	2.600	42.48	1.64
9	0.146	12.40	1.53	0.378	19.65	1.54
10	1.910	16.57	1.90	5.880	64.23	1.88

N o t e. Double-stranded DNA concentration (a Qubit fluorometer, Invitrogen, Life Technologies, USA) and total DNA concentration (a NanoDrop 8,000 device, Thermo Fisher Scientific, Inc., USA) were measured. $OD_{260/280}$ (absorption ratio at λ = 260 nm and λ = 280 nm, measured on the NanoDrop 8000 device) indicates purity of DNA preparation.

The obtained data show that the main factor affecting the success of DNA isolation is the safety of the sample, which is consistent with the results of other studies [28, 37, 38]. However, unlike some other papers [29], the authors obtained higher concentrations using the magnetic particle method. This is likely due to the severe degradation of DNA in the process of preparing skulls for deposition in collections. As a result, the extracted DNA consists of very short fragments that are better held by magnetic particles than by a silica gel membrane during the washing process.

Microsatellite profiles of historical animal samples of the Yaroslavl and Kholmogor breeds were obtained to assess the suitability of the selected DNA for molecular genetic research and to confirm that the laboratory meets the authenticity criteria. Genotypes of all 11 microsatellite loci studied were determined (Fig. 1). At the same time, it should be noted that the peak height decreases with increasing the allele length of microsatellites, which indicates a strong degradation of the resulting DNA, which is represented mainly by low-molecular fragments.

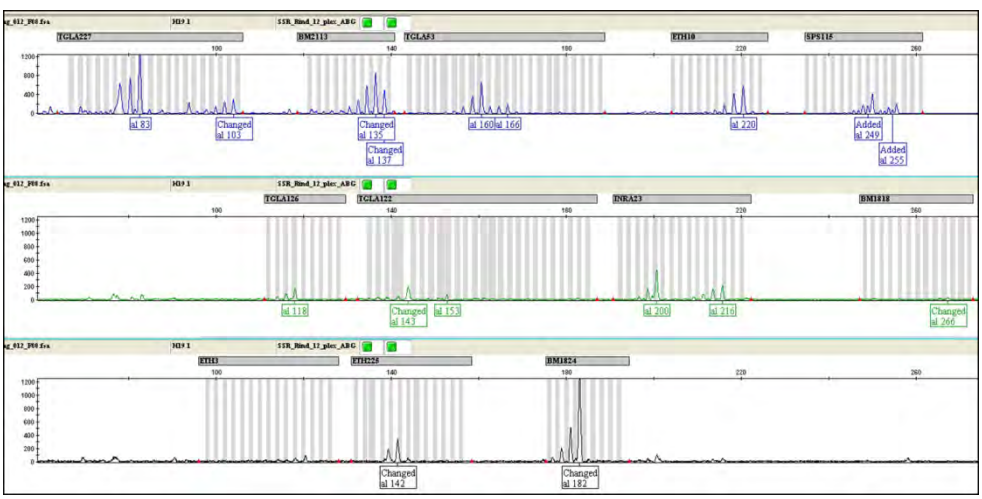

Fig. 1. Capillary electrophoresis-based multiplex analysis of microsatellite profile for 11 microsatellite loci of a Kholmogor animal historical craniological sample (H19.1**):** А — TGLA227 (alleles 83, 103), BM2113 (135, 137), TGLA53 (160, 166), ETH10 (220, 220), SPS115 (249, 255); Б — TGLA126 (118, 118), TGLA122 (143, 153), INRA23 (200, 216), BM1818 (266, 266); В — ETH225 (142, 142), BM1824 (182, 182). X axis corresponds to loci and fragments (peaks) detected; the size of the fragments (al 83, Changed al 103, Changed al 135, etc.) increases along the axis to the right). Y axis corresponds to the peak height. The color indicates different tags for the analyzer. See the full Figure 1 on http://www.agrobiology.ru.

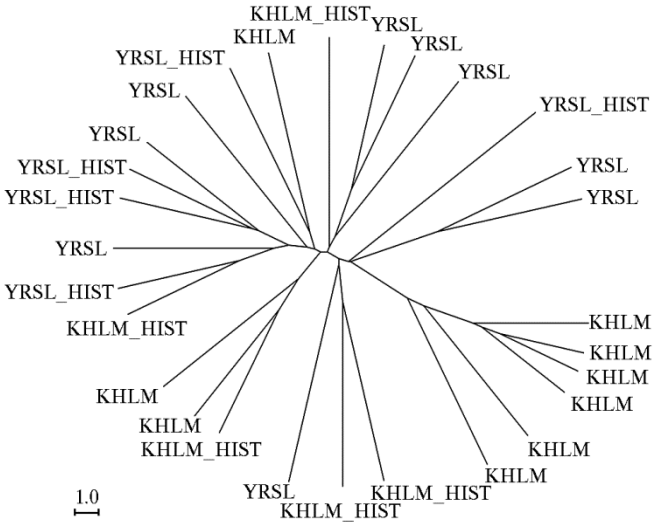

Fig. 2. Phylogenetic tree constructed from microsatellite markers based Nei [34] genetic distances (Ds) for modern and historical cattle samples: KHLM — modern Kholmogor breed, YRSL – modern Yaroslavl breed, KHLM_HIST — historical sample of Kholmogor breed, YRSL_HIST — historical sample of the Yaroslavl breed. UPGMA method, Populations program 1.2.32 [35], visualization with SplitsTree program 4.13.1 [36].

Analysis of microsatellite profiles of historical samples showed that each of the samples had its own unique genotype, which differed from the genotypes of other historical and modern samples. On a phylogenetic tree constructed on the basis of genetic distances of Nei [34] calculated from microsatellites, it was manifested in the formation of its independent branch by each sample (Fig. 2). In all five historical samples of the Yaroslavl breed and three of the five historical samples of the Kholmogor breed, private alleles were identified that were not present in other studied populations. The results of microsatellite analysis confirm that the laboratory where these studies were conducted meets the criteria for working with ancient DNA.

The efficiency of full-genomic genotyping of SNP when using DNA isolated using magnetic particles was significantly higher than when using DNA isolated using silicon spin-columns (Table 2). It may be because, for SNP analysis, the size of DNA fragments (as opposed to concentration) is not a critical factor. When analyzing microsatellite loci, the success of amplification is determined by the degree of DNA degradation [18].

2. Effectiveness of whole-genome genotyping DNA from Kholmogor cattle skull historical material with high-density Bovine HD BeadChip DNA chips (Illumina, Inc., USA)

Sample No.	Qubit, ng/μl	NanoDro, ng/μl	$OD_{260}/_{280}$	Call Rate	DNA extraction
1	5.32	35.68	1.87	0.958	Prep Filer™ BTA Forensic DNA Extraction Kit
2	4.52	26.19	1.96	0.970	Prep Filer™ BTA Forensic DNA Extraction Kit
3	6.84	33.10	1.83	0.970	Prep Filer™ BTA Forensic DNA Extraction Kit
4	4.94	65.71	1.90	0.977	Prep Filer™ BTA Forensic DNA Extraction Kit
5	2.94	21.64	1.96	0.641	QIAamp DNA Investigator Kit
6	1.18	21.15	1.86	0.533	QIAamp DNA Investigator Kit
7	6.84	35.15	1.85	0.775	QIAamp DNA Investigator Kit
8	4.48	24.75	2. 00	0.878	QIAamp DNA Investigator Kit

N o t e. Double-stranded DNA concentration (a Qubit fluorometer, Invitrogen, Life Technologies, USA) and total DNA concentration (a NanoDrop 8,000 device, Thermo Fisher Scientific, Inc., USA) were measured. $OD_{260/280}$ (absorption ratio at $\lambda = 260$ nm and $\lambda = 280$ nm, measured on the NanoDrop 8000 device) indicates purity of DNA preparation. Call Rate means the proportion of genotyped SNPs of the total number of SNPs on the DNA chip.

The main problems in molecular genetic studies of ancient and historical

samples are low DNA concentrations and high degradation in the extracts [13, 14, 18, 22]. The small length of the remaining target fragments may be a significant limitation for analyzing microsatellites with a longer fragment length (200 bps or more) [41, 42]. For unambiguous interpretation of the obtained profiles in some works [42, 43], it is recommended to amplify each sample at least three times. SNP markers are less demanding on the degree of DNA degradation. Studies have shown [29, 44] that high-performance sequencing and full-genomic genotyping of SNP on DNA chips will significantly expand the range of data processing procedures used and make more complete use of the information contained in historical DNA.

Thus, our findings show the possibility of successfully isolating DNA suitable for molecular genetic research from historical bovine skulls from craniological collections. Comparison of two different methods of DNA extraction based on the use of columns with a silica-gel membrane (QIAamp DNA Investigator Kit) and magnetic particles (Prep Filer™ BTA Forensic DNA Extraction Kit) revealed the advantage of the second method. In 8 of the 10 studied samples, the concentration of double-stranded DNA was higher when using the magnetic particle method, and in two samples — when using silica gel columns, while the average values of the DNA concentration were 2.75 ± 1.33 and 0.83 ± 0.23 ng/μl, respectively. Analysis of the genotypes of historical samples from 11 microsatellite loci shows that each of the 10 studied samples of the Kholmogor and Yaroslavl breeds has its own unique genotype that differs from other samples. The study of the effectiveness of full-genomic SNP genotyping shows the advantage of a set based on the use of magnetic particles (Prep Filer™ BTA Forensic DNA Extraction Kit). Assuming that the length of fragments in this type of analysis is not a critical factor, this method is preferred due to obtaining higher concentrations of DNA. In general, large-scale studies of historical samples using different types of DNA markers will be a significant addition to the results of studying modern representatives of breeds. It will help to clarify the origin and demographic history of domestic cattle breeds to develop effective programs for their preservation.

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0.83±0.23 ng/μl, respectively. Analysis of the genotypes of historical samples from 11 microsatellite loci shows that each of the 10 studied samples of the Kholmogor and Yaroslavl breeds has its own unique genotype that differs from other samples. The study of the effectiveness of full-genomic SNP genotyping shows the advantage of a set based on the use of magnetic particles (Prep Filer™ BTA Forensic DNA Extraction Kit). Assuming that the length of fragments in this type of analysis is not a critical factor, this method is preferred due to obtaining higher concentrations of DNA. In general, large-scale studies of historical samples using different types of DNA markers will be a significant addition to the results of studying modern representatives of breeds. It will help to clarify the origin and demographic history of domestic cattle breeds to develop effective programs for their preservation.

REFERENCES

1. Zinovieva N.A., Sermyagin A.A., Dotsev A.V., Boronetskaya O.I., Petrikeeva L.V., Abdelmanova A.S., Brem G. Animal genetic resources: developing the research of allele pool of Russian cattle breeds — minireview. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2019, 54(4): 631-641 (doi: 10.15389/agrobiology.2019.4.631eng).
2. Lindahl T. Instability and decay of the primary structure of DNA. *Nature*, 1993, 362(6422): 709-715 (doi: 10.1038/362709a0).
3. Orlando L., Ginolhac A., Zhang G., Froese D., Albrechtsen A., Stiller M., Schubert M., Cappellini E., Petersen B., Moltke I., Johnson P.L., Fumagalli M., Vilstrup J.T., Raghavan M., Korneliussen T., Malaspinas A.S., Vogt J., Szklarczyk D., Kelstrup C.D., Vinther J., Dolocan A., Stenderup J., Velazquez A.M., Cahill J., Rasmussen M., Wang X., Min J., Zazula G.D., Seguin-Orlando A., Mortensen C., Magnussen K., Thompson J.F., Weinstock J., Gregersen K., RшedK.H., Eisenmann V., Rubin C.J., Miller D.C., Antczak D.F., Bertelsen M.F., Brunak S., Al-Rasheid K.A., Ryder O., Andersson L., Mundy J., Krogh A., Gilbert M.T., Kjær K., Sicheritz-Ponten T., Jensen L.J., Olsen J.V., Hofreiter M., Nielsen R., Shapiro B., Wang J., Willerslev E. Recalibrating *Equus* evolution using the genome sequence of an early Middle Pleistocene horse. *Nature*, 2013, 99(7456): 74-78 (doi: 10.1038/nature12323).
4. Pääbo S., Higuchi R.G., Wilson A.C. Ancient DNA and the polymerase chain reaction: the emerging field of molecular archaeology (minireview). *The Journal of Biological Chemistry*, 1989, 264(17): 9709-9712.
5. Ward R., Stringer C. A molecular handle on the Neanderthals. *Nature*, 1997, 388: 225-226 (doi: 10.1038/40746).
6. Cooper A., Poinar H.N. Ancient DNA: do it right or not at all. *Science*, 2000, 289(5482): 1139 (doi: 10.1126/science.289.5482.1139b).
7. Lindahl T. Recovery of antediluvian DNA. *Nature,* 1993, 365(6448): 700 (doi: 10.1038/365700a0).
8. Handt O., Krings M., Ward R.H., Pääbo S. The retrieval of ancient human DNA sequences. *Am. J. Hum. Genet.*, 1996, 59: 368-376.
9. Cooper A. DNA from museum specimens. In: *Ancient DNA.* B. Herrmann, S. Hummel (eds.). Springer-Verlag, NY, 1993: 149-165 (doi: 10.1007/978-1-4612-4318-2_10).
10. Greenwood A.D., Capelli C., Possnert G., Pääbo S. Nuclear DNA sequences from late Pleistocene megafauna. *Molecular Biology and Evolution*, 1999, 16(11): 1466-1473 (doi: 10.1093/oxfordjournals.molbev.a026058).
11. Hagelberg E., Sykes B., Hedges R. Ancient bone DNA amplified. *Nature*, 1989, 342: 485 (doi: 10.1038/342485a0).
12. Hänni C., Brousseau T., Laudet V., Stehelin D. Isopropanol precipitation removes PCR inhibitors from ancient bone extracts. *Nucleic Acids Research*, 1995, 23(5): 881-882 (doi: 10.1093/nar/23.5.881).
13. Hansen H.B., Damgaard P.B., Margaryan A., Stenderup J., Lynnerup N., Willerslev E., Allentoft M.E. Comparing ancient DNA preservation in petrous bone and tooth cementum. *PLoS ONE*, 2017, 12(1): e0170940 (doi: 10.1371/journal.pone.0170940).
14. Höss M., Pääbo S. DNA extraction from Pleistocene bones by a silica-based purification method. *Nucleic Acids Research*, 1993, 21(16): 3913-3914 (doi: 10.1093/nar/21.16.3913).
15. Kalmár T., Bachrati C.Z., Marcsik A., Raskó I. A simple and efficient method for PCR amplifiable DNA extraction from ancient bones. *Nucleic Acids Research*, 2000, 28(12): e67 (doi: 10.1093/nar/28.12.e67).
16. Pääbo S. Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. *Proceedings of the National Academy of Sciences*, 1989, 86(6): 1939-1943 (doi:

10.1073/pnas.86.6.1939).
17. Hofreiter M., Jaenicke V., Serre D., von Haeseler A., Pääbo S. DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Research*, 2001, 29(23): 4793-4799 (doi: 10.1093/nar/29.23.4793).
18. Hansen A.J., Mitchell D.L., Wiuf C., Paniker L., Brand T.B., Binladen J., Gilichinsky D.A., Rønn R., Willerslev E. Crosslinks rather than strand breaks determine access to ancient DNA sequences from frozen sediments. *Genetics*, 2006, 173(2) 1175-1179 (doi: 10.1534/genetics.106.057349).
19. Rohland N., Hofreiter M. Comparison and optimization of ancient DNA extraction. *Biotechniques*, 2007, 42(3): 343-352 (doi: 10.2144/000112383).
20. Leonard J.A., Wayne R.K., Cooper A. Population genetics of ice age brown bears. *Proceedings of the National Academy of Sciences*, 2000, 97(4): 1651-1654 (doi: 10.1073/pnas.040453097).
21. Hofreiter M., Rabeder G., Jaenicke-Després V., Withalm G., Nagel D., Paunovic M., Jambrěsić G., Pääbo S. Evidence for reproductive isolation between cave bear populations. *Current Biology*, 2004, 14(1): 40-43 (doi: 10.1016/j.cub.2003.12.035).
22. Rohland N., Glocke I., Aximu-Petri A., Meyer M. Extraction of highly degraded DNA from ancient bones, teeth and sediments for high-throughput sequencing. *Nat. Protoc.*, 2018, 13: 2447-2461 (doi: 10.1038/s41596-018-0050-5).
23. Bowtell D.D. Rapid isolation of eukaryotic DNA. *Analytical Biochemistry*, 1987, 162(2): 463-465 (doi: 10.1016/0003-2697(87)90421-0).
24. Mohammadi A., Ghorbani Alvanegh A., Khafaei M., Habibi Azarian S., Naderi M., Kiyani E., Miri A., Bahmani H., Ramezani M., Tavallaei M. A new and efficient method for DNA extraction from human skeletal remains usable in DNA typing. *Journal of Applied Biotechnology Reports*, 2017, 4(2): 609-614.
25. Tolosa J.M., Schjenken J.E., Civiti T.D., Clifton V.L., Smith R. Column-based method to simultaneously extract DNA, RNA, and proteins from the same sample. *Biotechniques,* 2007, 43(6): 799-804 (doi: 10.2144/000112594).
26. Saiyed Z.M., Bochiwal C., Gorasia H., Telang S.D., Ramchand C.N. Application of magnetic particles (Fe_3O_4) for isolation of genomic DNA from mammalian cells. *Analytical Biochemistry,* 2006, 356(2): 306-308 (doi: 10.1016/j.ab.2006.06.027).
27. Suffys P., Vanderborght P.R., dos Santos P.B., Correa L.A.P., Bravin Y., Kritski A.L. Inhibition of the polymerase chain reaction by sputum samples from tuberculosis patients after processing using a silica-guanidiniumthiocyanate DNA isolation procedure. *Memórias do Instituto Oswaldo Cruz*, 2001, 96(8): 1137-1139 (doi: 10.1590/s0074-02762001000800019).
28. Palomo-Díez S., Martínez-Labarga C., Gomes C., Esparza-Arroyo Á., Rickards O., Arroyo-Pardo E. Comparison of two different DNA extraction methodologies for critical bone or teeth samples. *Forensic Science International: Genetics Supplement Series*, 2017, 6: E359-E361 (doi: 10.1016/j.fsigss.2017.09.110).
29. Gamba C., Hanghøj K., Gaunitz C., Alfarhan A.H., Alquraishi S.A., Al-Rasheid K.A., Bradley D.G., Orlando L. Comparing the performance of three ancient DNA extraction methods for high-throughput sequencing. *Molecular Ecology Resources*, 2016, 16(2): 459-469 (doi: 10.1111/1755-0998.12470).
30. Brauner A. *Porody sel'skokhozyaistvennykh zhivotnykh. Krupnyi rogatyi skot* [Breeds of farm animals. Cattle]. Odessa, 1922 (in Russ.).
31. Boronetskaya O.I., Barbosova M.E., Nikiforov A.I., Bykova A.V., Mikheenkov V.E., Rabadanova G.Sh., Petrikeeva L.V., Polurotova A.I., Rukavitsina E.A. Katalog kraniologicheskoi kollektsii akademika E.F. Liskuna /Pod. redaktsiei V.P. Panova [Catalog of the craniological collection of academician E.F. Liskuna. V.P. Panov (ed.)]. Moscow, 2012 (in Russ.).
32. Robin E.D., Wong R. Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *J. Cell. Physiol.*, 1988, 136(3): 507-513 (doi: 10.1002/jcp.1041360316).
33. Pääbo S., Poinar H., Serre D., Jaenicke-Després V., Hebler J., Rohland N., Kuch M., Krause J., Vigilant L., Hofreiter M. Genetic analyses from ancient DNA. *Annual Review of Genetics*, 2004, 38: 645-679 (doi: 10.1146/annurev.genet.37.110801.143214).
34. Nei M. Genetic distance between populations. *The American Naturalist*, 1972, 106(949): 283-292.
35. Langella O. *Populations 1.2.32 (02/13/2011): a population genetic software*. CNRS UPR9034. 1999. Available dostupa: http://bioinformatics.org/~tryphon/populations/. No date.
36. Huson D.H., Bryant D. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution,* 2006, 23(2): 254-267 (doi: 10.1093/molbev/msj030).
37. Vanek D., Silerova M., Urbanova V., Saskova L., Dubska J., Beran M., Genomic DNA extraction protocols for bone samples: the comparison of Qiagen and Zymo Research spin columns. *Forensic Science International: Genetics Supplement Series*, 2011, 3(1): e397-e398 (doi: 10.1016/j.fsigss.2011.09.060).
38. Scorrano G., Valentini F., Martínez-Labarga C., Rolfo M. F., Fiammenghi A., Lo Vetro D.,

Martini F., Casoli A., Ferraris G., Palleschi G., Palleschi A., Rickards O. Methodological strategies to assess the degree of bone preservation for ancient DNA studies. *Annals of Human Biology*, 2014, 42(1): 10-19 (doi: 10.3109/03014460.2014.954614).

39. Zinovieva N.A., Dotsev A.V., Sermyagin A.A., Wimmers K., Reyer H., Sölkner J., Deniskova T.E., Brem G. Study of genetic diversity and population structure of five Russian cattle breeds using whole genome SNP analysis. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2016, 51: 788-800 (doi: 10.15389/agrobiology.2016.6.788eng).
40. Sermyagin A.A., Dotsev A.V., Gladyr E.A., Traspov A.A., Deniskova T.E., Kostyunina O.V., Reyer H., Wimmers K., Barbato M., Paronyan I.A., Plemyashov K.V., Sölkner J., Popov R.G., Brem G., Zinovieva N.A. Whole-genome SNP analysis elucidates the genetic structure of Russian cattle and its relationship with Eurasian taurine breeds. *Genet. Sel. Evol.*, 2018, 50(1): 37 (doi: 10.1186/s12711-018-0408-8).
41. Petren K., Grant P.R., Grant B.R., Clack A.A., Lescano N.V. Multilocus genotypes from Charles Darwin's finches: biodiversity lost since the voyage of the Beagle. *Phil. Trans. R. Soc. B*, 2010, 365: 1009-1018 (doi: 10.1098/rstb.2009.0316).
42. Allentoft M., Heller R., Holdaway R., Bunce M. Ancient DNA microsatellite analyses of the extinct New Zealand giant moa (*Dinornis robustus*) identify relatives within a single fossil site. *Heredity*, 2015, 115: 481-487 (doi: 10.1038/hdy.2015.48).
43. Bourke B.P., Frantz A.C., Lavers C.P., Davison A., Dawson D.A., Burke T.A. Genetic signatures of population change in the British golden eagle (*Aquila chrysaetos*). *Conserv. Genet.*, 2010, 11(5): 1837-1846 (doi: 10.1007/s10592-010-0076-x).
44. Knapp M, Hofreiter M. Next generation sequencing of ancient DNA: requirements, strategies and perspectives. *Genes*, 2010, 1(2): 227-243 (doi: 10.3390/genes1020227).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1122-1134
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Physiology of adaptation

UDC 636:591.1:612.34

doi: 10.15389/agrobiology.2019.6.1122eng
doi: 10.15389/agrobiology.2019.6.1122rus

THE ADAPTATION OF PANCREATIC SECRETION AND METABOLISM IN ANIMALS WITH DIFFERENT DIGESTION TYPE TO CHANGES IN DIETARY PROTEIN INGREDIENTS

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Program of Basic Scientific Research of the Russian Academy of Sciences, subprogram "Studying the mechanisms of adaptation of the digestive system of mammalian animals and poultry to rations with different ingredient composition of feed" (Decree of the Presidium RAS No. 132 of July 5, 2017)
Received September 19, 2019

Abstract

The adaptation of the pancreas to diet composition is still a matter of discussion. The methodical difficulties related to the sampling of pure pancreatic juice resulted in the scarce and discrepant published data on the alterations of exocrine pancreatic secretion in response to changes in diet composition (A.D. Sineshchekov, 1965; P.P. Berdnikov, 1990; Ts.Zh. Batoev, 2001; A. Huget et al., 2006; V.I. Fisinin et al., 2017; K. Liu et al., 2018); the effects of individual dietary ingredients on the pancreatic secretion were not scrutinized to date. We present a comparative study on the alterations of exocrine pancreatic function in response to the change in dietary protein source (substitution of sunflower cake for soybean cake or meal) with the use of similar methodology for animals with different digestion type; this approach can reveal both specific and common patterns in the respective adaptive reactions. The study was performed on 18 laying hens (*Gallus gallus domesticus* L.; cross Hisex White) at 10-12 months of age including 3 hens with chronic fistulae of main pancreatic duct inserted according to the method of Ts.Zh. Batoev (2001), and on 9 piglets (*Sus scrofa domesticus* L.; hybrid of Danish Landrace and Danish Yorkshire breeds) at 5 months of age and 45-55 kg of live bodyweight including 3 intact piglets, 3 piglets with cannulated pancreatic duct, and 3 piglets with ileal cannulae. It was found that the most responsive enzymatic activity in chicken is lipase: in hens fed diet with sunflower cake the basal (preprandial) level of lipase activity was significantly higher by 37.7 % in compare to diet with soybean meal, postprandial lipase activity in 90 min after feeding higher by 46.6 %; in 150 min after feeding higher by 93.7 % ($p < 0.05$). The shift from soybean meal to sunflower cake resulted in the significant increase in crude fat digestibility by 3.5 % ($p < 0.05$). This shift was also found to increase the postprandial protease activity though similar response of crude protein digestibility was not found. To the contrary, the same shift in diet composition in pigs resulted in the significant decrease in postprandial protease activity by 43.8 % ($p < 0.05$); no alterations were found in the digestibility of crude protein probably due to the presence of trypsin inhibitor(s) in soybean meal. Regardless of the similar total volumes of pancreatic juice secreted in pigs during the sampling period with different dietary protein sources the dynamics of the postprandial pancreatic secretion substantially differed with these sources both in the complex-reflex and neurohumoral regulative phases. The exocrine pancreatic function in chicken was considerably more strenuous in compare to pigs: the relative (per 1 kg of live bodyweight) volume of pancreatic juice secreted in chicken was 2.2-fold higher, total amylase activity secreted was 94-fold higher, protease activity 145-fold higher, lipase activity 17-fold higher in compare to pigs. The tryptic activity in blood serum in chicken was 7-fold higher in compare to pigs evidencing higher metabolic intensity. Therefore, when changing the dietary ingredients the functional peculiarities of the adaptation of pancreatic

secretion to these shifts in the productive animals with different digestion type should be taken into account since exocrine pancreatic function and feed digestibility are correlated and can, in turn, jointly affect the productive performance.

Keywords: pancreas, exocrine pancreatic function, chicken, pigs, activities of digestive enzymes in blood serum

The adaptation of the pancreas to the diet composition in animals is still a matter of discussion. There is an opinion about parallel changes in the enzymatic activity in pancreatic juice. According to the parallel secretion hypothesis, the activity of pancreatic enzymes changes in the same proportions, regardless of the food components [1]. Such results were obtained in cannulated dogs that constantly lost pancreatic juice, in which the ability of the pancreas to adapt the enzymatic composition of the secret to the nature of the food was significantly impaired [2]. Such an imbalance occurs in diseases of the digestive system, as well as in the case of excess intake of nutrients, in particular fats [2]. However, there is a large amount of experimental data indicating the ability of the pancreas to change the enzymatic activity and juice production depending on the composition of the consumed food [3-6]; it is also described for birds [7-11]. Evidence of adaptation of the digestive glands to the quality of food was obtained using modern methods in the process of synthesis, transport, and isolation of zymogenic granules at the level of both individual acinar cells and acinuses of topographically different parts of the gland [12-14]. Currently, more and more attention is paid to molecular genetic approaches in the study of secretory function of the pancreas [15], and although these methods are not widely used in the study of pancreas adaptation to food quality, the few obtained results are consistent with biochemical data [16, 17]. The experiments on animals fitted with a cannula, which allows taking samples of pancreatic juice in vivo during the experiments and sending it to the intestine, are of particular interest. It should be noted that due to methodological difficulties in obtaining pure pancreatic juice, the available data on the adaptation of the secretory function of farm animals are few and contradictory [18, 19]. In the last century, the academician Pavlov developed a method for studying pancreatic juice in dogs and obtained experimental data on the adaptation of pancreatic secretion to bread, milk, and meat, which became the basis of the physiology of the digestive system [3]. Later, the adaptation of pancreatic secretion to various feeds and additives was studied in farm animals [7-9, 20], but the effect of certain feed ingredients on pancreatic secretion was not studied.

This paper describes for the first time quantitative changes in the secretory function of the pancreas when replacing one of the components of the diet. In addition, due to the use of similar methods for the study of pancreatic secretion (experiments on fistulated individuals), indicators were compared in animals with different types of digestion, which allowed us to identify both peculiar and general patterns of adaptive responses of the pancreas.

Our goal was to study, in a comparative aspect, the adaptation mechanisms of the digestive system of mammals and birds to diets with different protein ingredients.

Techniques. The study was performed on chickens (*Gallus gallus* L.) of Leghorn breed egg cross Hisex White aged 10-12 months with chronic pancreatic duct fistulas (3 birds), intact chickens (15 birds), piglets (*Sus scrofa domesticus* L.) cross between Danish Landrace and the Danish Yorkshire aged 5 months, the live weight of 45-55 kg (3 piglets with the cannulated pancreatic duct, 3 piglets with ileal cannulae, and 3 intact piglets).

To obtain "pure" pancreatic juice from a bird, a surgical operation was performed in the chronic experiment [7]. The essence of this operation was to create an isolated segment of the duodenum and transplant the main pancreatic duct into it, implanting two L-shaped fistulas and forming an external anastomosis making it possible to return the pancreatic juice to the duodenum if necessary. The piglets were operated according to the technique of Tkachyov [21]. From the duodenum, a 4-5-cm-long segment was cut out, into which the pancreatic duct flows, and a Y-shaped cannula was implanted (in an isolated segment of the intestine and the main intestine), allowing the pancreatic juice to return to the duodenum during the period outside the experiments.

Physiological experiments on poultry were performed using the method developed earlier [8]. The authors used PK-1 feed (All-Russian Research and Technological Poultry Institute RAS) with different protein ingredients. At least 3 experiments were performed on each bird to study digestion in each accounting period. Physiological experiments on piglets were started in the morning on an empty stomach after a 14-hour fast. The piglets were placed in a special machine in which they were kept for 3.5 hours. To collect pancreatic juice, a microtube was attached to the fistula through a special rubber adapter. In the first 30 minutes, the juice was collected after fast, then the pigs were given 500 g ($^1/_3$ of the daily norm) of compound feed (SK-4, Institute of Animal Physiology, Biochemistry and Nutrition, Russia) (as per Feeding norms and diets for agricultural animals. Handbook. Moscow, 2003), and the secretion was collected every 30 minutes for 180 minutes.

A Smith-Roe method in a modification for determining high enzyme activity [7] was applied for amylase analysis, proteolytic activity was assessed by Gammersten's hydrolysis of purified casein with colorimetric control (KFK-3, Zagorsk Optical and Mechanical Plant, Russia, $\lambda = 450$ nm) [7]. Lipolytic activity was assessed using a Sinnowa BS3000P biochemical analyzers (SINNOWA Medical Science & Technology Co., Ltd, China) and Screen Master LIHD113 (Hospitex Diagnostics S. r. L., Italy) with a set of veterinary diagnostic reagents for determining blood concentration of animal lipase (DIAKON-VET, Russia).

Blood of chickens was samples from the axillary vein, in pigs from the tail vein before feeding. Freshly prepared sodium citrate solution was added to the test tubes, the blood was centrifuged for 5 minutes at 5,000 rpm, and the resulting plasma was examined using a SINNOWA BS3000P flow biochemical semi-automatic analyzer (SINNOWA Medical Science & Technology Co., Ltd, China) using biochemical kits (DIAKON-VET, Russia). The activity of amylase and lipase was studied on a Chem Well 2900 (T) device (Awareness Technology, Inc., USA) using appropriate reagent kits (Human GmbH, Germany). Trypsin activity was evaluated using a semi-automatic biochemical analyzer Sinnowa BS-3000P (SINNOWA Medical Science & Technology Co., Ltd, China) [22].

Digestion trials of the digestibility of nutrients in the diet were performed by generally accepted methods (Methods of scientific and industrial research on poultry feeding. Molecular genetic methods for determining intestinal microflora. Sergiev Posad, 2013).

Statistical analysis was performed by ANOVA method (Statistica 10.0 software, StatSoft, Inc. USA; Microsoft Excel). The mean value (M) and standard errors of the means (±SEM) were calculated for the enzyme activity indicators. The reliability of differences was determined by Student's t-criterion, considering them statistically significant at $p < 0.05$.

Results. Table 1 describes the experiment design.

1. Design of the experiment on replacing dietary soybean cake/meal → sunflower cake in animals with different types of digestion

Investigation stage	Group (period)	Feed features
Hissex White Laying hens (*Gallus gallus* L.)		
Study of the pancreas secretory function	Control	Main diet (MD) with soybean cake (19.8%)
	Test	MD with sunflower cake (21.0%)
Study of the digestibility of feed nutrients	Control	MD with soybean cake (19.8%)
	Test	MD with sunflower cake (21.0%)
Determination of blood biochemical parameters	Control	MD with soybean cake (19.8%)
	Test	MD with sunflower cake (21.0%)
Crossbred (Danish Landrace and the Danish Yorkshire) piglets (*Sus scrofa domesticus* L.)		
Study of the pancreas secretory function	Control	MD with soybean meal (18.5%)
	Test	MD with sunflower cake (22.5%)
Study of the digestibility of feed nutrients	Control	MD with soybean meal (18.5%)
	Test	MD with sunflower cake (22.5%)
Determination of blood biochemical parameters	Control	MD with soybean meal (18.5%)
	Test	MD with sunflower cake (22.5%)

The composition and characteristics of the used main diets are shown in Tables 2 and 3

2. Composition (%) and quality indicators of diets for Hissex White laying hens (*Gallus gallus* L.)

Ingredient, indicator	Combined feed	
	control (feed 1)	test (feed 2)
Wheat	58.225	55.781
Sunflower cake	5.000	21.026
Soybean cake	19.784	8.912
Limestone (36%)	9.137	9.045
Soybean oil	1.936	3.026
Wheat bran	3.847	Absent
Monocalcium phosphate	1.149	1.233
Table salt	0.250	0.250
Lysine (98%)	0.073	0.214
Sodium sulphate	0.205	0.182
Feed methionine (98%)	0.214	0.151
Premix	0.180	0.180
In 100 g of feed:		
exchange energy, kcal	270.00	270.00
raw fiber, g	4.89	5.92
raw protein, g	16.70	17.20
raw fat, g	6.72	8.12
lysine, g	0.73	0.80
methionine, g	0.44	0.45
calcium, g	4.78	4.61
total phosphorus, g	0.89	0.90

3. Composition (%) and quality indicators of diets for crossbred piglets (*Sus scrofa domesticus* L.)

Ingredient, indicator	Combined feed	
	control (feed 3)	test (feed 4)
Wheat	50	47.4
Barley	11.84	11.2
Wheat bran	15	14.2
Soybean meal SP (42%)	18.55	Absent
Sunflower cake	Absent	22.5
Sunflower oil	1.45	1.0
Chalk	1.13	1.0
Tricalcium phosphate	0.79	0.7
Table salt	0.24	0.24
Lysine	Absent	0.22
Threonine	Absent	0.11
Premix KS-4-1	1	1.0
Total	100.0	100.0
1 kg of concentrate feed contains:		
energy feed units (EFU)	1.28	1.28
exchange energy, MJ,	12.8	12.8
net energy, MJ	9.5	9.5
dry matter, g	911.6	914.5
raw protein, g	170.5	170.0
raw fat, g	34.8	44.1
linoleic acid $C_{18:2}$, %	1.78	1.78
α- linolenic acid, %	0.19	0.19
raw fiber, g	50.4	99.0
crude ash, g	51.8	65.1
nitrogen-free extractives (NFE), g	638	683
lysine, g	7.8	7.8
methionine + cystine, g	5.60	6.22
threonine, g	5.9	5.9
calcium, g	7.53	7.50
total phosphorus, g	6.11	6.08

In the experiment on laying hens, concentrate feed was prepared in such a way that feed 1 had the prevalence of soybean cake and feed 2 — of sunflower cake. The analysis showed that feed 2 contained more raw fat (1.4%) and fiber (1.0%) than feed 1. In the study of the secretory pancreatic function in chickens (Fig. 1), we revealed an increase in lipolytic activity by 33.8% ($p < 0.05$) when replacing feed 1 with feed 2, which seems to be due to the quality of fat in sunflower cake [23], as well as an increase in the amount of raw fat in the experiment relative to the control. Protease activity increased by 28.1% ($p \geq 0.05$) as a result of changes in protein quality and a slight increase in the proportion of raw protein in the feed (by 0.5%), as well as the content of amino acids, which in total exceeded that in the control by 0.33%. From these re-

sults, it follows that the secretory function of the pancreas is adapted to the quality of the consumed feed.

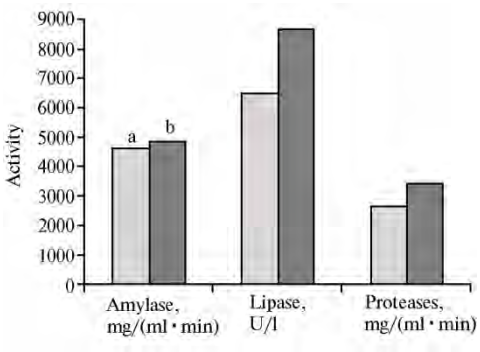

Fig. 1. Activity of pancreatic enzymes in laying Hissex White hens (*Gallus gallus* L.) depending on dietary protein ingredients: a — feed 1, b — feed 2 (n = 20, lab test on fistulated poultry; see Table 2 for the composition of diets, the values of protease activity increased by 10 times).

To understand the mechanisms of adaptation of pancreatic enzyme production to the changing composition of the diet, we studied the dynamics of the pancreas secretory function of chickens (Fig. 2).

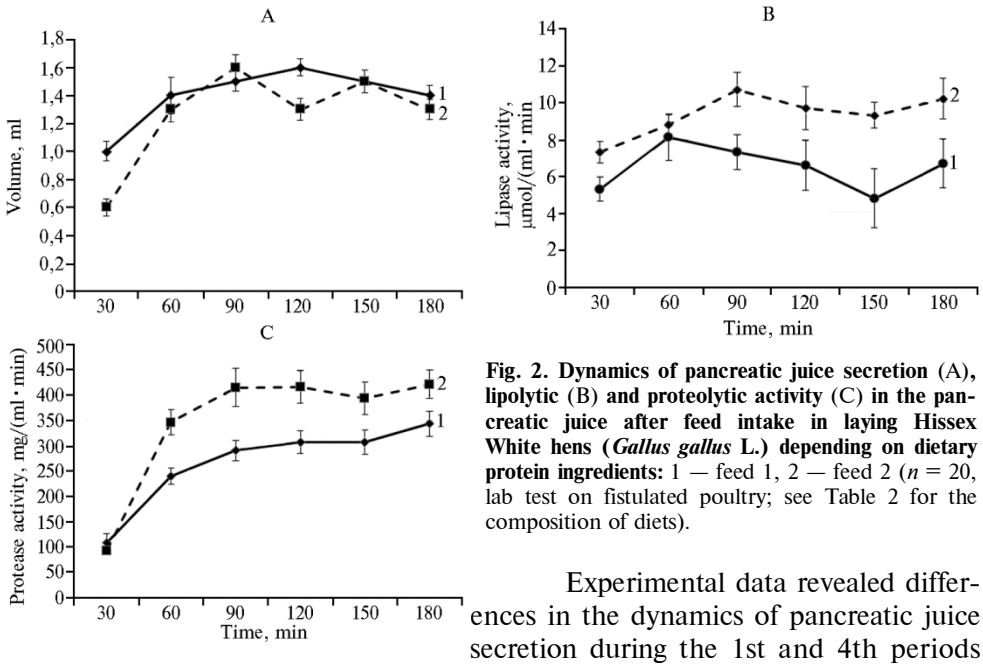

Fig. 2. Dynamics of pancreatic juice secretion (A), **lipolytic** (B) **and proteolytic activity** (C) **in the pancreatic juice after feed intake in laying Hissex White hens (*Gallus gallus* L.) depending on dietary protein ingredients:** 1 — feed 1, 2 — feed 2 (n = 20, lab test on fistulated poultry; see Table 2 for the composition of diets).

Experimental data revealed differences in the dynamics of pancreatic juice secretion during the 1st and 4th periods of the experiment depending on the diets (see Fig. 2, A), which is associated with the complex reflex and neurochemical phases of regulation of the external secretory function of the pancreas [7, 8].

The changes were the most apparent in the activity of lipase (see Fig. 2, B): the increase in the 1st period (before feeding) was by 37.7% ($p < 0.05$), in the 90th min by 46.6% ($p < 0.05$) when using sunflower cake, and in the 150th min by 93.7%. The difference in the basal activity of enzymes when using different protein supplements indicates a long-term adaptation to feed 2, which causes increased lipolytic activity. The main difference in the dynamics is that the intensive growth of lipase activity in the presence of sunflower cake in the feed continued until the 90th min of the experiment, and then its value remained until the end of the experiment.

The dynamics of proteolytic activity in chicken pancreatic juice when replacing soybean meal with sunflower meal in the diet (see Fig. 2, C) had no fundamental differences, but the curve of enzyme activity was higher in using dietary sunflower cake. If feeding activity parameters were not significantly different, in the 60th, 90th and 120th minutes of the experiment, the activity of proteases when

adding the sunflower meal was significantly higher than indicators for the diet with soybean meal. The first 30-60 minutes of the postprandial period correspond to the complex reflex phase of regulation of pancreatic secretion, which is determined by both conditional and unconditional reflexes (due to the presence of taste receptors in the oral cavity of chickens) [24-26], and from the 90th minute by hormonal factors that also affect the secretory function of the pancreas [27].

The digestibility of feed nutrients is associated with chicken pancreas secretory function (Table 4).

4. Digestibility and utilization of feed nutrients in laying Hissex White hens (*Gallus gallus* L.) depending on dietary protein ingredients (M±SEM, n = 10, lab test on intact poultry)

Diet	Digestibility, %				Availability, %		Utilization, %		
	protein	dry matter	fat	fiber	Ca	P	N	lysine	methionine
Feed 1	88.3±0.43	73.0±0.73	90.1±0.67	24.1±2.42	62.8±2.90	27.3±3.51	55.5±1.94	90.0±1.16	95.1±0.32
Feed 2	86.9±0.46	71.5±0.86	93.6±0.58*	11.8±2.74*	64.2±2.82	32.3±4.06	51.9±1.87	90.9±0.25	93.7±0.28

N o t e. Feed 1 — control, feed 2 — test. See Table 2 for diet compositions.
* Difference from the control is statistically significant at $p < 0.05$.

Analysis of the digestibility and availability of feed nutrients shows that replacing soybean meal with sunflower worsens the digestibility of fiber by 12.3% ($p < 0.05$), feed dry matter by 1.5%, protein by 1.4%, and methionine by 1.4%. In feed 2, raw fat was digested 3.5% better ($p < 0.05$) than in feed 1, which is consistent with the lipolytic activity of pancreatic juice (see Fig. 2).

5. Blood biochemical parameters of laying Hissex White hens (*Gallus gallus* L.) depending on dietary protein ingredients (M±SEM, n = 15, lab test on intact poultry)

Indicator	Control (feed 1)	Test (feed 2)	To control, %
Trypsin, U/l	154±16.8	105±10.8*	–31.8
Amylase, U/l	166±9.3	202±27.8	+21.7
Lipase, U/l	38±2.5	32±2.2	–15.8
Total protein, g/l	23.5±1.40	38.6±3.40*	+64.2
Uric acid, μmol/l	95±10.3	109±14.9	+14.7
Glucose, mmol/l	2.8±0.58	4.1±0.91	+46.4
Alkaline phosphatase, U/l	853±85.0	503±81.1*	–41.0
Cholesterol, mmol/l	2.6±0.70	3.9±0.90	+50.0
Triglycerides, mmol/l	5.6±1.63	8.7±2.76	+55.3

N o t e. See Table 2 for diet compositions.
* Difference from the control is statistically significant at $p < 0.05$.

Biochemical study of blood of laying hens (Table 5) revealed significant changes in indicators related to protein metabolism. The trypsin activity during experiment decreased by 31.8% ($p < 0.05$). In the case of a control diet with soybean cake, the activity of trypsin in the blood was high. The transition of poultry to a diet similar in the protein level, but with a different ingredient composition (replacing soybean cake with sunflower cake) led to a decrease in trypsin activity in the blood, apparently due to an increase in the activity of common proteases in the intestine [28]. It may be due to the presence of chlorogenic acid in sunflower oil cake that inhibits trypsin and lipase [23]. The total protein content in the control was lower than optimal, which is probably due to a deficit of full-fledged proteins and limiting amino acids in the diet. It is no accident that in the experiment, the use of concentrated feed caused an increase (within the margin of error) in the uric acid indicator and an increase in the total protein content in the blood by 64.2% ($p < 0.05$), which corresponds to its normal physiological value for laying hens. A decrease in alkaline phosphatase activity by 41.0% ($p < 0.05$) indicates a change in liver function, which produces this enzyme that hydrolyzes phosphorus bonds.

In the diets of experimental piglets, the used concentrate feed varied in raw fat and fiber content: in feed 4, compared to feed 3, the content of these components was higher by 9.3 and 21.1%, respectively; the diets did not differ in the amount of raw protein. The composition of limiting amino acids and their content also did not differ significantly due to adjustments using different amounts of synthetic amino acids. The excess of methionine and cystine in feed 4 (even without adding synthetic methionine to feed) was due to their increased content in sunflower cake.

6. Pancreatic secretory function of 5-month-old crossbred (Danish Landrace and Danish Yorkshire) piglets (*Sus scrofa domesticus* L.) depending on feed composition (M±SEM, n = 3, lab test on fistulated animals)

Indicator	Control (feed 3)	Test (feed 4)
Amount of pancreatic juice per experiment, ml	139,5±1,50	134,9±7,16
Enzyme activity in 1 ml juice:		
amylase, mg/(ml · min)	1564±267,0	1800±92
lipase, μmol/(ml · min)	14,8±1,01	15,1±1,16
proteases, mg /(ml · min)	88,1±9,92	49,6±6,04*

N o t e. See Table 3 for diet compositions.
* Difference from the control is statistically significant at $p < 0.05$.

The study of the pancreas secretory function of piglets (Table 6) showed that when replacing soybean protein with sunflower, the lipase activity did not change, although the fat content in this feed was 9.3% higher compared to the control diet. Perhaps the reason is the presence of chlorogenic acid in sunflower cake, which serves as an inhibitor of trypsin and lipase. We found no significant differences in amylase activity. The number of proteases decreased by 43.7% in the experimental period with the same content of protein and amino acids in compound feeds 3 and 4. Therefore, in piglets (as in chickens), the secretory function of pancreas adapts to the feed quality. We compared the dynamics of pancreatic juice release and enzyme activity after feeding by analyzing the mechanisms of the pancreas adaptation to the new feed (Fig. 3, A, B).

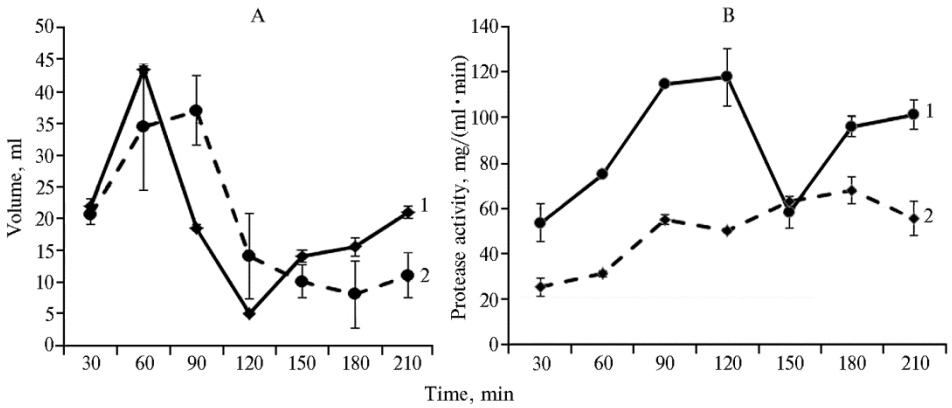

Fig. 3. Dynamics of pancreatic juice secretion (A) **and proteolytic activity** (B) **in pancreatic juice after feed intake in 5-month-old crossbred (Danish Landrace and Danish Yorkshire) piglets (*Sus scrofa domesticus* L.) depending on feed composition:** 1 — feed 3, 2 — feed 4 (n = 20, **lab test on** fistulated animals; see Table 3 for diet compositions 3).

The data indicate (see Fig. 3, A) that in piglets in the first 30 minutes after receiving the feed, the secretion of pancreatic juice increased by 2.0 and 1.3 times when using soybean or sunflower components in the diet, respectively. Further on, by the 90th minute of the experiment in the version with soybean meal, the amount of pancreatic juice decreased to the initial value, with sunflower cake – continued to increase to the 90th minute of the experiment and became 2 times higher compared

to the previous period. From the 120th to 210th minute of the experiment, an increase in secretions in the first period was observed; in the second period (when adding sunflower cake in the diet), significant changes in the amount of secretions were not observed. Therefore, despite similar gross amounts of pancreatic juice for the experiment, in variants with different protein feed ingredients, the dynamics of secretions after food intake differed significantly in the complex-reflex and neurohumoral phases of pancreatic function regulation.

Since there were significant changes in the activity of proteases, the authors analyzed the dynamics of this indicator in the postprandial period with different sources of protein in the feed of piglets (see Fig. 3, B). Proteolytic activity in the control (feed 3) increased in the postprandial phase of digestion up to the 120th minute of the experiment, followed by a decline and a new rise, typical for the neurochemical phase of regulation of pancreatic secretion. When using sunflower cake in the diet of piglets, the dynamics of protease activity was characterized by a 2.2-fold increase within 60 minutes after feeding (as in the control), but since the basal level of activity was 2 times lower, the value of this indicator in the postprandial period significantly differed at all points of the curve, except for the 150th minute (intersection of graphs). Therefore, in piglets, when replacing one protein component in the diet with another, the pancreas reacts by changing the predominantly proteolytic activity, which indicates that the secretory function of the pancreas is adapted to the feed quality.

No significant changes in the dynamics of amylase and lipase activity were observed in piglets after replacing the protein component of the feed.

Thus, the replacement of soybean meal with sunflower meal in the diet of piglets is reflected in the dynamics of pancreatic juice release and protease activity in the postprandial period of digestion, which indicates the adaptation of the pancreas to individual feed components with the same content of raw protein in concentrate feeds. The study of visceral organs in weaning pigs using common and low-oligosaccharide soybean meal revealed no differences [29]. At the same time, molecular genetic methods lead to the conclusion that the content of fats and carbohydrates in the diet of pigs significantly affects gene expression [30]. Therefore, the use of cannulated animals to study the adaptation of pancreatic secretion has a perspective.

When replacing soybean meal with sunflower meal, neither visible nor ileal digestibility of protein was significantly changed (Table 7), although the ileal method is considered the most objective when assessing the availability of nutrients in the diet [31]. Even with a higher content of fiber and fat in the second diet (experiment), there was a significant increase in their digestibility, by 11.7 and 28.7%, respectively ($p < 0.05$). At the same time, ash and nitrogen-free extractives were digested worse.

7. Digestibility and utilization of feed nutrients in 5-month-old crossbred (Danish Landrace and Danish Yorkshire) piglets (*Sus scrofa domesticus* L.) depending on feed composition ($M \pm$SEM, $n = 10$)

Diet	Digestibility, %								
	Pv	P	DM	OM	Fib	F	A	NFE	GE
Control	78.42±0.90	75.4±1.89	78.3±0.25	80.4±0.24	36.7±0.78	61.2±0.50	43.0±0.75	88.1±0.19	79.6±0.32
Test	81.21±1.87	77.0±2.12	77.1±0.32	80.0±0.47	41.0±0.56*	78.8±1.82*	37.6±0.80*	87.3±0.54	78.4±0.64

N o t e. Pv — protein (visible digestibility), Pi — protein (ileal digestibility), DM — dry matter, OM — organic matter, Fib — fiber, F — fat, A — ash, NFE — nitrogen-free extractives, GE — gross energy. See Table 3 for the composition of the diets.

* Differences from the indicator for feed 3 (control) are statistically significant at $p < 0.05$.

Biochemical studies have not revealed significant changes in the activity of digestive blood enzymes and biochemical parameters in piglets when using various protein supplements in the diet (Table 8).

8. Blood biochemical parameters of 5-month-old crossbred (Danish Landrace and Danish Yorkshire) piglets (*Sus scrofa domesticus* L.) depending on feed composition (M±SEM, n = 10)

Indicator	Control (feed 3)	Test (feed 4)
Trypsin, U/l	690±56.0	620±34.1
Amylase, U/l	179.5±83.9	141.7±32.7
Lipase, U/l	51.0±12.75	76.5±22.08
Total protein, g/l	57.03±2.59	62.2±2.24
Urea, mmol/l	4.3±0.29	5.2±0.45
Alanine aminotransferase, U/l	89.6±9.94	71.8±10.1
Aspartate aminotransferase, U/l	70.2±12.9	57.5±7.67

N o t e. See Table 3 for the composition of the diets.

Since the digestive systems of birds and mammals differ morphologically and functionally, the biological mechanisms of feed adaptation are not the same. For the first time in world practice, we have undertaken a comparative study of the features of such adaptation to changes in the protein component of the diet when replacing soybean meal with sunflower meal on fistulated animals.

The research showed that the amount of pancreatic juice during the experiment (per 1 kg of live weight) was almost 2.2 times higher in chickens than in piglets. Amylase activity, when used feed 1 for chickens, was 4,620±253.1, in piglets 1,564±267.0 mg/(ml· min), which in absolute values is almost 3 times lower than in chickens. When using feed 2, the amylase activity in chickens increases to 4,855±290.0, and in piglets to 1,800±92.0 mg/(ml· min).

The lipolytic activity of pancreatic juice in piglets in absolute values was higher than that in chickens by almost 2 times and amounted to 14.8±1.01 and 6.5±0.51, respectively, when the control feed was fed, and 15.1±1.16 and 8.7±0.62 mmol/(ml· min) when replacing the protein component (soybean cake/meal → sunflower cake). However, per 1 kg of live weight, the ratio of lipolytic activity in chickens and piglets was 17:1.

Protein hydrolysis in chickens was more intense. The activity of proteases in animals receiving the control feed was 3 times higher in chickens than in piglets – 267±17.9 vs 88.1±9.92 mg/(ml· min). Sunflower meal increased protease activity in chickens to 342±61.3 mg/(ml· min) but decreased it in pigs to 49.6±6.04 mg/(ml· min). Taking in mind that the digestibility of raw protein does not change significantly, one can assume the presence of trypsin inhibitors in soybean meal, which increases the activity of proteases. This assumption is confirmed in the works of Tarasenko [31], indicating higher availability of amino acids in sunflower meal (25.4%) compared to soybean. Our data are also partially consistent with the results of comparative studies of the physical and chemical properties and enzymatic activity of pancreatic juice in different animals [32].

The activity of digestive enzymes in the blood plasma of animals with different types of digestion significantly differed in trypsin: as calculated per 1 kg of live weight, the activity was 100 U/l in chickens and 14 U/l in pigs. Consequently, the metabolic processes in chickens are more intense than in pigs, and the trypsin activity index (trypsin activity/live weight) can serve as a criterion for evaluating the activity of metabolism.

It is known that the provision of feed is the most expensive area in the livestock economy, but the largest reserves are hidden here [20]. Knowledge of the biological effects of feed ingredients forms the basis for the formation of balanced diets for farm animals. The data obtained in this paper characterize the main digestive and nutrition-related metabolic processes comprehensively. This approach makes it possible to interpret the results of the study in both theoretical and practical aspects.

Thus, the data of the original experiments carried out on fistulated animals

allow drawing the following conclusions. A distinctive feature of the exocrine function of the pancreas of birds is its high intensity: the amount of pancreatic juice per unit of live weight in chickens is 2.2 times higher than in pigs, the amylase activity is 94 times higher, protease activity 145 times higher, and lipase 17 times higher. The trypsin activity index in the blood plasma of chickens is significantly (7 times) higher than in pigs, which indicates increased metabolism. Adaptation of pancreatic secretion in chickens when replacing soybean meal with sunflower meal is characterized by an increase in lipase activity in pancreatic juice by 33.8%. In this case, significant differences are observed in the basal and postprandial period 60 minutes after feeding. As a result, the raw fat digestibility increases by 3.5% in feeding sunflower cake. In pigs, when replacing soybean meal with sunflower meal, the external secretory function of the pancreas reacts by 43.8% reducing the proteolytic activity. At the same time, the digestibility of raw protein does not change due to the presence of a trypsin inhibitor in soybean meal. In animals, regardless of the type of digestion, the adaptation of pancreatic secretion occurs in the case of non-parallel changes in the enzymatic activity in response to qualitative changes in feed ingredients.

REFERENCES

1. Gilliland E.L., Glazer G. Parallel secretion of enzymes by the rabbit pancreas. *The Journal of Physiology*, 1980, 303(1): 33-41 (doi: 10.1113/jphysiol.1980.sp013268).
2. Fomina L.S. V sbornike: *Fiziologiya pishchevareniya* /Pod redaktsiei A.V. Solov'eva [Digestion physiology. A.V. Solov'ev (ed.)]. Leningrad, 1974: 360-369 (in Russ.).
3. Pavlov I.P. *Fiziologiya pishchevareniya. Tom 5, kniga 1* [Digestion physiology. Vol. 5, book 1]. Moscow-Leningrad, 1952 (in Russ.).
4. Rothman S., Leibow C., Isenman L. Conservation of digestive enzymes. *Physyological Reviews*, 2002, 82(1): 1-18 (doi: 10.1152/physrev.00022.2001).
5. Ren L.Q., Zhao F., Tan H.Z., Zhao J.T., Zhang J.Z., Zhang H.F. Effects of dietary protein source on the digestive enzyme activities and electrolyte composition in the small intestinal fluid of chickens. *Poultry Science*, 2012, 91: 1641-1646 (doi: 10.3382/ps.2011-02081).
6. Keomanivong F.E., Grazul-Bilska A.T., Redmer D.A., Bass C.S., Kaminski S.L., Borowicz P.P., Kirsch J.D., Swanson K.C. The impact of diet and arginine supplementation on pancreatic mass, digestive enzyme activity and insulin-containing cell cluster morphology during the estrous cycle in sheep. *Domestic Animal Endocrinology*, 2017, 59: 23-29 (doi: 10.1016/j.domaniend.2016.10.001).
7. Batoev Ts.Zh. *Fiziologiya pishchevareniya ptits* [Physiology of bird digestion]. Ulan-Ude, 2001: 10-63 (in Russ.).
8. Fisinin V.I., Vertiprakhov V.G., Grozina A.A., Andrianova E.N., Shevyakov A.N., Khasanova L.V., Anshakov D.V. *Veterinariya i kormlenie*, 2018, 5: 4-7 (doi: 10.30917/ATT-VK-1814-9588-2018-5-1) (in Russ.).
9. Perepelkina L.I., Berdnikov P.P., Samsonenko I.A. *Vestnik Altaiskogo gosudarstvennogo agrarnogo universiteta*, 2012, 7(93): 67-68 (in Russ.).
10. Vertiprakhov V.G. Pancreatic exocrine function in hens with addition of lysine and methionine to their ration. *Russian Agricultural Sciences*, 2015, 41(2-3): 171-174 (doi: 10.3103/S1068367415020275).
11. Fisinin V.I., Vertiprakhov V.G., Grozina A.A., Khasanova L.V. Pancreatic secretion and intestinal digestibility of amino acids in chicken at different dietary protein level and quality. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2017, 52(2): 374-381 (doi: 10.15389/agrobiology.2017.2.374eng).
12. Korot'ko G.F. *Rossiiskii zhurnal gastroenterologii, gepatologii, koloproktologii*, 2014, 3: 4-12 (in Russ.).
13. Rothman S., Liebow C., Grendell J. Nonparallel transport and mechanisms of secretion. *Biochimica et Biophysica Acta*, 1991, 1071(2): 159-173 (doi: 10.1016/0304-4157(91)90023-P).
14. Mozheiko L.A. *Novosti mediko-biologicheskikh nauk*, 2017, 15(1): 72-76 (in Russ.).
15. Brannon PM. Adaptation of the exocrine pancreas to diet. *Annual Review of Nutrition*, 1990, 10: 85-105 (doi: 10.1146/annurev.nu.10.070190.000505).
16. Swanson K.C., Matthews J.C., Woods C.A., Harmon D.L. Postruminal administration of partially hydrolyzed starch and casein influences pancreatic α-amylase expression in calves. *The Journal of Nutrition*, 2002, 132(3): 376-381 (doi: 10.1093/jn/132.3.376).

17. Sans M.D., Williams J.A. Translational control of protein synthesis in pancreatic acinar cells. *Int. J. Gastrointest. Cancer*, 2002, 31(1-3): 107-115 (doi: 10.1385/IJGC:31:1-3:107).
18. Huguet A., Savary G., Bobillier E., Lebreton Y., Le Huërou-Luron I. Effects of level of feed intake on pancreatic exocrine secretions during the early postweaning period in piglets. *Journal of Animal Science*, 2006, 84(11): 2965-2972 (doi: 10.2527/jas.2006-044).
19. Liu K., Shen J., Cao Y., Cai C., Yao J. Duodenal infusions of isoleucine influence pancreatic exocrine function in dairy heifers. *Archives of Animal Nutrition*, 2018, 72(1): 31-41 (doi: 10.1080/1745039X.2017.1396144).
20. Sineshchekov A.D. *Biologiya pitaniya sel'skokhozyaistvennykh zhivotnykh* [Biology of farm animals' nutrition]. Moscow, 1965 (in Russ.).
21. Tkachev I.Z. *Kompleksnoe (fiziologicheskoe i zootekhnicheskoe) izuchenie protsessov pitaniya u svinei* [Comprehensive (physiological and zootechnical) study of nutrition processes in pigs]. Moscow, 1988: 25-26 (in Russ.).
22. Vertiprakhov V.G., Grozina A.A. *Veterinariya*, 2018, 12: 51-54 (doi: 10.30896/0042-4846.2018.21.12.51-54) (in Russ.).
23. Andrianova E.N., Egorov I.A., Prisyazhnaya L.M., Grigor'eva E.N., Rebrakova T.M. *Ptitsa i ptitseprodukty*, 2016, 2: 39-41 (in Russ.).
24. Kudo K., Nishimura S., Tabata S. Distribution of taste buds in layer-type chickens: scanning electron microscopic observations. *Animal Science Journal*, 2008, 79(6): 680-685 (doi: 10.1111/j.1740-0929.2008.00580.x).
25. Kudo K., Shiraishi J., Nishimura S., Bungo T., Tabata S. The number of taste buds is related to bitter taste sensitivity in layer and broiler chickens. *Animal Science Journal*, 2010, 81(2): 240-244 (doi: 10.1111/j.1740-0929.2009.00729.x).
26. Wang Z., Yoshida Y., Kramer N.E., Kawabata F., Tabata S., Woo K. Kim W.K., Liu H.-X. Abundant proliferating cells within early chicken taste buds indicate a potentially "built-in" progenitor system for taste bud growth during maturation in hatchlings. *Histology and histopathology*, 2019, 34(5): 503-511 (doi: 10.14670/HH-18-055).
27. Chey W.Y. Regulation of pancreatic exocrine secretion. *Int. J. Pancreatol.*, 1991, 9(1): 7-20 (doi: 10.1007/BF02925574).
28. Fisinin V.I., Vertiprakhov V.G., Grozina A.A. The exocrine pancreatic function in chicken (*Gallus gallus* L.) fed diets containing different ingredients. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2018, 53(4): 811-819 (doi: 10.15389/agrobiology.2018.4.811eng).
29. Pangeni D., Jendza J.A., Meno D.R., Anil L., Yang X., Baidoo S.K. Effect of replacing conventional soybean meal with low oligosaccharide soybean meal fed to weanling piglets. *Journal of Animal Science*, 2016, 95(1): 320-326 (doi: 10.2527/jas.2016.0780).
30. Mentzel C.M.J., Cardoso T.F., Lex A.M.J., Sørensen D.B., Fredholm M., Cirera S. Fat and carbohydrate content in the diet induces drastic changes in gene expression in young Gцttingen minipigs. *Mamm. Genome*, 2017, 28: 166-175 (doi: 10.1007/s00335-017-9690-y).
31. Tarasenko O.A., Omarov M.O., Golovko E.N., Kashirina M.V. *Zhivotnovodstvo Rossii*, 2007, 10: 27-28 (in Russ.).
32. Smolin S.G. *Fiziko-khimicheskie pokazateli i aktivnost' fermentov soka podzheludochnoi zhelezy u kur, svinei i sobak: monografiya* [Physico-chemical parameters and activity of pancreatic juice enzymes in chickens, pigs and dogs: a monograph]. Krasnoyarsk, 2008 (in Russ.).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1135-1143
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.52/.58:636.018

doi: 10.15389/agrobiology.2019.6.1135eng
doi: 10.15389/agrobiology.2019.6.1135rus

COMPARATIVE EVALUATION OF THE PECULIARITIES OF STRESS REACTIVITY OF THE RUSSIAN WHITE BREED CHICKEN WITH *sw+* MUTATION AND AMROX IN HYPOTHERMIA CONDITIONS DURING EMBRYONAL AND EARLY POSTNATAL PERIODS OF ONTOGENESIS

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The authors declare no conflict of interests
Acknowledgements:
The work has been carried out under the State Task AAAA-A18-118021590129-9.
Received June 21, 2019

Abstract

Modern programs of conservation of poultry genetic resources suggest the need to study specific characteristics of breeds for their further use in breeding. The ability of chickens to adapt and maintain productivity over a wide range of temperatures is an important economically significant feature, since maintaining a temperature optimum to realize the genetic potential of productivity requires significant energy costs. Regarding this, the unique long-term experiment of RRIFAGB on the population of Russian white breed chickens, started by A.N. Sokolova, is of interest. This population was created through selection for resistance to low temperatures in the first days of life (15-22 °C in the first 5 days with a gradual decrease to 14-11 °C to 21-30-day-old age) and the keeping of adult chickens in winter at a temperature below 0 °C. As a result, genotypes appeared, differing not only in thermal resistance, but also possessing an increased resistance to diseases of the leukemia-sarcoma complex and white embryonic down (so-called «snow whites»). The aim of this work was to investigate the degree of adaptive response of Russian white embryos (*Gallus gallus domesticus*) and neonatal chicks, homozygous for the gene *sw+*, to a lower ambient temperature compared to the breed Amrox, resulting in the first demonstration of interbreed differences in epigenetic adaptation of chickens in early ontogeny. The thermal stability of *sw+* homozygous Russian white chickens («snow white» population) and Amrox breeds in prenatal (12.5 days of incubation) and early neonatal periods (when removed from the incubator) was studied in a comparative aspect. Thermal stability in embryos (n = 70) was assessed by changes in the volume of extraembryonic fluid; in chickens (n = 60) — by changes in body temperature and characteristic behavioral reactions under hypothermia. Body surface temperature was determined using Thermal Expert FL 13mm f/1.0 thermal imaging camera, and rectal temperature was determined with an electric thermometer. Interbreeding differences in the response to low temperatures in embryos and 1-day-old chickens were revealed. Cooling of incubated eggs for 6 hours at +20 °C caused a slight expected decrease in the embryo weight in both breeds, but led to an increase in the volume of allantois-amniotic fluid (as a protective mechanism). The decrease in temperature caused an increase in the amount of fluid in embryos of both breeds, but in «snow whites» by 8.2 % and in Amrox embryos to a lesser extent, by 6.7 %. It is found that neonatal «snow white» chicks, as a result of a 2-hour exposure at 16 °C, lose body heat less intensively (the body temperature range of 32,4-17,0 °C) than Amrox chickens (the body temperature range of 28,2-14,5 °C) with no critical decrease in rectal temperature allowed. The rectal temperature of Amroxes reduced by 12.7 % or 5.1 °C ($p < 0.001$) compared to that at the moment of removal from the incubator, while in «snow whites» — only by 3.7 % or 1.4 °C. The «snow white» chicks are mobile, whereas the Amrox chicks are torpid. Thus, the chickens of the Russian white breed, homozygous for *sw+* gene, along with the snow-white color of the down at 1-day age, have more perfect mechanisms of thermoregulation (mainly physical, not chemical) and are better adapted to the conditions of low temperatures during embryonic and early postnatal periods.

Keywords: *Gallus gallus domesticus*, chickens, thermoregulation, embryos, extraembryonic fluid, *sw+* gene, neonatal chickens, hypothermic stress

Modern programs for the conservation of genetic resources of poultry require the study of specific characteristics of breeds for further use in breeding

purposes. The ability to adapt and maintain productivity over a wide temperature range is an important economically significant feature, since maintaining a temperature optimum to implement the genetic potential of productivity requires significant energy costs. When solving the problem of thermal resistance of poultry, two approaches are considered, the first one is based on the study of epigenetic adaptation (including due to the dosed temperature effect in sensitive periods of embryogenesis) [1-3] and the second is genetic selection. The possibility of selection in order to obtain resistance to extreme housing temperatures has been proved [4-6]. Candidate genes associated with thermal resistance are identified both in birds of gene pool breeds and of industrial lines [7, 8].

A large number of studies all over the world are focused on the issues of chickens' thermal resistance. However, the overwhelming majority of them relate to bird resistance to tropical and subtropical climate conditions [2, 6], while the problem of adaptation to lower temperatures for a significant part of Russia is more relevant.

In this regard, a unique long-term experiment on a population of Russian White breed chickens, started by Sokolova in 1954 (All-Russian Research Institute of Farm Animals Genetics and Breeding, RRIFAGB) is of interest. The Russian White breed chickens were bred in the USSR on the basis of crossing the white Leghorn breed with local outbred chickens and approved in 1953. The population of Russian White chickens bred in the RRIFAGB Genetic Collection of Rare and Endangered Breeds of Chickens was created by selection for resistance to low temperatures in the first days of life (15-22 °C in the first 5 days with a gradual decrease to 14-11 °C to the age of 21-30 days) with adult animals kept in winter at temperatures below 0 °C [4, 5]. Sokolova [4, 5] found an uneven response of 1-day-old Russian White chickens to a decrease in ambient temperature. At 30 °C, the body temperature of all neonatal chickens was 39.6–40.0 °C. Under conditions of lowering the temperature to 21 °C, some individuals developed hypothermia, which led to death; in others, the temperature remained the same or decreased slightly (to 37-38 °C), and they maintained normal viability. It was also found that increased thermal resistance was inherited according to the dominant type, and thermal stability had monogenic inheritance. In generations of selection for thermal resistance, the role of chemical thermoregulation decreased, but the role of the physical one, aimed at reducing heat transfer, in particular by reducing the relative mass of the lungs, increased. As a result of selection under hypothermic stress, genotypes appeared that differed from the original bird not only by the thermal resistance of young animals but also by increased resistance to Marek's disease, leukemia (confirmed by experimental infection), as well as white embryonic down hair (the so-called "snow white"), the proportion of which was approximately 25% of the total number of chickens at hatch [4, 5]. The remaining chicks had predominantly ordinary yellow down hair, with the exception of a small number of individuals with an intermediate type of color. Possibly, the selection of neonatal chicks according to their degree of reactivity to such a stressful factor as sublethal low temperature influenced the mechanism of neuroendocrine regulation of melanogenesis, which is controlled by the pituitary, thyroid, steroid and sexual hormones [9], which resulted in a mutation in the color of the down hair of chicks.

The authors' assumption about the influence of the neuroendocrine status on the color of plumage in chickens is confirmed by the studies of Dmitriev [10, 11]. Thus, when assessing 10-week-old chickens from the Russian White population that had snow-white coloration of down hair at 1 day old, according

to the index of functional reserves of the adrenal glands (FRAG), detected by the introduction of adrenocorticotropic hormone (ACTH), it was found that the average concentration of corticosterone in the blood of "snow whites" in response to the introduction of ACTH was 82.6 ng/ml. Within the same experiment, 10-week-old chicks from the Aurora Blue population obtained by selection of black-motley Australorps with a high FRAG index were evaluated (this selection led to the individuals with a blue plumage color). The average concentration of corticosterone in the blood of chickens in the Aurora Blue population was 84.8 ng/ml. In other breeds of the collection, it was significantly lower (24.0-52.3 ng/ml). It is known that the increase in oxidative processes associated with increased thyroid function causes decomposition of melanin (in particular, these results in white color of arctic animals) [12].

At present, selection for resistance to low temperatures is not carried out, but as a result of selection according to the snow-white color of the down hair, a population of Russian White chickens homozygous for the sw^+ gene was created (snow white down). From the authors' point of view, this experimental population is of significant interest as a model for studying the genetic and physiological mechanisms that determine the thermoregulation processes in chickens in the embryonic and early postnatal periods of ontogenesis. The effect of cold stress on the morphophysiological parameters of embryos in the second half of the incubation period, including in terms of epigenetic adaptation, was studied as early as the 1950s and 1960s [13, 14], but without taking into account the pedigree features of the embryos.

This paper for the first time shows interbreeds differences in epigenetic adaptation of chickens in early ontogenesis.

Our goal was to study the degree of the adaptive response to a low ambient temperature in embryos and neonatal chicks of the Russian White breed homozygous for the sw^+ gene in comparison with the Amrox breed.

Techniques. Studies were carried out in 2018 on embryos (n = 35) and neonatal chicks (n = 30) of Russian White breed chickens (*Gallus gallus domesticus*), homozygous for the sw^+ gene, and the Amrox breed (respectively n = 35 and n = 30), bred in the Genetic Collection of Rare and Endangered Breeds of Chickens (RRIFAGB). Hens of both breeds were kept in individual cages with individual registration of egg-laying capacity.

Eggs were obtained from chickens at the age of 49 weeks. Eggs, as well as embryos and chickens, were weighed using HL-400 EX electronic scales (A&D Company Ltd., Japan). The yolk diameter was determined without violating the integrity of the shell using an ultrasound portable scanner Raskan (Rateks NPP, Russia) [15]. The eggs were incubated in laboratory conditions at the accepted temperature conditions for hens of the gene pool (1-2 days – 38.0 °C, 3-10 days – 37.8 °C, 17-21 days – 37.2 °C) in the incubator and hatcher Remil-Ts (Ramil NPP, Russia).

In the experiment, eggs with 5.5-day-old embryos were cooled to 20 °C for 6 hours; in the control group, they continued to be incubated under the standard regimen. The volume of extraembryonic fluid (allantoic and amniotic) was determined at the age of 12.5 days, when it reached its maximum value [15] using a measuring cylinder; the result was taken into account in absolute terms and as an output relative to the egg mass (volume/mass).

In neonatal chickens from the control group, the external and rectal body temperature was measured immediately when removed from the incubator. Body temperature in the region of the head and paws was determined using a

Thermal Expert FL 13mm f/1.0 thermal imager (Thermal Expert, South Korea), and rectal temperature was determined using a Microlife MT 3001 electronic thermometer (Microlife, China).

To avoid undesirable behavioral response to lower air temperatures (crowding), 1-day-old chickens were placed in individual cells of hatcher trays. The experimental group I (n = 10) was there for 2 hours at 24 °C, the experimental group II (n = 10) — for 1 hour at 16 °C. In young animals of both groups, the external and rectal temperatures were measured, then they were placed in a common tray and behavioral reactions were evaluated at room temperature (24 °C). The control bird (n = 10) was kept at a temperature of 30-32 °C optimum for chicks of this age.

Statistical processing of the results was carried out in Microsoft Excel. The group means (M) and the standard error of the means (± SEM) were determined. The significance of differences was evaluated using Student's t-test. Differences were considered statistically significant at $p < 0.05$.

Results. Amrox chickens were used for comparison given the fact that their productivity is the closest to that of Russian White chickens, 17.0±0.7 eggs per the month preceding the experiment, with a weight of 60.0±0.5 g (in Russian White, 21.0±0.5 eggs per month and 59.6±0.3 g, respectively). In addition, Amroxes were not subjected to selection for resistance to low ambient temperatures. The age of 5.5 days was chosen for cooling, because it was found that during this period (before the start of functioning of the neuroendocrine system) the resistance of embryos to cold stress was especially high (data not shown).

Interbreed differences in the degree of response of embryos to cold stress were identified in comparison with the generally accepted incubation mode (Table 1). Since the volume of extraembryonic fluid is largely dependent on the weight of the egg, its absolute and relative values are presented.

1. Egg weight, egg weight loss, embryo development and extraembryonic fluid output in chickens (*Gallus gallus domesticus*) of *sw*$^+$ gene homozygous Russian "snow white" breed and Amrox depending on the incubation mode (M±SEM)

Порода	Egg mass, g	Weight loss, %	Embryo, g	Fluid	
				ml	ml/g
Russian "snow white" (n = 35):					
control	58.9±0.70	6.9±0.10	8.9±0.10	12.2±0.20	0.207±0.0030[a]
experiment (cooling)	58.9±0.60	6.6±0.20	8.1±0.10	13.2±0.40	0.224±0.0040[c]
Amrox (n = 35):					
control	60.6±0.70	6.1±0.20	8.8±0.10	11.8±0.20	0.194±0.0020[b]
experiment (cooling)	60.7±0.70	6.0±0.20	8.4±0.10	12.6±0.30	0.207±0.0030[d]

N o t e. For a description of the groups (control and experience), see the Techniques section. Extraembryonic fluid output and development were evaluated in 12.5-day-old embryos.

[ab, cd, ac, bd] Differences are statistically significant at $p < 0.001$.

Cooling caused a slight decrease in the mass of embryos of both breeds, but led to an increase in the volume of allantoic-amniotic fluid: in "snow white" by 8.2%, in Amroxes by 6.7%. The increase in fluid volume in itself is consistent with Romanoff [14], who found that lowering the incubation temperature caused an increase in the size of the amnion. Probably, this is how its protective function is implemented.

Interbreed differences are, from the authors' point of view, of greater interest, since at this stage of development, they were identified for the first time and testify to the particularity of the reaction of "snow white" embryos to temperature stress. This phenomenon can be explained, on the one hand, by the result of many years selection of the population for adaptation to cold stress conditions, and, on the other, by the known differences in the thermal tolerance of adult chickens of different breeds. According to the literature, Leghorns better adapt to

heat stress compared to other breeds, and the Russian White breed was selected using Leghorns [14].

The theoretical prerequisites for conducting studies on neonatal chicks were the evidence that they are considered poikilothermic and only at an older age react with an increase in blood corticosteroids in response to exposure to low temperatures [16-18]. Chicks at the age of 1 day are not capable of this because of the imperfect mechanism of thermoregulation [19-21].

2. Reaction to temperature stress in neonatal chicks (*Gallus gallus domesticus*) of *sw*$^+$ gene homozygous Russian "snow white" breed and Amrox depending on differences in egg quality (M±SEM)

Indicator	Breed	
	Russian "snow white" (n = 10)	Amrox (n = 10)
Live weight, g	37.8±1.2	39.4±1.0
When removed from the incubator, 37.2 °C		
Temperature, °C:		
head	39.2±0.05[a]	38.3±0.06[d]
legs	38.5±0.12	38.4±0.17
rectal	37.7±1.2	40.1±0.69
1-day-old chickens after 2 hours at 24 °C (group I)		
Temperature, °C:		
head	38.7±0.36	38.9±0.25
legs	34.2±0.38[a]	32.5±0.31[c]
rectal	37.3±0.42[a]	38.5±0.13[b]
Behavioral response	Actively moving, looking for feed	Crowd in the corner of the tray, clinging to each other
1-day-old chickens after 1 hour at 16 °C (group II)		
Temperature, °C:		
head	33.1±0.32[a]	30.7±0.50[d]
legs	21.9±0.24[a]	19.1±0.83[c]
rectal	36.3±0.42[a]	35.0±0.39[b]
Behavioral response	Active, despite muscle trembling, eyes open, running, looking for feed, trying to get out of the tray	Numbness, muscular tremors, eyes closed, chicks fall sideways
Egg quality parameters (30 eggs for each breed)		
Egg weight, g	54.7±0.80	57.6±0.50
Yolk diameter, cm	2.88±0.010[a]	2.95±0.020[c]

[ab, ac, ad] Differences are statistically significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

It is known that under conditions of low temperatures in neonatal chickens, compensatory reactions of the body are activated, primarily behavioral: young animals erect feathers, trying to keep warm. Muscle tremors appear, with muscle contraction additional heat is released to heat the body [22-24]. If the effect of lowered temperatures is maintained, the body temperature drops by 3-4 °C or more, the chickens fall on their side, they develop a coma, and then death occurs. In the authors' studies, interbreed differences in the response of chickens to hypothermic stress were found.

When removed from the incubator, there were almost no statistically significant differences between breeds in live weight and temperature in the chickens' body (Table 2). Young animals of "snow white" in comparison with Amrox chicks had a lower rectal temperature (2.4 °C) and a higher temperature in the head area (0.9 °C). When the neonatal chickens were kept for 2 hours at a temperature of 24 °C, the thermoregulation differences became noticeable: in "snow white", there was only a slight decrease in limb temperature while maintaining the values of the remaining indicators, while Amrox chickens more intensively lost heat through their paws, their rectal temperature also decreased by 1.6 °C ($p < 0.05$). In an attempt to maintain body temperature, the chickens crowded in the corner of the tray, while the young animals of the Russian White breed were active and comfortable.

After 1 h at 16 °C, the differences turned out to be even more significant (Fig. 1). Amrox chickens heat losses became critical: rectal temperature decreased by 12.7% (or 5.1 °C) ($p < 0.001$) compared to that measured at the time

of removal from the incubator, while in “snow white” it was only 3.7% (by 1.4 °C); heat losses in the head and legs of the Amrox were also significantly higher.

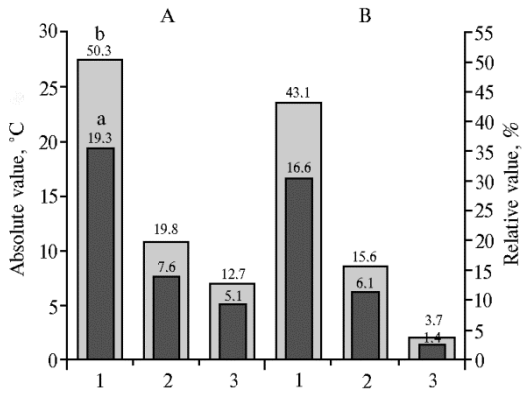

Fig. 1. Absolute (a) **and relative** (b) **decrease in temperature of neonatal chicks (*Gallus gallus domesticus*) of *sw*$^+$ gene homozygous Russian “snow white” breed and Amrox after 1 hour at 16 °C:** 1 — legs, 2 — head, 3 — rectal.

After 2 h at 16 °C, muscular tremors and drowsiness were observed in Amrox chickens, by the end of the period the young animals fell into a state of stupor. Russian White chickens reacted to cooling with muscle tremors, but were generally active (Fig. 2).

At a low temperature, Amrox chickens more intensively lost heat over the entire surface of the body (temperature range 28.2-14.5 °C) in comparison to “snow white”, which had lower heat range (32.4-17.0 °C), and areas of intense heating had limited localization (Fig. 3). Probably, this is also an adaptation feature of Russian White chickens.

Fig. 2. Behavioral response of neonatal chicks (*Gallus gallus domesticus*) of *sw*$^+$ gene homozygous Russian “snow white” breed and Amrox after 2 hours at 16 °C.

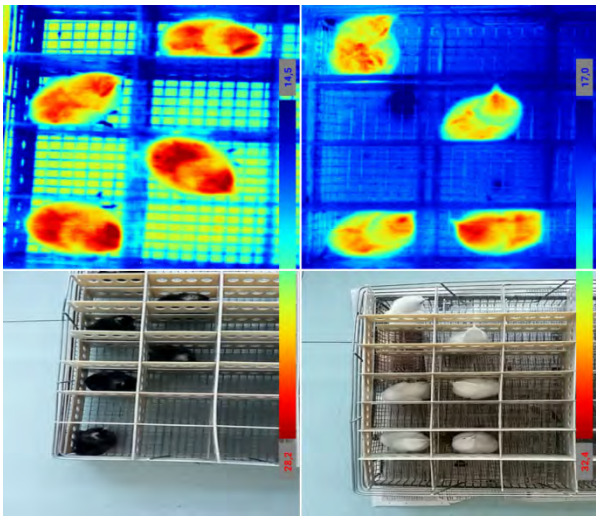

Рис. 3. Heat irradiation intensity neonatal chicks (*Gallus gallus domesticus*) of *sw*$^+$ gene homozygous Russian “snow white” breed (on the right; body temperature range 32.4-17.0 °C) **and Amrox** (on the left; 28.2-14.5 °C) **after 1 hour at 16 °C.**

According to the literature, at lower temperatures (20 °C for 12 h), the absorption of the yolk sac and the amount of fatty acids in the blood of neonatal chickens are increased [25]. Neonatal chickens are considered poikilothermic, the ability to maintain body temperature appears only at the age of 4 days (for example, 6-day-old chickens are able to tolerate the effects of 4 °C for 24 hours), they

become fully homeothermic by the age of 10 days [26]. In chickens from 4 days of age, the content of corticosteroids in the blood plasma increases in response to exposure to low temperatures. In 1-day-old chicks, this does not happen [26]. At the age of 1 day, a response to a lower temperature is also an increase in lipolysis processes, which is accompanied by an increase in the content of free fatty acids in the blood [27-29].

Similar results were obtained in our studies on the Plymouth Rock chickens of the B2 line of the domestic cross-country Baros 123 when growing an experimental group of chickens up to 6 days of age at 24 °C. As compared to the control, chickens from the experimental group showed a statistically significant decrease in the content of triglycerides in the blood (by 38%) and protein in the dry matter of the pectoral muscle (by 5%) with the same amount of dry matter [30, 31]. However, it is obvious that the Amrox chickens, which had the starting advantage in the form of a larger yolk in the egg (see Table 1), were not able to compensate for heat loss due to this mechanism.

Thus, chickens of the Russian White breed homozygous for sw^+ gene, along with snow-white coloration of down hair at 1 day old, have more advanced thermoregulation mechanisms (mainly physical, rather than chemical) and are better adapted to low-temperature conditions in the embryonic and early postnatal periods. This feature can be used in further breeding of the line, as well as in the creation of a hybrid bird with increased adaptive abilities in conditions of hypothermic stress.

REFERENCES

1. Mortola J.P. Metabolic response to cooling temperatures in chicken embryos and hatchlings after cold incubation. *Comparative Biochemistry and Physiology, Part A, Molecular & Integrative Physiology*, 2006, 145(4): 441-448 (doi: 10.1016/j.cbpa.2006.07.020).
2. Zabudskii Yu.I., Golikova A.P., Fedoseeva N.A. Heat training for prenatal period of ontogenesis as a method to increase the thermotolerance in poultry (review). *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2012, 4: 14-21 (in Russ.).
3. Black J.L., Burggren W.W. Acclimation to hypothermic incubation in developing chicken embryos (*Gallus domesticus*). *Journal of Experimental Biology*, 2004, 207: 1543-1552 (doi: 10.1242/jeb.00909).
4. Sokolova A.N. *Genetiko-selektsionnye metody sozdaniya populyatsii kur s povyshennoi ustoichivost'yu k neoplazmam. Avtoreferat kandidatskoi dissertatsii* [Genetic and selection methods for creating a chicken population with increased resistance to neoplasms. PhD Thesis]. St. Petersburg, 1999 (in Russ.).
5. Sokolova A.N. *Materialy XIII Vsemirnogo kongressa po ptitsevodstvu* [Proc. XIII World Poultry Congress]. Kiev, 1966: 151-155 (in Russ.).
6. Fisinin V.I., Kavtarashvili A.Sh. Heat stress in poultry. II. Methods and techniques for prevention and alleviation (review). *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2015, 50(4): 37-49 (doi: 10.15389/agrobiology.2015.4.431eng).
7. Nadaf J., Pitel F., Gilbert H., Duclos M.J, Vignoles F., Beaumont C., Vignal A., Porter T.E., Cogburn L.A., Aggrey S.E., Simon J., Le Bihan-Duval E. QTL for several metabolic traits map to loci controlling growth and body composition in an F_2 intercross between high- and low-growth chicken lines. *Physiological Genomics*, 2009, 38(3): 241-249 (doi: 10.1152/physiolgenomics.90384.2008).
8. Pinard-van der Laan M.H., Bed'hom B., Coville J.L., Pitel F., Feve K., Leroux S., Legros H., Thomas A., Gourichon D., Repérant J.M., Rault P. Microsatellite mapping of QTLs affecting resistance to coccidiosis (*Eimeria tenella*) in a Fayoumi × White Leghorn cross. *BMC Genomics*, 2009, 10: 31 (doi: 10.1186/1471-2164-10-31).
9. Kosimov R.B. *Biokhimicheskie osobennosti ingibirovaniya melanogeneza shersti u ovets. Doktorskaya dissertatsiya* [Biochemical features of the inhibition of wool melanogenesis in sheep. DSc Thesis]. Dushanbe, 2009 (in Russ.).
10. Lu L., Dmitriev V.B., Stanishevskaya O.I., Dementyeva N.V., Turlova Yu.G., Lapa M.A. Destabilizing effect of selection in chicken: factors of variability. *Proc. XXV World Poultry Conference.* Beijing, 2016: 345.
11. Dmitriev V.B. *Funktsional'nye endokrinnye rezervy v selektsii sel'skokhozyaistvennykh zhivotnykh* [Functional endocrine reserves in farm animal breeding]. St. Petersburg, 2009 (in Russ.).
12. Dement'ev G.P. *Zoologicheskii zhurnal*, 1948, 23(5): 189-197 (in Russ.).
13. Rol'nik V.V. *Biologiya embrional'nogo razvitiya ptits* [Biology of the embryonic development of birds]. Leningrad, 1968 (in Russ.).

14. Romanoff A.L. Membrane growth and function. *Annals of the New York Academy of Science*, 1952, 55: 2-288.
15. Lapa M.A. *Kriterii otsenki i otbora ptitsy s tsel'yu povysheniya pishchevykh i biotekhnologicheskikh kachestv yaits. Doktorskaya dissertatsiya* [Evaluation and selection criteria for poultry in order to improve the nutritional and biotechnological qualities of eggs. DSc Thesis]. St. Petersburg, 2015 (in Russ.).
16. Freake H.C., Oppenheimer J.H. Thermogenesis and thyroid function. *Annual Review of Nutrition,* 1995, 15: 263-291 (doi: 10.1146/annurev.nu.15.070195.001403).
17. Tachibana T., Takahashi H., Oikawa D., Denbow D.M., Furuse M. Thyrotropin-releasing hormone increased heat production without the involvement of corticotropin-releasing factor in neonatal chicks. *Pharmacology Biochemistry and Behavior*, 2006, 83(4): 528-532 (doi: 10.1016/j.pbb.2006.03.012).
18. Decuypere E., Iqbal A., Michels H., Kühn E.R., Schneider R., Abd el Azeem A. Thyroid hormone response to thyrotropin releasing hormone after cold treatment during pre- and postnatal development in the domestic fowl. *Hormone and Metabolic Research,* 1988, 20: 484-489.
19. Arancibia S., Rage F., Astier H., Tapia-Arancibia L. Neuroendocrine and autonomous mechanisms underlying thermoregulation in cold environment. *Neuroendocrinology*, 1996, 64(4): 257-267 (doi: 10.1159/000127126).
20. Debonne M., Baarendse P.J.J., Van Den Brand H., Kemp B., Bruggeman V., Decuypere E. Involvement of the hypothalamic pituitary-thyroid axis and its interaction with the hypothalamic-pituitary-adrenal axis in the ontogeny of avian thermoregulation: a review. *World's Poultry Science Journal*, 2008, 64(3): 309-321 (doi: 10.1017/S0043933908000056).
21. Laurberg P., Andersen S., Karmisholt J. Cold adaptation and thyroid hormone metabolism. *Hormone and Metabolic Research*, 2005, 37(9): 545-549 (doi: 10.1055/s-2005-870420).
22. DiMicco J.A., Zaretsky D.V. Physiology and Pharmacology of Temperature Regulation. The dorsomedial hypothalamus: a new player in thermoregulation. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 2007, 292(1): 47-63 (doi: 10.1152/ajpregu.00498.2006).
23. Mujahid A., Furuse M. Behavioral responses of neonatal chicks exposed to low environmental temperature. *Poultry Science*, 2009, 88(5): 917-922 (doi: 10.3382/ps.2008-00472).
24. Wentworth B., Cigan J., Schaaf T. Tolerance of Japanese quail embryos and young chicks to hypothermia. *Poultry Science*, 2009, 88(5): 1040-1043 (doi: 10.3382/ps.2008-00195).
25. Mujahid A., Furuse M. Oxidative damage in different tissues of neonatal chicks exposed to low environmental temperature. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, 2009, 152(4): 604-608 (doi: 10.1016/j.cbpa.2009.01.011).
26. Mujahid A. Acute cold-induced thermogenesis in neonatal chicks (*Gallus gallus*). *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, 2010, 156(1): 34-41 (doi: 10.1016/j.cbpa.2009.12.004).
27. Mujahid A., Furuse M. Homeothermy in neonatal chicks exposed to low environmental temperature with or without intracerebroventricular administration of corticotropin-releasing factor. *FEBS Letters*, 2008, 582(20): 3052-3060 (doi: 10.1016/j.febslet.2008.07.050).
28. Takahashi H., Iigo M., Ando K., Tachibana T., Denbow D.M., Furuse M. Regulation of body temperature by thyrotropin-releasing hormone in neonatal chicks. *Developmental Brain Research*, 2005, 157(1): 58-64 (doi: 10.1016/j.devbrainres.2005.03.004).
29. Hirabayashi M., Ijiri D., Kamei Y., Tajima A., Kanai Y. Transformation of skeletal muscle from fast- to slow-twitch during acquisition of cold tolerance in the chick. *Endocrinology*, 2005, 146(1): 399-405 (doi: 10.1210/en.2004-0723).
30. Stanishevskaya O.I., Toritsina E.S. *Ptitsevodstvo*, 2005, 1: 15-17 (in Russ.).
31. Toritsina E.S. *Biologicheskaya rol' zheltka yaits v povyshenii geneticheskogo potentsiala kur po khozyaistvenno-poleznym priznakam. Avtoreferat kandidatskoi dissertatsii* [The biological role of egg yolk in increasing the economically useful traits' genetic potential of chickens. PhD Thesis]. St. Petersburg, 2005 (in Russ.).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1144-1153
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Dietary additives

UDC 636.2:636.085.8:579

doi: 10.15389/agrobiology.2019.6.1144eng
doi: 10.15389/agrobiology.2019.6.1144rus

INFLUENCE OF ZASLON®-FITO ENTEROSORBENT OF MYCOTOXINS ON RUMEN MICROBIOME AND PRODUCTIVITY OF DAIRY COWS

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Foundation for Basic Research, grant No. 18-016-00207
Received June 4, 2019

Abstract

One of the global problems arising from the extension of opportunistic microflora in canned foods is toxin accumulation. Mycotoxins, the secondary metabolites of micromycetes, suppress immune system, disrupt the functioning of the rumen, intestines, liver, kidneys, reproductive and nervous system of cows, which leads to premature cow disposal. When developing preventive measures to increase the safety level of feed of own procurement, it should be borne in mind that the basis of the modern concept of control pathogens and their toxins is the use of natural environmentally friendly technologies. Modification of sorbents with natural essential oils can help restore the cows' rumen microbiome disrupted due to the toxins impact. The article presents the results of a study of unidentifiable rumen microorganisms in cattle by T-RFLP (terminal restriction fragment length polymorphism) method. The purpose of the study was to identify the effects of a phytobiotic enterosorbent Zaslon®-Fito on the composition of rumen microbiome, blood biochemical parameters and productivity. Farm test of Zaslon®-Fito enterosorbent was carried out on dairy cows (AO PZ "Plamya", Leningrad Province, Gatchina District). Two groups of dairy black and white mottled Holstein cows (*Bos taurus taurus*) of the 2-3rd lactation were formed, 15 animals in each group, for a 71-day experiment. The enterosorbent Zaslon®-Fito was added to the diet by mixing with feed, 20 g per animal per day. At the first step of the investigation, the ELISA method showed that these feeds were contaminated with aflatoxins (9 μg/kg, or 2.4 times higher than the maximum permissible concentration), zearalenone (264 μg/kg, or 2.6 times higher than MPC), and deoxynivalenol (310 μg/kg, MPC = 1000 μg/ml). The use of Zaslon®-Fito had a pronounced positive effect on the milk productivity of cows with a 5.5 % increase in the average daily milk yield per 1 animal (up to 1.8 kg). Analysis of the main biochemical parameters of dairy cows' blood as influenced by Zaslon®-Fito detected a tendency to total protein level optimization; in the control animals there was a decrease in glucose concentration (to the lower limit), and in the experimental group, we found out an increase in the amount of inorganic phosphorus compared to the control ($p \leq 0.05$). The counts of chytridiomycetes (class *Neocallimastigales*) and archaea in the rumen of cows fed dietary Zaslon®-Fito additive did not change, while the absolute number of bacteria increased to $1.25 \times 10^{11} \pm 5.43 \times 10^{9}$ genome equivalent per gram. Thus, our investigation shows that Zaslon®-Fito as the dietary additive has a direct positive impact on the animal health and productivity. This is due to multifunctional properties of Zaslon®-Fito, including both high adsorption of mycotoxins and regulation of the rumen microbiota due to some essential oils contained in the composition. The use of enterosorbent Zaslon®-Fito in the diet of dairy cows at the daily rate of 20 g per animal can reduce the risks of the harmful effects of feed mycotoxins and improve the production performance and the quality of the obtained products.

Keywords: unidentifiable rumen bacteria, mycotoxins, enterosorbent, cattle, T-RFLP analysis

Feed contamination by mold toxins remains one of the most urgent issue in dairy farming, since mycotoxins reduce animal resistance, cause metabolic disorders, digestive problems, and nervous system problems, impair reproductive

functions and increase the risk of abortion [1]. However, studies on the effects of mycotoxins on the health and livestock performance of cows are limited [2, 3]. One of the most problematic diseases in cattle is aflatoxicosis, which is associated with pathology of the liver and kidneys and a risk of carcinogenesis.

Mycotoxins in cattle rumen are metabolized to form other toxic compounds. So, aflatoxin B_1 is metabolized to aflatoxicol [4], which is no less toxic than its precursor. The remaining part of aflatoxin B_1 is absorbed in the gastrointestinal tract through passive diffusion and is hydroxylated in the liver to aflatoxin M_1 [5]. Residues of aflatoxin M_1 in milk constitute 1-6.2% of the amount of aflatoxin B_1 eaten [1, 6, 7]. Aflatoxin M_1 is resistant to high temperatures and does not decompose during pasteurization of dairy products. The highest inflow of aflatoxin from feed to milk is characteristic of highly productive cows at the peak of lactation [7].

Currently used sorbents have a number of negative properties: the ability to desorb mycotoxins, bind and excrete essential bioactive substances (EAS), i.e. micro and macro elements, vitamins and other nutrients from the body. In addition, the dosages for their inclusion in the diet are very high (up to 20-30 kg/t of feed). In this regard, the search for new sorbents that do not have such disadvantages is relevant.

In vitro experiments have shown a rather high degree of sorption of aflatoxin B_1, aflatoxin M_1, sterigmatocystin, T-2 toxin, zearalenone and ochratoxin A by diatomite [8]. In this case, the degree of sorption of aflatoxin B_1 was 100%. One hundred sixty broilers from 1 to 42 days old were used to evaluate the effectiveness of diatomite administration against the background of feeds that were significantly affected by aflatoxin B_1 [9]. When using diatomite, poultry body weight increased by 9.5%, feed intake by 7.4%, serum albumin content by 22.6%, lactate dehydrogenase activity by 44.4%. Despite a number of useful properties of diatomite, experiments demonstrating its effect on the sorption of mycotoxins in ruminant feeds have not been conducted previously.

Natural feed additives, the essential oils, are also popular as a substitute for antibiotics in livestock production [10]. Essential oils are a variety of EAS with a wide spectrum of action [11-13]. The prospect of their use in livestock production is associated with the proven ability to increase resistance, suppress unwanted microflora, stimulate the secretion of digestive enzymes and blood circulation, exhibit antioxidant effects, and improve the absorption of useful nutrients of feed [14, 15]. It is assumed that essential oils can positively affect protein metabolism and reduce the production of ammonia in the rumen [16, 17].

The present article for the first time shows that the use of dietary Zaslon®-Fito complex enterosorbent based on diatomite enriched with essential oils positively affects the health and productivity of dairy cows. The effect of this feed additive on the microflora pattern in rumen content is demonstrated for the first time.

The purpose of the study is to identify the characteristics of the effect of the phytobiotic enterosorbent Zaslon®-Fito on the composition of the rumen microbiome, blood biochemical parameters, and productivity of dairy cows (*Bos taurus taurus*).

Techniques. Scientific and economic tests were carried out at AO PZ Plamya (Leningrad Province, Gatchina District) in 2017. According to the principle of analogs, two groups (experimental and control) of dairy black and white mottled Holstein cows of the 2nd-3rd lactation were assigned, 15 animals in each. Cows were kept on a leash. At the beginning of the experiment, all animals were apparently healthy, were in the same conditions of feeding and keeping. In total, cows consumed 38.0 kg of natural feed, which corresponded to

10.8 MJ of metabolizable energy in dry matter. The duration of the experiment was 71 days. Cows in the control group consumed the main diet. Zaslon®-Fito (BIOTROF LLC, Russia) was introduced into the main diet of animals of the experimental group by mixing with feed at the rate of 20 g · animal^{-1} · days^{-1}.

Bulk feed samples were collected in triplicate using an aseptic sampler. The depth of immersion of the sampler in the trench was 1 m. Mycotoxins, except for deoxynivalenol (DON), were extracted from feed samples using 70% methanol, and DON was extracted using distilled water.

Blood for analysis was sampled in the morning in the fasting state and analyzed using standard methods. Indicators of milk productivity were determined in all animals. At the end of the experiment, rumen chyme samples were taken using a sterile probe and stored at –20 °C until molecular analysis.

The mass fraction of fat in milk was determined according to GOST 5867-90, protein in milk according to GOST 23327-98. The content of somatic cells in milk was determined (a SOMATOS device, Sibagropribor VPK LLC, Russia).

Mycotoxins in feed and milk were quantified by enzyme-linked immunosorbent assay (ELISA) (AgraQuant test systems, omer Labs, Inc., Austria) according to the attached protocols.

For ELISA analysis, an extract of the feed or milk sample (or standards) and mycotoxins conjugated with the enzyme were mixed, poured into microwells with antibodies, washed and the enzyme substrate was added. The intensity of the substrate staining was inversely proportional to the content of mycotoxin in a sample or standard. Then, stopping solutions were added, the 10% hydrochloric acid for zearalenone and T-2 toxin and 10% phosphoric acid for other mycotoxins. Optical density was measured at λ = 450 nm (a StatFax 303+ microstrip photometer, Awareness Technology, Inc., USA), comparing the sample and standards.

Total DNA from chyme samples was isolated using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania). Microorganisms were identified by T-RFLP (terminal restriction fragment length polymorphism) method. Polymerase chain reaction (PCR, a Maxygen thermal cycler, Life Technologies, Inc., USA) was performed according to amplification mode 3 min at 95 °C; 30 s at 95 °C, 30 s at 55 °C, 60 s at 72 °C (35 cycles); 60 s at 72 °C. Primers to 16S rRNA gene fragment of bacteria 63f (5′-CAGGCCTAACACATGCAAGTC-3′) and 1492r (5′-TACGGHTACCTTGTTACGACTT-3′), and those of archaea A109f (5′-ACKGCTCAGTAACACGT-3′), A934r (5′-GTGCTCCCCCGCCAATTCCT-3′) were 5′-labeled with Sy5 fluorophore (Beagle, Russia).

Fluorescently labeled amplicons were purified [18], and HaeIII, HhaI, and MspI restriction enzymes were applied to 30-50 ng of PCR products following the instructions of the manufacturer (Fermentas, Lithuania). The resultant fragments were analyzed (a CEQ 8000, Beckman Coulter, USA). Microorganisms were taxonomically attributed using Fragment Sorter program (http://www.oardc.ohiostate.edu/trflpfragsort/index.php). The total number of bacteria, *Archaea*, and *Neocallimastigales* class micromycetes was determined by quantitative PCR method with a kit for real-time PCR in the presence of intercalating dye EVA Green (ZAO Syntol, Russia) and primers F — 5′-ACTCCTACGGGAGGCAGCAG-3′, R — 5′-ATTACCGCGGCTGCTGG-3′ (bacteria), F — 5′-AGGAATTGGCGGGGGAGCAC-3′, R — 5′-GCCATGCACCWCCTCT-3′ (*Archaea*), F — 5′-GCACTTCATTGTGTGTACTG-3′, R — 5′-GGATGAAACTCGTTGACTTC-3′ (fungi) in the following 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) (a detecting thermal cycler DT Lite-4, NPO DNK-Tekhnologiya LLC, Russia).

Mathematical and statistical processing of the results was performed with

Microsoft Excel 2010 and PAST software (http://folk.uio.no/ohammer/past/). Mean values (*M*) and standard errors of the mean (±SEM) were determined. The significance of differences was assessed by Student's *t*-test.

1. Diets of dairy black and white mottled Holstein cows (*Bos taurus taurus*) in control and test groups (AO PZ Plamya (Leningrad Province, Gatchina District, 2017)

Ingredient, indicator	Control	Test
Compound feed K60-1-89, kg	12.0	12.0
Corn, kg	1.0	1.0
Oilcake, kg	0.5	0.5
Hay, kg	2.0	2.0
Silo, kg	20.0	20.0
Beet pulp, kg	0.5	0.5
Molasses, kg	1.5	1.5
Minvit®-3-1 Se, kg	0.2	0.2
Salt, g	70.0	70.0
Propylene glycol, kg	0.25	0.25
Sorbent Barrier®-Fito, g · animal $^{-1}$ · days $^{-1}$	–	20.0
Common food elements:		
feed units	23.41	23.41
ME, MJ	259.09	259.09
dry matter, kg	22.08	22.08
crude protein, g	4057.1	4057.1
BP, g	370.21	370.21
NBP, g	146.89	146.89
CDP, g	3103.9	3103.9
crude fat, g	1214.1	1214.1
crude fiber, g	3590.6	3590.6
NDF, g	1605.0	1605.0
starch, g	3044.5	3044.5
sugar, g	1954.4	1954.4
NFES, g	2802.0	2802.0

Note. ME — metabolizable energy, BP — breakdown protein, NBP — non-breakdown protein, CDP — digestible protein of cattle, NDF — neutral detergent fiber, NFES — nitrogen-free extractive substances. A dash means that the absence of the component.

Results. Zaslon®-Fito is a feed additive consisting of a mineral carrier of organic origin (diatomite), and blends of essential oils. The high content of porous silica (about 90%) in an amorphous form gives a high specific surface area (up to 40 ha/kg) and, as a result, a significant sorption capacity. A mixture of essential oils of carvacrol, thymol, limonene, linalool, citral, allicin, salvin, cineol, and linoleic acid was applied to diatomite to give additional phytobiotic properties. The content of the mixture of essential oils in the finished preparation based on diatomite was 0.5%.

Table 1 shows the composition of the main cows' diet. The feed used in the study was affected by aflatoxins (AFLA) at 9 μg/kg, 2.4 times higher than threshold limit value, zearalenone (ZEN) at 264 μg/kg, 2.6 times higher than threshold limit value) and DON at 310 μg/kg vs. threshold limit value of 1000 μg/ml (Table 2).

2. Mycotoxins (mg/kg dry matter) **in components of diets of dairy black and white mottled Holstein cows (*Bos taurus taurus*) in control and test groups** (AO PZ Plamya (Leningrad Province, Gatchina District, 2017)

Mycotoxin	Compound feed		Feed mixture from the hopper		Silage		Hay	
	mg/kg	ratio to TLV	mg/kg	ratio to TLV	mg/kg	ratio to TLV	mg/kg	ratio to TLV
AFLA	0.0038	na	0.0063	> 1.58-fold	0.8400	> 2.10-fold	0.7200	> 1.80-fold
OTA	0.002	na	0.0017	na	–	na	–	na
T-2	0.0795	> 1.33-fold	0.07	> 1.17-fold	0.0360	na	0.0042	na
ZEN	0.0401	na	0.0225	na	0.1087	> 1.09-fold	0.0387	na
DON	2.1	> 2.10-fold	1.5	> 1.50-fold	2.1	> 2.10-fold	1.7	> 1.70-fold

Note. AFLA — total amount of aflatoxins in the rumen, OTA — ochratoxin A, T-2 — T-2 toxin, ZEN — zearalenone, DON — deoxynivalenol; TLV — threshold limit value. TLVs are given according to the requirements of the Commission of the Customs Union. [19], na — not above TLV. Dashes mean that mycotoxin was not detected.

Application of the Zaslon®-Fito preparation for 71 days contributed to an increase in the average daily natural milk yield per animal by 5.5% relative to the control group ($p \leq 0.05$) (up to 1.8 kg) (Table 2). A similar pattern was observed when recalculating data for milk with 4% fat content (see Table 2). In this case, the daily milk yield increased by 8.4% ($p \leq 0.05$), or 2.7 kg/animal. The fat and protein content in milk in the cows of the experimental groups tended to increase (by 2.6% and 3.0%, respectively) compared with the same values in the cows of the control group (the differences are not statistically sig-

nificant).

The main identified positive effects of the use of Zaslon®-Fito include a marked decrease in the content of aflatoxin M_1 (by 16%) ($p \leq 0.05$) and the number of somatic cells (by 30%) ($p \leq 0.05$) in milk in cows of the experimental group compared with control animals. At the same time, the content of dry fat-free residue and lactose remained almost unchanged in all groups (Table 3).

3. Milk constituents and productivity of dairy black and white mottled Holstein cows (*Bos taurus taurus*) upon application of dietary Zaslon®-Fito phyrobiotics (*M*±SEM, AO PZ Plamya (Leningrad Province, Gatchina District, 2017)

Indicator	Control (n = 15)	Test (n = 15)
Daily natural milk yield, kg	32.8±0.6	34.6±0.5**
Daily 4% fat milk yield, kg	31.5±0.6	34.2±0.5
Protein, %	3.2±0.1	3.3±0.1
Fat, %	3.8±0.1	3.9±0.1
AFLAM1, ng/kg	47.3±2.4	39.8±1.1**
The number of somatic cells, thousand/cm^3	239±8	169±8*
Urea, mg/100 ml	27.6±1.3	26.1±0.9
Dry fat-free residue, %	8.7±0.2	8.8±0.1
Lactose, %	4.8±0.1	4.8±0.2

N o t e. For description of groups see the Techniques section; AFLAM1 — aflatoxin M_1.
*, ** Differences with the control are statistically significant at $p \leq 0.001$ and $p \leq 0.05$, respectively.

4. Blood biochemistry of dairy black and white mottled Holstein cows (*Bos taurus taurus*) upon application of dietary Zaslon®-Fito phyrobiotics (*M*±SEM, AO PZ Plamya (Leningrad Province, Gatchina District, 2017)

Indicator	Control (n = 5)	Test (n = 5)	Norm
Total protein, g/l	90.1±4.30	86.1±4.10	72.0-86.0
Total bilirubin, μmol/l	1.7±0.07	1.7±0.08	0.2-5.10
Glucose, mmol/l	2.4±0.09	3.4±0.18*	2.2-3.30
Calcium, mmol/l	2.2±0.08	2.2±0.12	2.5-3.13
Ketone bodies	nd	nd	nd
Urea, mmol/l	3.9±0.23	3.9±0.20	3.3-6.70
Reserve alkalinity, vol.% CO_2	54.0±2.50	56.0±2.70	46-66
Phosphorus, mmol/l	1.6±0.05	2.1±0.10*	1.5-1.94

N o t e. For description of groups see the Techniques section; nd — not detected.
* Differences with the control are statistically significant at $p \leq 0.05$.

A decrease in the content of AFLAM1 in milk of cows may indicate the removal of aflatoxin B1 from their body, since it was previously reported that absorption of aflatoxicol (a metabolite of aflatoxin B1) and aflatoxin M_1 in the body was rather fast, as a result of which, usually, they are detected after several hours in milk [20]. The maximum concentration of these substances in milk is observed 1 day after the animals ate the affected feed [1].

Biochemical analysis of blood of the test cows revealed a tendency to optimized total protein as compared to control (Table 4). Deviations in the total protein content are recorded in metabolic disorders and liver diseases [21]. Therefore, the deviation of this indicator from reference values in control animals could be associated with the detrimental effect of toxins on the body (see Table 4).

A decrease in glucose was detected in the blood of cows in the control group (to the lower limit of normal). Data significantly differed between the control and experimental groups ($p \leq 0.05$). It is known that many diseases of cows, including ketosis, are characterized by low glucose level due to impaired carbohydrate metabolism, glycogen stores in muscles and liver [22].

An increase in the amount of inorganic phosphorus was recorded in the group of cows, in the diet of which the sorbent was introduced, in comparison with the control ($p \leq 0.05$) (see Table 4). It is known [21] that its content in the

blood can be used as an indicator of the state of metabolic processes.

5. Rumen microflora composition (%) (according to T-RFLP analysis data) in dairy black and white mottled Holstein cows (*Bos taurus taurus*) upon application of dietary Zaslon®-Fito phyrobiotics (*M*±SEM, AO PZ Plamya (Leningrad Province, Gatchina District, 2017)

Microorganisms	Control (n = 3)	Test (n = 3)
Family *Eubacteriaceae*	2.0±0.08	3.72±0.19**
Пор. *Selenomonadales*	5.7±0.16	8.5±0.38**
Family *Bacillaceae*	9.1±0.35	10.6±0.42
Family *Bifidobacteriaceae*	0.5±0.05	0.7±0.05**
Family *Lactobacillaceae*	2.5±0.11	1.3±0.15**
Family *Enterobacteriaceae*	5.4±0.26	1.1±0.06*
Family *Clostridiaceae*	9.7±0.71	6.5±0.42**
Staphylococcus sp. and *Fusobacterium* sp.	4.6±0.18	3.9±0.24
Unidentified phylotypes:	19.0±0.95	37.9±1.57*
158 bp	0.1±0.01	1.1±0.05*
202 bp	0.8±0.09	1.6±0.27
225 bp	1.5±0.29	blrd
226 bp	blrd	0.9±0.06
425 bp	0.1±0.02	0.6±0.07**
433 bp	blrd	0.9±0.09
440 bp	0.2±0.01	0.4±0.03**
463 bp.	5.5±0.34	21.6±0.98*
576 bp.	0.6±0.04	2.2±0.18**
Other	1.8±0.12	1.5±0.08

N o t e. For description of groups see the Techniques section; blrd – below the limit of reliable determination
*, ** Differences with the control are statistically significant at $p \le 0.001$ and $p \le 0.05$, respectively.

A pronounced change in the rumen microflora composition of cows occurred under the influence of the sorbent (Table 5). In the experimental group, the proportion of VFA-synthesizing bacteria of the *Selenomonadales* order, forming acetic acid and other volatile fatty acids (VFAs), increased significantly (by 49%) ($p \le 0.05$) [23, 24]. This explains the data on the increase in fat in milk in animals fed with Zaslon®-Fito, since acetic acid is a precursor of milk fat. At the same time, in animals in the experimental group, in comparison with the control group, the number of lactic acid synthesizing bacteria of the *Lactobacillales* order ($p \le 0.05$), which have the ability to decompose monosugar in the rumen to lactate, decreased. A high content of lactobacilli is often associated with rumen acidification, a decrease in the proportion of bacteria synthesizing cellulases, impaired fixation of plant foods and lactic acidosis by an animal [23-25].

According to some researchers, the correction of the ratio of lactate-utilizing and lactate-producing bacteria can help reduce the risk of lactic acidosis. Feeding cows with bacteria of order *Selenomonadales* led to an increase in the pH of the rumen content and a decrease in the concentration of lactic acid during a rapid transition to a highly concentrated diet [26].

The number of lactate-producing bacteria in the rumen of ruminants can be controlled by adding antibiotics to the diet [26]. In addition, probiotics are often included in cattle diets to prevent lactic acidosis and increase animal productivity. To date, a number of probiotic preparations having various mechanisms of action have been described. Often, microorganisms that stimulate the growth of lactate-utilizing bacteria are used as probiotics [27]. Positive results when using *Megasphaera elsdenii* lactate-utilizing bacteria as a probiotic have been described by Elam et al. [28]. The main limitation to the use of *Megasphaera elsdenii* lactate-utilizing bacteria as a probiotic is the need for strict anaerobiosis to maintain a viable culture. Nevertheless, such promising results in the prevention of lactic acidosis are an incentive for further research.

This study showed a decrease in the content of *Staphylococcus* sp. and *Fusobacterium* sp., among which pathogens of inflammatory diseases, including mastitis, are often found. Perhaps this led to the healing of the udder epithelium,

elimination of possible foci of inflammation and a decrease in the number of somatic cells in milk that were observed. Previously, other authors have shown the positive effect of plant essential oils in animals due to the antimicrobial effect, which can lead to a change in the fermentation processes in the rumen [17]. Most essential oils resemble ionophores in terms of antimicrobial properties, at the same time they selectively inhibit gram-positive bacteria. The reasons for selectivity are similar to those of ionophores, i.e. the hydrophobic nature of essential oils allows them to interact with the bacterial membrane, changing transmembrane ion transport [17]. Such selectivity is the main property of ionophores, which explains the decrease in the number of gram-positive bacteria producing lactate and the increase in the content of gram-negative bacteria forming propionate [26]. Given the similarity of essential oils and ionophores in terms of selectivity and mechanism of action, it could be expected that one of the main effects of essential oils would be a shift in the proportions of VFAs in the rumen. Nevertheless, a number of researchers reported that this did not always happen, and essential oils either did not affect the amount of VFAs in the rumen [29] or contributed to an increase or decrease in the amount of propionate [30]. Moreover, the degree of influence of essential oils on the concentration of a number of VFAs in the rumen largely depended on the diet, amount and type of essential oils. In addition, by inhibiting lactate-producing bacteria, ionophores generally reduce the risk of lactate acidosis. Ionophores also reduce the synthesis of ammonia in the rumen, which, in turn, leads to a more efficient use of feed protein and a reduction in methane emission [31].

A decrease in the amount of urea in the milk of cows fed with Zaslon®-Fito can have an indirect relationship with the regulation of the rumen microflora composition, in particular, with the optimization of the number of protozoans synthesizing proteases. It is known [23] that the protein fraction of feed under the influence of microflora, primarily protozoa, breaks down in the rumen to ammonium and then turns into a microbial protein, which positively affects the physiology of animals. The remaining ammonia is transported to the liver, where it is transformed into urea, which is excreted with urine. Part of the urea is returned back to the rumen. In the case of rumen dysbiosis, a significant portion of the feed protein is transformed through ammonia into urea, resulting in a lack of protein.

It was also found that ionophores had an effect on rumen anaerobic fungi [32, 33]. Elliott et al. [32] suggested that an increase in the concentration of propionic acid in the rumen could be due to the antifungal rather than antibacterial action of ionophores. In these studies, the number of chytridiomycetes of the *Neocallimastigales* class and *Archaea* in the rumen of cows fed with Zaslon®-Fito did not change compared to the control ($3.22\times10^7\pm9.17\text{-}110^4$ and $3.21\times10^8\pm1.42\times10^6$ genome equiv/g, respectively). Moreover, the phytobiotics led to an increase in the absolute number of bacteria to $1.25\times10^{11}\pm5.43\times10^9$ genome equiv/g.

Thus, the dietary Zaslon®-Fito preparation increases the average daily milk yield by 5.5% and reduces the aflatoxin M_1 level (by 16%) ($p \le 0.05$) and the number of somatic cells (by 30%) ($p \le 0.05$). In blood of cows from the test group, the glucose level is higher and the amount of inorganic phosphorus increases ($p \le 0.05$). Therefore, the dietary sorbent Zaslon®-Fito applied to dairy cows at 20 $g \cdot animal^{-1} \cdot day^{-1}$ minimizes the negative effect of feed toxins, improving livestock performance and feed hygiene. The positive influence may be due to the multifunctional effect of the preparation, including high sorption of mycotoxins and regulation of the rumen microbiota pattern by essential oils.

REFERENCES

1. Diaz D.E., Hagler W.M. Jr., Blackwelder J.T., Eve J.A., Hopkins B.A, Anderson K.L., Jones F.T., Whitlow L.W. Aflatoxin binders II: reduction of aflatoxin M_1 in milk by sequestering agents of cows consuming aflatoxin in feed. *Mycopathologia*, 2004, 157(2): 233-241 (doi: 10.1023/B:MYCO.0000020587.93872.59).
2. Driehuis F. Silage and the safety and quality of dairy foods: a review. *Agricultural and Food Science*, 2013, 22(1): 16-34 (doi: 10.23986/afsci.6699).
3. Gallo A., Giuberti G., Frisvad J.C., Bertuzzi T., Nielsen K.F. Review on mycotoxin issues in ruminants: occurrence in forages, effects of mycotoxin ingestion on health status and animal performance and practical strategies to counteract their negative effects. *Toxins (Basel)*, 2015, 7(8): 3057-111 (doi: 10.3390/toxins7083057).
4. Trucksess M.W., Richard J.L., Stoloff L., McDonald J.S., Brumley W.C. Absorption and distribution patterns of aflatoxicol and aflatoxins B1 and M1 in blood and milk of cows given aflatoxin B_1. *American Journal of Veterinary Research*, 1983, 44(9): 1753-1756.
5. Kuilman M.E.M., Maas R.F.M., Judah D.J., Gremmels J. Bovine hepatic metabolism of aflatoxin B_1. *Journal of Agricultural and Food Chemistry*, 1998, 46(7): 2707-2713 (doi: 10.1021/jf980062x).
6. Diaz D. *Mikotoksiny i mikotoksikozy* [Mycotoxins and mycotoxicoses]. Moscow, 2006 (in Russ.).
7. Veldman A., Meijst J.A.C., Borggreve G.J., Heeres-van der Tol J.J. Carry-over of aflatoxin from cows' food to milk. *Animal Science*, 1992, 55(2): 163-168 (doi: 10.1017/S0003356100037417).
8. Natour R.M., Yousef S.M. Adsorption efficiency of diatomaceous earth for mycotoxin. *Arab Gulf Journal of Scientific Research*, 1998, 16(1): 113-127.
9. Modirsanei M., Mansoori B., Khosravi A.R., Kiaei M.M., Khazraenia P., Farkhoy M., Masoumi Z. Effect of diatomaceous earth on the performance and blood variables of broiler chicks during experimental aflatoxicosis. *Journal of the Science of Food and Agriculture*, 2008, 88(4): 626-632 (doi: 10.1002/jsfa.3127).
10. Simitzis P.E., Deligeorgis S.G. The effects of natural antioxidants dietary supplementation on the properties of farm animal products. In: *Animal feed: types, nutrition, safety*. Nova Science Publishers, Inc., New York, 2011: 155-168.
11. Adil S., Magray S.N. Impact and manipulation of gut microflora in poultry: a review. *Journal of Animal and Veterinary Advances*, 2012, 11(6): 873-877 (doi: 10.3923/javaa.2012.873.877).
12. Jang I.S., Ko Y.H., Kang S.Y., Lee C.Y. Effect of a commercial essential oil on growth performance, digestive enzyme activity and intestinal microflora population in broiler chickens. *Animal Feed Science and Technology*, 2007, 134(3-4): 304-315 (doi: 10.1016/j.anifeedsci.2006.06.009).
13. Jamroz D., Wiliczkiewicz A., Wertelecki T., Orda J., Skorupińska J. Use of active substances of plant origin in chicken diets based on maize and locally grown cereals. *British Poultry Science*, 2005, 46(4): 485-493 (doi: 10.1080/00071660500191056).
14. Windisch W., Schedle K., Plitzner C., Kroismayr A. Use of phytogenic products as feed additives for swine and poultry. *Journal of Animal Science*, 2008, 86(Suppl. 14): E140-E148 (doi: 10.2527/jas.2007-0459).
15. Zeng Z., Zhang S., Wang H., Piao X. Essential oil and aromatic plants as feed additives in non-ruminant nutrition: a review. *Journal of Animal Science and Biotechnology*, 2015, 6(1): 7 (doi: 10.1186/s40104-015-0004-5).
16. McIntosh F.M., Williams P., Losa R., Wallace R.J., Beever D.A., Newbold C.J. Effects of essential oils on ruminal microorganisms and their protein metabolism. *Applied and Environmental Microbiology*, 2003, 69(8): 5011-5014 (doi: 10.1128/AEM.69.8.5011-5014.2003).
17. Calsamiglia S., Busquet M., Cardozo P.W., Castillejos L., Ferret A. Invited review: Essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science*, 2007, 90(6): 2580-2595 (doi: 10.3168/jds.2006-644).
18. Maniatis T., Frich E., Sembruk Dzh. *Molekulyarnoe klonirovanie* [Molecular cloning]. Moscow, 1984 (in Russ.).
19. *Veterinarno-sanitarnye trebovaniya Tamozhennogo soyuza «O primenenii veterinarno-sanitarnykh mer v tamozhennom soyuze»*. Utv. resheniem KTS ot 18.06.2010 № 317 [Veterinary and sanitary requirements of the Customs Union «On the application of veterinary and sanitary measures in the Customs Union» dated 18.06.2010 № 317].
20. Polan S.E., Hayes J.R., Campbell T.C. Consumption and fate of aflatoxin B, by lactating cows. *Journal of Agricultural and Food Chemistry*, 1974, 22(4): 635-638 (doi: 10.1021/jf60194a027).
21. Kazartsev V.V., Ratoshnyi A.N., Kazartsev V.V. *Zootekhniya*, 1986, 3: 323-330 (in Russ.).
22. Vasil'eva E.A. *Klinicheskaya biokhimiya sel'skokhozyaistvennykh zhivotnykh* [Clinical biochemistry of farm animals]. Moscow, 2000: 359 (in Russ.).
23. Hungate R.E. *The rumen and its microbes*. Academic Press, NY, 1966.
24. Tarakanov B.V. *Metody issledovaniya mikroflory pishchevaritel'nogo trakta sel'skokhozyaistvennykh zhivotnykh i ptitsy* [Methods to study microflora of the digestive tract of farm animals and poultry]. Moscow, 2006 (in Russ.).
25. Il'ina L.A. *Izuchenie mikroflory rubtsa krupnogo rogatogo skota na osnove molekulyarno-biologicheskogo metoda T-RFLP s tsel'yu razrabotki sposobov ee optimizatsii. Kandidatskaya dissertatsiya* [The study of cattle rumen microflora based on the molecular biological method T-RFLP with the aim of its optimization. PhD Thesis]. St. Petersburg, 2012 (in Russ.).

26. Nagaraja T.G., Newbold C.J., Van Nevel C.J., Demeyer D.I. Manipulation of ruminal fermentation. In: *The rumen microbial ecosystem*. P.N. Hobson, C.S. Stewart (eds.). Springer, Dordrecht, 1997: 523-632 (doi: 10.1007/978-94-009-1453-7_13).
27. Krehbiel C.R., Rust S.R., Zhang G., Gilliland S.E. Bacterial direct-fed microbials in ruminant diets: performance response and mode of action. *Journal of Animal Science*, 2003, 81(14, Suppl. 2): E120-E132 (doi: 10.2527/2003.8114_suppl_2E120x).
28. Elam N.A., Gleghorn J.F., Rivera J.D., Galyean M.L., Defoor P.J., Brashears M.M., Younts-Dahl S.M. Effects of live cultures of *Lactobacillus acidophilus* (strains NP45 and NP51) and *Propionibacterium freudenreichii* on performance, carcass, and intestinal characteristics, and *Escherichia coli* strain O157 shedding of finishing beef steers. *Journal of Animal Science*, 2003, 81(11): 2686-2698 (doi: 10.2527/2003.81112686x).
29. Meyer N.F., Erickson G.E., Klopfenstein T.J., Greenquist M.A., Luebbe M.K., Williams P., Engstrom M.A. Effect of essential oils, tylosin, and monensin on finishing steer performance, carcass characteristics, liver abscesses, ruminal fermentation, and digestibility. *Journal of Animal Science*, 2009, 87(7): 2346-2354 (doi: 10.2527/jas.2008-1493).
30. Cardozo P.W., Calsamiglia S., Ferret A., Kamel C. Screening for the effects of natural plant extracts at different pH on in vitro rumen microbial fermentation of a high-concentrate diet for beef cattle. *Journal of Animal Science*, 2006, 83(11): 2801-2808 (doi: 10.2527/2005.83112572x).
31. Guan H., Wittenberg K.M., Ominski K.H., Krause D.O. Efficacy of ionophores in cattle diets for mitigation of enteric methane. *Journal of Animal Science*, 2006, 84(7): 1896-1906 (doi: 10.2527/jas.2005-652).
32. Elliott R., Ash A.J., Calderon-Cortes F., Norton B.W., Bauchop T. The influence of anaerobic fungi on rumen volatile fatty acid concentrations in vivo. *The Journal of Agricultural Science*, 1987, 109(1): 13-17 (doi: 10.1017/S0021859600080928).
33. Newbold C.J., Wallace R.J., Watt N.D., Richardson A.J. Effect of the novel ionophore tetronasin (IC1 139603) on ruminal microorganisms. *Applied and Environmental Microbiology*, 1988, 54(2): 544-547.

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1154-1166
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.93:636.085.8:591.1

doi: 10.15389/agrobiology.2019.6.1154eng
doi: 10.15389/agrobiology.2019.6.1154rus

PHYSIOLOGICAL STATUS AND FUR QUALITY OF YOUNG SILVER FOXES (*Vulpes vulpes* L.) UNDER USE OF ANTIPARASITIC DRUG NIACID-GRANULES PLUS AND BIOACTIVE KERATIN AS A FEED ADDITIVE

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The authors declare no conflict of interests
Received September 29, 2019

Abstract

Recently, among carnivores, including silver-black foxes, cases of nematode infestation have become more frequent, causing significant economic damage. From helminthic infestations, youngsters suffer the most. Currently, in the veterinary world practice, anthelmintics of the new generation, in particular, avermectin-based products are recognized the most promising. To date, neither in Russia nor abroad, keratin preparations in combination with anthelmintics have been used in fur farming. In the present work, it was experimentally proved for the first time that a new-generation anthelmintic Niacid-Granules Plus combined with a sulfur-containing feed additive Bioactive Keratin improve fur grading indices (size, amount of fur, completeness of fur, damage to fur, damage to skin etc.) in silver-black foxes (*Vulpes vulpes* L.). This paper documents physiological effects and fur quality parameters in young silver-black foxes due to the combined use of Bioactive Keratin and an avermectin-based anthelmintic. Niacid-Granules Plus (LLC SPA Ecobiovet, Russia, and Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology, Russia) are brown granules, 300-400 g per pack, with 85 % abamectin B_{1a} and 15 % abamectin B_{1b} contents and the allowed shelf life for at least 21 months. Bioactive Keratin (Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology, Russia) is a monoproduct derived from tow of wool and containing 98 % of native protein, a preparative form of homogeneous liquid mass, color from light gray-beige to dark gray-beige, with a specific smell, mass fraction of dry matter 3-10 %, mass fraction of keratin 95-98 %, and a shelf life of not more than 2 years. The preparations Niacid-Granules Plus and Bioactive Keratin belong to the IV class of toxicity. It is shown that the combined use of Niacid-Granules Plus and Bioactive Keratin significantly enhances the effect of each drug used separately. In farm tests (AO Saltykovskii Breeding Farm, Moscow Province, 2017-2018), it was found that deworming young silver-black foxes at the age of 2 months with Niacid-Granules Plus and the subsequent use of dietary Bioactive Keratin as a sulfur-containing feed additive significantly affect body weight gain in foxes. Under the combined use of Niacid-Granules Plus and Bioactive Keratin, the live body weight of pups is 9.1 % higher compared to the control ($t_{fact.} = 7.40 \geq t_{tab.} = 2.1$ at $p = 0.05$ and $t_{fact.} = 0.81 \leq t_{tab.} = 2.1$ at $p = 0.05$, respectively). Niacid-Granules Plus together with Bioactive Keratin also improved the complete blood counts during the investigation, thereby contributing to a faster normalization of the physiological status of young silver-black foxes. When using both drugs, the weight of young silver-black foxes increased by 30.4 %, the area of the obtained furs by 18.4 %, the hair density by 24.6 % compared to these parameters in intact animals. This confirms the effectiveness of the joint use of the studied preparations. The use of Niacid-granules plus are allowed to animals at the age of 2 months, twice at 10-day interval, and the dietary Bioactive Keratin should be added to feed in six cycles (5-day cycles

with 10-day intervals).

Keywords: *Vulpes vulpes* L., silver fox, young animals, Niacid-granules plus, avermectin, bioactive keratin, body weight gain, blood indicators, fur quality

In fur-bearing animals [1], the formation of animal hair and the quality of skin due to breed characteristics depend on the physiological state of animals, which, in turn, is determined by feeding conditions [2], maintenance [3], preventive and therapeutic measures [4]. In recent years, among carnivores, including silver-black foxes, cases of nematode infestations have become more frequent. Young animals suffer especially severely from helminthic infestations. As a rule, helminthic diseases cause exhaustion, the reproductive ability of animals decreases, growth and development of young silver-black foxes and other carnivores decelerates [5, 6]. In fur-bearing animals, more than 70 species of various nematodes can parasitize [7]. Toxocariasis, toxascaridosis, and trichinosis are of the greatest economic importance. Despite the fact that at present there is a fairly wide range of anthelmintic agents (for example, Ivomek®, ZAO Nita-PHARM, Russia; Nilverm®, Pliva, Croatia; Albamelin®, AO Veterinary Preparations, Russia), helminthic infestations cannot be completely defeated. It is known that any anthelminthic preparation destroys only adult worms, while helminth eggs remain in the body even after deworming. Therefore, with the established fact of helminthic infestation, it is required to conduct 2-fold treatment of animals with an interval of 10 days so that by the time of the second treatment, immature animals that are not able to produce their offspring will have appeared from eggs [5].

The most promising anthelmintic preparations of the new generation are, in particular, avermectin-based products [8-10]. The Niacid-Granules Plus preparation (LLC SPA Ecobiovet, Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology, Russia) is one of the latest developments in the line of anthelmintics of the avermectin series [10], however, it has not yet been used in animal farming. According to the mechanism of action, this is a neurotoxin-type drug (the active substance blocks and inhibits the transmission of a nerve impulse, causing paralysis and then death of nematodes) [8].

Earlier, the authors examined various areas of practical application of keratin-containing preparations [11], in particular, in cosmetology and medicine [12-14]. Preparations of hydrolyzed keratin can be used in animal husbandry [15], as well as in fur breeding as active feed additives that affect the condition of the hair of the skin [11]. The hydrolyzed keratin contains the amino acid cysteine, which refers to thiol compounds. The mechanism of biochemical action of thiols consists in the ability to restore disulfide bonds in pathological disorders, inactivate toxic agents, and also increase the content of sulfhydryl groups, providing an antioxidant effect [16, 17]. Thiol compounds are primarily exposed to active oxygen radicals, which protects the functional groups of biological molecules and cell membranes from their influence [18, 19].

To date, keratin preparations in combination with anthelmintic preparations have not been used in the industrial breeding of fur-bearing animals, although the complex use of biologically active substances [20-22] and veterinary drugs is recognized as highly effective (Vidal Veterinarian 2019. Medicines for Veterinary Applications in Russia. Moscow, 2018). However, the data on the effectiveness of these substances, their doses [23], and the characteristics of the effect on fur-bearing animals, in particular silver-black foxes, are extremely limited and quite fragmented (24). In individual publications, the separate use of keratin on minks was reported (O.V. Barantseva, Technology for the preparation of keratin-containing feed additives and assessment of its effect on the quality of

mink's skin. Cand. Dis. Moscow, 2011), as well as the use of keratin, collagen and melanin on ferrets [11].

In the present work, it was experimentally proved for the first time that a new-generation anthelmintic Niacid-granules plus combined with a sulfur-containing feed additive Bioactive keratin, the keratin-containing waste recycling product, improve fur grading indices (size, amount of fur, completeness of fur, damage to fur, damage to skin etc.) in silver-black foxes

The purpose of the work is to determine the physiological effect of the combined use of bioactive keratin and anthelmintic based on avermectin in the growing young silver-black foxes and to evaluate the quality of the obtained skin products.

Techniques. In the experiment, we used the preparation Niacid-Granules Plus (LLC SPA Ecobiovet) and Bioactive Keratin (Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology) from tow of fine sheep wool (reusable waste from wool processing in the textile industry) [25].

The toxicity of the drug Niacid-Granules Plus and the feed additive Bioactive Keratin was evaluated according to GOST 12.1.007-76 Harmful Substances. Classification and General Safety Requirements, as well as on the basis of the requirements set forth in GOST 32296-2013 Methods of Testing Chemical Products on the Human Body. Basic Requirements for Conducting Tests to Assess Acute Toxicity for Intragastric Administration by the Fixed-Dose Method. As experimental laboratory animals, 70 random-bred white mice and 70 outbred Wistar rats assigned to groups of analogs by live weight were used. The experiments were conducted in accordance with the protocols of the Geneva Convention and the principles of proper laboratory practice (National Standard of the Russian Federation GOST R 53434-2009), as well as according to the recommendations of The Guide for the Care and Use of Laboratory Animals (National Academy Press Washington, DC 1996). Mean lethal doses were calculated [26].

To evaluate the effectiveness of separate and combined use of anthelmintic preparation and bioactive feed additive, 2-month-old silver-black foxes (*Vulpes vulpes* L.) were divided into 4 groups of 10 animals by the methods of analogs using birth dates and live weight (AO Saltykovskii Breeding Farm, 2017). The drugs were given to animals in accordance with the following scheme: in Group I, the anthelmintic preparation Niacid-Granules Plus was added twice to the animals' BD accepted at the farm (AI 200 μg/granule per 1 kg of animal weight, with an interval of 10 days, starting from the 1st day of the experiment); in Group II, the anthelmintic preparation Niacid-Granules Plus in the same dose and the preparation Bioactive Keratin in the amount of 0.2% of the daily protein norm (6 cycles, including 5-day courses with 10-day intervals) were added to the BD; Group III (control) consisted of intact animals that did not receive the anthelmintic preparation and the feed additive, Group IV was intact animals, in the BD of which the drug Bioactive Keratin was added in an amount of 0.2% of the daily protein norm (6 cycles including 5-day courses with 10-day intervals).

During the observation period, young animals were weighed individually with an accuracy of 0.01 kg (electronic scales MP VD (Zh) A F-2, OOO MIDLiK, Moscow, Russia).

The physiological state of the animals was assessed by the results of the general clinical analysis of whole blood (performed at the laboratory of Moscow Station for the Control of Animal Diseases). The samples were taken from the lateral saphenous vein of a thigh (in the morning after a night's sleep before feeding and drinking) from 2-month-old animals before the start of the experiment and from 7-month-old animals at the end of the experiment. The hemo-

globin content, the total number of erythrocytes and leukocytes, the content and ratio of the main subpopulations of granulocytic leukocytes (lymphocytes, monocytes, eosinophils, neutrophils, and basophils) included in the leukocyte formula were determined.

At the end of the experiment, the obtained skins were subjected to primary processing and a quality assessment commission in accordance with GOST 2790-88, and the basic properties of the skins (area, length, and thickness of hair of various categories) were determined. The thickness of the hair on the rump was determined by direct counting and by the number of hair roots in horizontal sections. Histological sections were prepared according to the standard method with hematoxylin and eosin staining, fixing them at the final stage under a coverslip with Canadian balsam [11], and examined at ×400 magnification (ZEISS Axio Lab. A1, Zeiss AG, Germany).

During statistical processing of the obtained results, the arithmetic mean (M) and standard error of the mean (±SEM) were determined. The statistical significance of differences in means was evaluated by Student's t-test [27, 28].

Results. Niacid-Granules Plus granules are feed bait supplemented with antiparasitic ingredient (200 μg AI/kg). Characterization of organoleptic, physicochemical and biological properties of the preparation was performed according to the requirements of the State Pharmacopoeia XI [29] (Table 1).

1. Characterization of anthelmintic Niacid-Granules Plus drug (LLC SPA Ecobiovet) [29]

Indicator	Description
Appearance, color	Brown granules weighing 300-400 mg
Authenticity of AI (abamectin):	
avermectin B_{1a}, % (not less)	85
avermectin B_{1b}, % (not more)	15
Mass fraction of abamectin, %	200±20 rg/granule
Toxicity, rat test-dose	IV class of toxicity
Foreign mechanical inclusions	Not allowed
Storage time	Not less than 21 months

As follows from Table 1, the active ingredient of the drug Niacid-Granules Plus is a complex of natural non-hydrogenated avermectins B_{1a} and B_{1b}, which are stable only if the relevant established storage modes are observed [29].

2. Characterization of Bioactive Keratin dietary additive (Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology, Russia) [29]

Indicator	Description
Appearance	Homogeneous liquid mass
Color	From light gray beige to dark gray beige
Smell	Specific
Hydrogen indicator	6.5-7.5
Authenticity	Positive reaction to keratin
Mass fraction of dry matter, %	3.0-10.0
Mass fraction of keratin calculated on the dry matter, %	95.0-98.0
The total number of mesophilic aerobic and facultative anaerobic microorganisms, CFU/1 g (cm^3) of product	Not more than 1×10^2
Yeast, yeast-like, mold fungi in 1 g/cm^3 of product	Absent
Bacteria of the family *Enterobacteriaceae* in 1 g/cm^3 of product	
Bacteria of the species *Pseudomonas aeruginosa* in 1 g/cm^3 of product	Absent
Bacteria of the species *Staphylococcus aureus* in 1 g/cm^3 of product	Absent
Toxicity, rat test-dose	IV class of toxicity
Storage time	No more than 2 years

Bioactive Keratin is a sulfur-containing protein that is unique in composition and properties. Keratin preparations are actively used in medicine and

cosmetology [12-14, 29]. However, this protein, obtained from the secondary products of the fur-processing industry, may be in demand in animal husbandry as a biologically active additive to the BD of fur-bearing animals. Bioactive Keratin from tow of sheep wool has the following characteristics (Table 2).

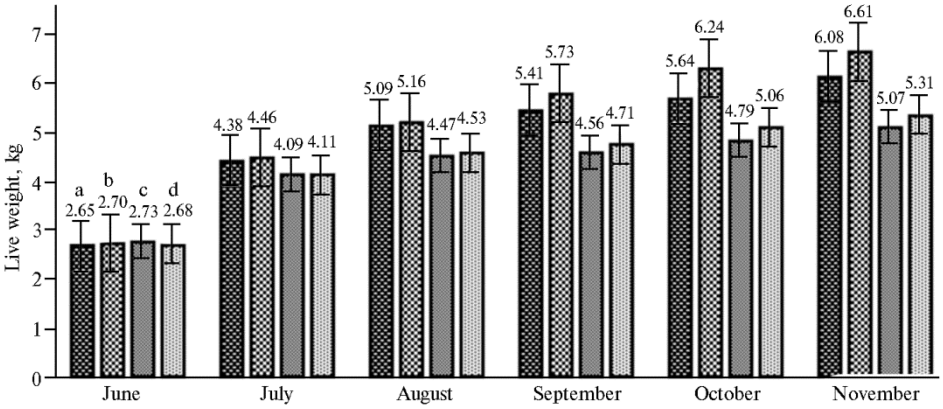

Fig. 1. Body weight dynamics of young silver-black foxes (*Vulpes vulpes* L.) upon application of anthelmintic Niacid-Granules Plus and sulfur-containing feed additive Bioactive Keratin: a — Niacid-Granules Plus, b — Niacid-Granules Plus + Bioactive Keratin, c — intact animals (control), d — Bioactive Keratin (n = 10 per group, AO Saltykovskii Breeding Farm, 2017).

In June, before the start of the experiment, the mass of 2-month-old animals in all groups was approximately the same (Fig. 1), statistically significant differences between the groups were not revealed. After 1 month, the live weight of animals in all groups increased on average by 1.50-1.65 times compared with the previous indicators. At the same time, significant differences appeared between the mass of animals from Group I who received Niacid-Granules Plus and intact animals from Group III (control) ($t_{fact.} = 3.05 > t_{table} = 2.1$ at $p = 0.05$). When comparing weight of animals from Groups II and III, it was found that with the combined use of Niacid-Granules Plus and Bioactive Keratin, the mass of young animals in Group II was 9.1% higher than in the control group and approximately 2% higher than the indicator in the case of using only the anthelmintic preparation (respectively, $t_{fact.} = 7.40 > t_{table} = 2.1$ at $p = 0.05$ and $t_{fact.} = 0.81 < t_{table} = 2.1$ at $p = 0.05$). Comparison of the live weight of animals in Groups II and IV also made it possible to establish statistically significant differences between them ($t_{fact.} = 5.46 > t_{table} = 2.1$ at $p = 0.05$). There were no such differences between Groups III and IV ($t_{fact.} = 0.34 < t_{table} = 2.1$ at $p = 0.05$). The obtained results reliably indicate the maximum positive effect (increase in the live weight of young animals) with the combined use of the preparations Niacid-Granules Plus and Bioactive Keratin. By the end of the observation period, when comparing the average weight of animals from Group I receiving the anthelmintic preparation and from the control Group III, reliable ($t_{fact.} = 9.34 > t_{table} = 2.1$ at $p = 0.05$) differences (19.9% excess) were revealed. Between Groups II (a combination of two preparation) and IV (using only Bioactive Keratin), there were also significant ($t_{fact.} = 10.11 > t_{table} = 2.1$ at $p = 0.05$) differences in favor of the joint use of the preparations (24.5% increase).

The indicators characterizing the growth rate of young silver-black foxes are presented in Table 3.

A general clinical blood test, which allows making a general assessment of the physiological status of animals, showed (Table 4) that at the beginning of the experiment (before using the preparations), despite some fluctuations, the

indices in the groups did not differ significantly. Thus, the content of hemoglobin, which is responsible for the transport of oxygen from the lungs to tissues and organs, ranged from 7.5 to 8.6%, red blood cells from 4.0 to 4.6 million/ml, white blood cells from 13.1 to 14.9 thousand/ml. It should be noted that at the beginning of the experiment, the young animals were not yet treated with anthelmintic drugs and the obtained values, in all probability, indicate the presence of a weak toxocariasis infestation, the cause of which could be infection both in utero and with mother's milk or through swallowing infective eggs with feed or water [30]. It is known that during helminthiases, the content of hemoglobin in the blood of fur-bearing animals decreases due to blood loss because of posthemorrhagic anemia caused by Toxocara feeding on blood, which damage the intestinal membrane and other internal organs [17, 31].

3. Body weight gain in young silver-black foxes (*Vulpes vulpes* L.) upon application of anthelmintic Niacid-Granules Plus and sulfur-containing feed additive Bioactive Keratin (M±SEM, n = 10 per group, AO Saltykovskii Breeding Farm, 2017)

Groups	Absolute,kg	Relative, %	Daily, g
I (Niacid-Granules Plus)	3.4±0.1	78.6±5.7	114.3±9.0
II (Niacid-Granules Plus + Bioactive Keratin)	3.9±0.2	84.0±6.4	130.3±11.5
III (intact animals, control)	2.3±0.1	60.0±5.3	78.0±5.5
IV (Bioactive Keratin)	2.6±0.1	65.8±5.6	87.7±5.7

N o t e. For a description of drug application scheme in the groups, see the Techniques section.

4. Clinical blood test results of young silver-black foxes (*Vulpes vulpes* L.) upon application of anthelmintic Niacid-Granules Plus and sulfur-containing feed additive Bioactive Keratin (M±SEM, n = 10 per group, AO Saltykovskii Breeding Farm, 2017

Groups	Начало опыта (1-е сут)			Окончание опыта (210-е сут)		
	HGB, g/%	RBC,$\times10^6$/ml	WBC, $\times10^3$/ml	HGB, g/%	RBC, $\times10^6$/ml	WBC, $\times10^3$/ml
I (NGP)	7.9±0.7	4.2±0.3	13.8±0.9	13.2±1.1	7.3±0.6	6.4±0.5
II (NGP + BK)	8.4±0.6	4.0±0.4	13.6±1.2	13.9±1.2	7.7±0.6	5.3±0.3
III (IA, control)	8.2±0.6	4.4±0.2	14.4±1.0	7.0±0.5	3.9±0.4	14.8±1.0
IV (BK)	8.0±0.3	4.3±0.3	14.1±1.2	7.3±0.4	4.0±0.4	14.4±1.0

N o t e. NGP — Niacid-Granules Plus, NGP + BK — Niacid-Granules Plus + Bioactive Keratin, IA — intact animals, BK — Bioactive Keratin; HGB — hemoglobin, RBC — red blood cells, WBC — white blood cells. For a description of drug application scheme in the groups, see the Techniques section.

After anthelmintic treatment with Niacid-Granules Plus (separately and in combination with a sulfur-containing keratin additive), hematological parameters began to change. In Group I, where only anthelmintic preparation was used, the hemoglobin content increased by 1.7 times, while in Group III (control), by day 210, it decreased by 14.6% compared with the initial one ($t_{fact.} = 1.30 < t_{table} = 2.1$ at $p = 0.05$). The established fact indicates an increase in infestation without deworming of young animals. In Group II, where bioactive keratin was used together with the preparation Niacid-Granules Plus, the hemoglobin level increased by 1.9 times. In Group IV, where animals received only bioactive keratin, the hemoglobin level by the end of the experiment decreased by 8.75% ($t_{\text{fact.}} = 1.40 < t_{table} = 2.1$ at $p = 0.05$) from the initial one.

The obtained results indicate that the preparation Bioactive Keratin, used as a feed additive, somewhat inhibited the destruction of red blood cells, and also acted as a detoxifier. Red blood cells fulfill the transport function, providing the organism with oxygen and utilizing carbon dioxide. The study of red blood cells is included in the general blood test during the initial diagnosis of many diseases, including parasitic ones [17, 31].

According to the obtained data, the number of red blood cells in the blood

of 2-month-old silver-black foxes prior to deworming ranged from 4.0-4.6 million/ml. Apparently, these values characterize the presence of anemia as a consequence of pathological processes in the body of young animals that are most susceptible to the effects of toxocariasis pathogens. In this case, anemia can also develop due to a lack of iron, vitamin B_{12}, and folic acid [4].

Upon anthelmintic use, the red blood cell counts in of young silver-black foxes began to increase. Thus, in Group I (Niacid-Granules Plus), by the end of the experiment, it increased by 73.8% ($t_{fact.} = 4.62 > t_{table} = 2.1$ at $p = 0.05$), while in Group III (control), it decreased by 9.3% ($t_{fact.} = 1.12 < t_{table} = 2.1$ at $p = 0.05$). With the combination of anthelmintic and feed additives, the same indicator increased by 66.7% ($t_{fact} = 5.13 > t_{table} = 2.1$ at $p = 0.05$). In Group IV, where animals received only a feed additive, the number of red blood cells in the blood decreased by 7.0% by the end of the experiment ($t_{fact} = 0.60 < t_{table} = 2.1$ at $p = 0.05$) from the initial one. Obviously, the peptides formed during the hydrolytic cleavage of bio-additives in the intestines of fur-bearing animals prevent the destruction of red blood cells.

The number of leukocytes in the blood is one of the most important indicators of an animal's state of health. The main function of leukocytes is to detect a foreign agent and neutralize it; these blood cells are the first line of defense of the body encountered by the pathogens colonizing it, including helminths [30, 31]. The content of leukocytes in the blood of young animals before the start of the experiment ranged from 13.4 to 14.4 thousand/ml (see Table 4). The authors consider these initial data as an indication of the presence of a pathological process in the body of young animals, which is associated with the presence of helminths (since the anthelmintic treatment of animals at that time was not yet conducted). In Groups I and II (see Table 4), the number of leukocytes gradually decreased. In Group IV, where animals received only Bioactive Keratin, the number of leukocytes was 2.1% higher than at the beginning of the experiment ($t_{fact} = 0.19 < t_{table} = 2.1$ at $p = 0.05$), but at the same time, it is 2.7% lower than that of animals from Group III ($t_{fact} = 0.28 < t_{table} = 2.1$ at $p = 0.05$). With the combined use of the anthelmintic preparation and bio-additive (Group II), the content of leukocytes in the blood of young animals decreased by 2.6 times. In addition, it is noteworthy that bioactive keratin, together with an antihelminthic preparation, contributes to the normalization of the composition of blood corpuscles.

Niacid-Granules Plus had a pronounced effect on skin area, it increased by 18.1% ($t_{fact} = 22.60 > t_{table} = 2.1$ at $p = 0.05$) compared to Group III (control), (Table 5).

5. Skin area of young silver-black foxes (*Vulpes vulpes* L.) upon application of anthelmintic Niacid-Granules Plus and sulfur-containing feed additive Bioactive Keratin (*M*±SEM, *n* = 10 per group, AO Saltykovskii Breeding Farm, 2018)

Groups	Area, cm^2
I (Niacid-Granules Plus)	2152.0±9.5
II (Niacid-Granules Plus + Bioactive Keratin)	2198.0±11.0*
III (intact animals, control)	1856.0±9.0
IV (Bioactive Keratin)	1909.0±10.3

N o t e. For a description of drug application scheme in the groups, see the Techniques section.
* Differences with control are statistically significant at $p = 0.05$.

Combination of bioactive keratin and anthelmintic preparation was more effective in terms of skin area, it was 18.4% higher ($t_{fact} = 19.20 > t_{table} = 2.1$ at $p = 0.05$) than in Group III (control). Dietary bioactive keratin without deworming ensured an increase in the area of skins by 2.7% ($t_{fact} = 3.87 > t_{table} = 2.1$ at

p = 0.05) compared to Group III. Between Groups II and IV, this indicator differed by 15.1% statistically significantly ($t_{fact.} = 24.60 > t_{table} = 2.1$ at p = 0.05).

The use of drugs affected the quality of the hair. The greatest length of all hair categories was characteristic of the lateral area of the skins in all groups, the smallest length in fur and transient hair was noted in the rump area, in the guard and aligning hair in the spinal part (Table 6).

6. Skin hair length (mm) **of young silver-black foxes (*Vulpes vulpes* L.) upon application of anthelmintic Niacid-Granules Plus and sulfur-containing feed additive Bioactive Keratin (*M*±SEM, *n* = 50, AO Saltykovskii Breeding Farm, 2018)**

Groups	Category of hair	Locations		
		spine	side	rump
I (Niacid-Granules Plus)	Down hair	44.7±0.1*	46.3±0.5*	43.0±0.8*
	Awn hair	52.8±0.3	53.2±0.3	52.4±0.6
	Guard hair	69.8±0.8	75.7±0.8	70.5±0.9
	Guide hair	73.0±0.7	82.0±0.7	75.7±0.6
II (Niacid-Granules Plus + Bioactive Keratin)	Down hair	46.9±0.7*	49.8±0.4*	45.9±0.8*
	Awn hair	53.6±0.4	56.9±0.5	53.2±0.3
	Guard hair	72.2±0.7	77.2±0.6	72.9±0.7
	Guide hair	74.1±0.3	84.5±0.2	82.3±0.6
III (intact animals, control)	Down hair	31.4±0.2	32.8±0.4	32.0±0.4
	Awn hair	40.2±0.1	41.7±0.6	42.1±0.2
	Guard hair	37.8±0.3	26.6±0.2	27.2±0.4
	Guide hair	62.1±0.2	54.3±0.7	57.4±0.6
IV (Bioactive Keratin)	Down hair	36.6±0.1	38.8±0.6	37.4±0.4
	Awn hair	44.0±0.2	45.6±0.4	44.1±0.2
	Guard hair	48.3±0.2	36.2±0.2	31.8±0.7
	Guide hair	65.5±0.4	58.7±0.6	60.2±0.4

N o t e. For a description of drug application scheme in the groups, see the Techniques section.
* Differences with control are statistically significant at p = 0.05.

The length of down hair on the side of the skins in Groups I and III significantly increased by 4.5% ($t_{fact.} = 2.80 > t_{table} = 2.1$ at p = 0.05) as influenced by using only anthelmintic preparation. In Groups II and IV with the combination of an anthelmintic preparation and a bio-additive, an increase in the length of down hair reached 4.2% ($t_{fact.} = 3.50 > t_{table} = 2.1$ at p = 0.05). Differences were also characteristic of the remaining topographic areas in which the length of the hair was analyzed (see Table 6).

7. Hair thickness (microns) **on the skins of young silver-black foxes (*Vulpes vulpes* L.) upon application of anthelmintic Niacid-Granules Plus and sulfur-containing feed additive Bioactive Keratin (*M*±SEM, *n* = 50, AO Saltykovskii Breeding Farm, 2018)**

Groups	Category of hair	Locations		
		spine	side	rump
I (Niacid-Granules Plus)	Down hair	20.4±0.2	20.3±0.3	21.8±0.4
	Awn hair	47.2±0.7	45.5±0.5	50.2±0.5
	Guard hair	76.9±0.7	72.6±0.7	80.3±0.6
	Guide hair	87.9±0.7	73.8±0.2	94.5±0.6
II (Niacid-Granules Plus + Bioactive Keratin)	Down hair	21.4±0.2	21.2±0.4	22.1±0.3
	Awn hair	49.9±0.8	45.3±0.6	50.0±0.7
	Guard hair	80.3±0.7	75.6±0.6	85.7±0.9
	Guide hair	96.8±0.5	95.7±0.7	97.8±0.9
III (intact animals, control)	Down hair	22.4±0.5	21.7±0.2	23.6±0.7
	Awn hair	36.9±0.2	35.7±0.4	37.1±0.5
	Guard hair	58.4±0.6	59.6±0.6	60.1±0.4
	Guide hair	68.5±0.4	61.1±0.7	59.8±0.6
IV (Bioactive Keratin)	Down hair	24.3±0.6	23.8±0.3	24.8±0.4
	Awn hair	37.6±0.4	37.7±0.3	39.7±0.7
	Guard hair	61.8±0.9	65.1±0.9	69.9±0.3
	Guide hair	71.4±0.6	70.8±0.3	71.8±0.6

N o t e. For a description of drug application scheme in the groups, see the Techniques section.

The thickness of hair (Table 7) in various topographic areas of the skins was studied on the same samples on which the length of the hair was measured. The obtained data enable to conclude that neither the anthelmintic preparation Niacid-Granules Plus, nor its combination with the feed additive Bioactive Keratin affected the thickness of the hair of the skins in comparison with the control ($t_{fact.} = 1.8 < t_{table} = 2.1$ and $t_{fact.} = 1.3 < t_{table} = 2.1$, respectively, at $p = 0.05$). The same was noted for Group IV.

The density of hair on skin was estimated by the number of primary and secondary follicles, as well as by direct counting of all hair categories per unit area (1 cm^2) of samples taken from the rump part of the skin (Table 8).

8. Hair density on the skins of young silver-black foxes (*Vulpes vulpes* L.) upon application of anthelmintic Niacid-Granules Plus and sulfur-containing feed additive Bioactive Keratin (*M*±SEM, *n* = 10, AO Saltykovskii Breeding Farm, 2018)

Groups	Average number of hair follicles per microscope field of view		Total hair numberper unit area (1 cm^2)
	primary	secondary	
I (Niacid-Granules Plus)	14.3	1.4	14322±468
II (Niacid-Granules Plus + Bioactive Keratin)	15.7	1.6	15670±580*
III (intact animals, control)	11.1	1.0	12576±462
IV (Bioactive Keratin)	12.3	1.1	13009±359

N o t e. For a description of drug application scheme in the groups, see the Techniques section.
* Differences with control are statistically significant at p = 0.05.

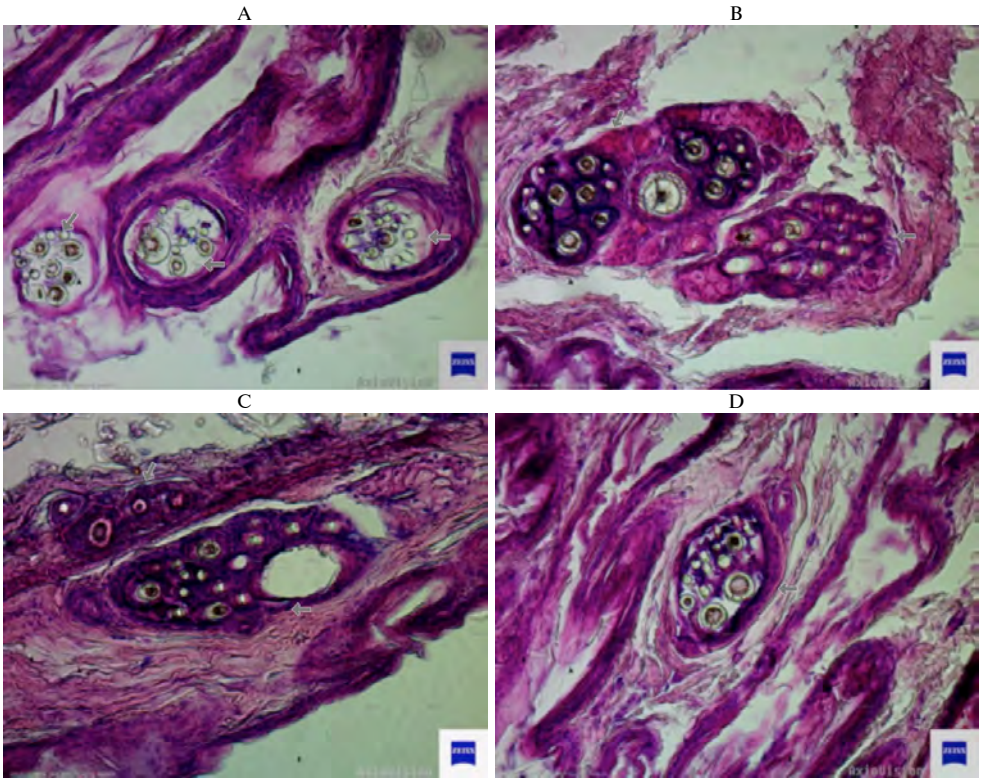

Fig. 2. Horizontal histological sections of the skin tissue of silver-black fox (*Vulpes vulpes* L.) pelt as influenced by anthelmintic Niacid-Granules Plus (A)**, Niacid-Granules Plus in combination with the sulfur-containing feed additive Bioactive Keratin** (B)**, in intact animals (control)** (C) **and when using Bioactive Keratin** (D) AO Saltykovskii Breeding Farm, 2018). Staining with hematoxylin and eosin, magnification ×400; arrows indicate bunches of hair.

The use of Niacid-Granules Plus had a positive effect on the downiness of the skins: the total number of all hair categories per 1 cm^2 was 13.9% higher

($t_{fact.}$ = 2.66 > t_{table} = 2.1 at p = 0.05) than in control. Combination of the anthelmintics and bioactive keratin significantly ($t_{fact.}$ = 4.17 > t_{table} = 2.1 at p = 0.05) increased hair density by 24.6% compared to control, whereas bioactive keratin only by 3.4% ($t_{fact.}$ = 0.74 < t_{table} = 2.1 at p = 0.05). Consequently, the combined application of the studied preparations had the greatest positive effect. It is obvious that the anthelmintic drug contributed to the healing of the young silver-black foxes, due to which bioactive keratin stimulated the growth of the thicker hair to the maximum extent.

Many authors note that it is keratin that stimulates hair growth, increasing their density due to the activation of embryonic follicles [11]. To test this hypothesis, horizontal histological sections were prepared from samples of the same skins taken from the rump part (Fig. 2).

Our findings substantiate application of Niacid-Granules Plus in combination with Bioactive Keratin as an element of the technology for growing slaughtered young silver-black fox to improve the physiological condition of animals and obtain more qualitative skins. Niacid-Granules Plus is recommended to be used 2 times with a 10-day interval at the age of 2 months, and Bioactive Keratin should be added to the main diet in six cycles (5-day courses with 10-day intervals).

Thus, deworming of young silver-black foxes ages 2 months using a new generation of Niacid-Granules Plus drug followed by application of dietary Bioactive Keratin contribute to the accelerated normalization of the physiological status of young silver-black foxes, which is confirmed by clinical indicators of blood, and significantly affect the live weight gain. When using both preparations, the weight of young silver-black foxes increased by 30.4%, the area of the obtained skins by 18.4%, the density of the hair by 24.6% compared to the same parameters for intact animals. This allows us to recommend Niacid-Granules Plus in combination with feed additive Bioactive Keratin for practical use in animal husbandry to improve the well-being of animals and the quality of marketable products obtained from them.

REFERENCES

1. *Canids: foxes, wolves, jackals and dogs. Status survey and conservation action plan*. C. Sillero-Zubiri, M. Hoffmann, D.W. Macdonald (eds.). IUCN, Gland, Switzerland and Cambridge, UK, 2004.
2. Zhang T., Zhong W., Sun W., Wang Z., Sun H., Fan Y., Li G. Effects of dietary fat: carbohydrate ratio on nutrient digestibility, serum parameters, and production performance in male silver foxes (*Vulpes vulpes*) during the winter fur-growing period. *Canadian Journal of Animal Science*, 2017, 97(2): 199-206 (doi: 10.1139/CJAS-2015-0167).
3. Juokslahti T., Korhonen T., Oksanen A. Coccidiosis in farmed silver foxes (*Vulpes vulpes*) and blue foxes (*Alopex lagopus*) in Finland: a case report. *Acta Veterinaria Scandinavica*, 2010, 52(supplement 1): S18 (doi: 10.1186/1751-0147-52-S1-S18).
4. Özkan B. Parasitary infestation in a fox. *Journal of Istanbul Veterinary Sciences*, 2018, 2(1): 14-22 (doi: 10.30704/http-www-jivs-net.388610).
5. Lankas G.R., Gordon L.R. *Toxicology*. In: *Ivermectin and avermectin*. W.C. Campbell (ed.). Springer-Verlag, NY, 1989: 89-112.
6. Taira K., Saeed I., Kapel C.M. Dose-dependent egg excretion in foxes (*Vulpes vulpes*) after a single infection with *Toxocara canis* eggs. *Parasitology Reserch*, 2002, 88(10): 941-943 (doi: 10.1007/s00436-002-0680-x).
7. Magi M., Guardone L., Macchioni F., Prati M.C., Mignone W. Extraintestinal nematodes of the red fox *Vulpes vulpes* in north-west Italy. *Journal of Helminthology*, 2015, 89(4): 506-511 (doi: 10.1017/S0022149X1400025X).
8. Bobova T.A., Kolobov A.V., Zavarzin I.V., Dzhafarov M.Kh., Plakhtinskii V.V., Kuleshova E.S. *Tezisy XIX Mendeleevskogo s"ezda po obshchei i prikladnoi khimii* [Proc. XIX Mendeleev Congress on General and Applied Chemistry]. Volgograd, 2011, vyp. 1: 130 (in Russ.).

9. Dyary H.O. Veterinary anthelmintics and anthelmintic drug resistance. *Journal of Zankoy Sulaimani. Part-A (Pure and Applied Sciences)*, 2016, 18(1): 191-206 (doi: 10.17656/jzs.10463).
10. Prichard R., Ménez C., Lespine A. Moxidectin and the avermectins: consanguinity but not identity. *International Journal of Parasitology: Drugs and Drug Resistance,* 2012, 2: 134-153 (doi: 10.1016/j.ijpddr.2012.04.001).
11. Vasilevich F.I., Sapozhnikova A.I., Okutin A.S., Gordienko I.M., Ruchkina Z.S., Bordachev V.N. Improvement of ferret fur pelt quality by using products of recycling raw materials of animal origin. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2017, 6(52): 1214-1225 (doi: 10.15389/agrobiology.2017.6.1214eng).
12. Mogosanu G.D., Grumezescu A.M., Chifiriuc M.C. Keratin-based biomaterials for biomedical applications. *Current Drug Targets*, 2014, 15(5): 518-530 (doi: 10.2174/1389450115666140307154143).
13. Kirsner R.S., Cassidy S., Marsh C., Vivas A., Kelly R.J. Use of a keratin-based wound dressing in the management of wounds in a patient with recessive dystrophic epidermolysis bullosa. *Advances in skin and wound care*, 2012, 25(9): 400-403 (doi: 10.1097/01.ASW.0000419404.44947.de).
14. Rouse J.G., Van Dyke M.E. A review of keratin-based biomaterials for biomedical applications. *Materials*, 2010, 3: 999-1014 (doi: 10.3390/ma3020999).
15. Van Heugten E., Van Kempen T.A. Growth performance, carcass characteristics, nutrient digestibility and fecal odorous compounds in growing-finishing pigs fed diets containing hydrolyzed feather meal. *Journal of Animal Science*, 80(1): 171-178 (doi: 10.2527/2002.801171x).
16. Poole L.B. The basics of thiols and cysteines in redox biology and chemistry. *Free Radical Biology and Medicine*, 2015, 80: 148-157 (doi: 10.1016/j.freeradbiomed.2014.11.013).
17. Golubkov V.A. V sbornike: *Ekologiya Yuzhnoi Sibiri i sopredel'nykh territorii* [In: Ecology of Southern Siberia and adjacent territories]. Moscow, 2016: 12 (in Russ.).
18. Dröge W., Breitkreutz R. Glutathione and immune function. *Proceedings of the Nutrition Society*, 2000, 59(4): 595-600 (doi: 10.1017/s0029665100000847).
19. Ghezzi P. Role of glutathione in immunity and inflammation in the lung. *International Journal of General Medicine*, 2011, 4: 105-113 (doi: 10.2147/IJGM.S15618).
20. Wang B., Yang W., McKittrick J., Meyers M.A. Keratin: structure, mechanical properties, occurrence in biological organisms, and efforts at bio inspiration. *Progress in Materials Science*, 2016, 76: 229-318 (doi: 10.1016/j.pmatsci.2015.06.001).
21. Ramms L., Fabris G., Windoffer R., Schwarz N., Springer R., Zhou C., Lazar J., Stiefel S., Hersch N., Schnakenberg U., Magin T.M., Leube R.E., Merkel R., Hoffmann B. Keratins as the main component for the mechanical integrity of keratinocytes. *PNAS USA*, 2013, 110(46): 18513-18518 (doi: 10.1073/pnas.1313491110).
22. Windoffer R., Beil M., Magin T.M., Leube R.E. Cytoskeleton in motion: the dynamics of keratin intermediate filaments in epithelia. *J. Cell Biol.*, 2011, 194(5): 669-678 (doi: 10.1083/jcb.201008095).
23. Dzhafarov M.Kh., Vasilevich F.I. Ecological, physiological and biochemical adaptation in helminth: trends in evolution of anthelminthic chemical agents (review). *Advances in Pharmacology and Pharmacy*, 2014, 2: 30-45.
24. Peng Y., Shi Q., Wang Y., Zhang F., Ji Z., Zhang J. Dietary probiotics have different effects on the composition of fecal microbiota in farmed raccoon dog (*Nyctereutes procyonoides*) and silver fox (*Vulpes vulpes fulva*). *BMC Microbiology*, 2019, 19(1): Article number 109 (doi: 10.1186/s12866-019-1491-x).
25. Sapozhnikova A.I., Kaspar'yants S.A., Mesropova N.V., Gordienko N.M. *Sposob polucheniya keratina. A.c. 2092072 (RF) MKI[6] A 23 K 1/10. № 95117245-13.* Zayavl. 06.10.95. Opubl. 10.10.97. Byul. № 28 [The method of obtaining keratin. A.c. 2092072 (RF) MKI[6] A 23 K 1/10. № 95117245-13. Appl. 06.10.95. Publ. 10.10.97. Bul. № 28] (in Russ.).
26. Prozorovskii V.B., Prozorovskii M., Demchenko V.M. *Farmakologiya i toksikologiya*, 1978, 41(4): 497-502 (in Russ.).
27. Borovikov V. *STATISTICA. Iskusstvo analiza dannykh na komp'yutere* [STATISTICA. The art of computer data analysis]. St. Petersburg, 2003 (in Russ.).
28. Borovkov A.A. *Matematicheskaya statistika. Otsenka parametrov. Proverka gipotez* [Math statistics. Estimation of parameters. Hypothesis testing]. Moscow, 1984 (in Russ.).
29. Esepenok K.V., Sapozhnikova A.I., Mirzayev M.N. Safety assessment of the "Niacid-granules plus" product and the "Bioactive keratin" sulfur-containing feed additive. *Materials of the International Conference «Scientific research of the SCO countries: synergy and integration»*. Beijing, 2019: 140-148 (doi: 10.34660/INF.2019.16.36671).
30. Napisanova L.A., Zhdanova O.B., Okulova I.I., Ashikhmin S.P., Berezina Yu.A., Chasovskikh O.V. *Rossiiskii parazitologicheskii zhurnal*, 2016, 2: 210-216 (in Russ.).
31. Sanzhieva S.E. *Agrarnyi vestnik Urala*, 2010, 4: 56-58 (in Russ.).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1167-1176
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Northern reindeer herding

UDC 636.294:575.174:575.113:577.2.08

doi: 10.15389/agrobiology.2019.6.1167eng
doi: 10.15389/agrobiology.2019.6.1167rus

DEVELOPMENT AND VALIDATION OF A LOW DENSITY SNP PANEL FOR ASSESSMENT OF GENETIC DIVERSITY OF THE REINDEER (*Rangifer tarandus*) POPULATIONS

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The authors declare no conflict of interests

Acknowledgements:
The equipment of the Sharing Center for Farm Animal Bioresources and Bioengineering (FSC for Animal Husbandry) was used.
Supported financially by Russian Science Foundation, project 16-16-10068-P
Received August 6, 2019

Abstract

Reindeer (*Rangifer tarandus*) is a valuable member of the Arctic ecosystems and the main livestock species of the Russian North, which require the analysis of the genetic structure and the possibility of addressing the differences between wild and domestic forms, breeds and populations using modern molecular genetic approaches. The use of DNA chips based on parallel genotyping of hundreds of thousands of SNP markers is an effective approach to study the reindeer genome, but at the same time due to a high price, it is not beneficial for wide practical application. In this regard, the aim of our work is to select the optimal number of SNP markers that allow conducting population and genetic studies of reindeer without loss of bio-informatics content. The sample collection included wild deer (WLD, $n = 83$) inhabiting the Taimyr Peninsula and the Republic of Sakha (Yakutia), and domestic deer of the Nenets breed from the Nenets Autonomous Okrug (NEN, $n = 100$) and the Murmansk Region (MUR, $n = 19$), as well as from Even and Evenki breeds from the Republic of Sakha (Yakutia) (YAK, $n = 19$). All deer were genotyped using a high-density DNA chip BovineHD BeadChip (777,962 SNPs). After quality control and filtering, 4456 polymorphic SNP markers remained in the analysis. In the TRES program, using the Delta method, 368 of the most informative SNP markers were selected. Data processing was performed in the Admixture 1.3, PLINK 1.9 programs and R packages (ggplot2, adegenet 1.3-1, pophelper, diveRsity). It was shown that 70 % from 368 selected SNPs had a high minor allele frequency (MAF ≥ 0.3), while about 50 % from set including 4456 markers had MAF ≤ 0.1. Comparing the results of principal component analysis (PCA), discriminant principal component analysis (DAPC), and cluster analysis, no loss of information value was found for 368 SNPs compared to using the set of 4456 markers. Comparing

pairwise F_{ST} values between the studied groups of reindeer, the similarity of the interpopulation linkages was demonstrated, based on 4456 and 368 SNP markers, respectively. Thus, the selected panel of SNP markers is an informative, universal for both wild and domestic deer and a cheap approach for creating a custom DNA chip for reindeer.

Keywords: *Rangifer tarandus*, reindeer, SNP markers, DNA chips

Reindeer (*Rangifer tarandus*) is a unique species, among which both the wild and the domestic forms coexist and are valuable members of the Arctic ecosystems [1, 2]. Both the forms require the development of rational programs to conserve their genetic diversity. Rapid improvements and increased availability of new-generation sequencing technologies (next-generation sequencing) have led to an increase in the numbers of full genomes determined for various animal species each year [3, 4].

Over the past few years, numerous studies have been performed to map the genome of the reindeer, which has revealed three versions of the genome assembly. In 2017, a group of Chinese researchers introduced the first version of the domestic reindeer genome [5]. DNA was extracted from the blood of one female of the Evenk breed from a population which had been introduced into the mountains of Inner Mongolia in China, and sequencing of the sample was performed on the HiSeq 4000 platform (Illumina, San Diego, CA, USA).

This assembly showed that the total genome size of the domestic reindeer was 2.64 Gb. A total of 21,555 protein-coding genes were decoded, and 3803 genes were completely reconstructed (92.6% of the genome size). However, it was later discovered that this genome assembly was fragmented and contained a high percentage of missing data [6].

In 2019, the genome of the caribou, the North American relative of *R. tarandus* [6] with a genome size of 2.205 Gb, was announced. According to Taylor et al. [6], the caribou genome includes 33,177 protein-coding genes, including 3820 (93.1%) completely reconstructed genes. However, since the wild and the domestic forms of *R. tarandus* are clearly differentiated by single-nucleotide polymorphism (SNP) markers [8], this described genome assembly may not be relevant to the domestic reindeer. Finally, a group of Scandinavian researchers [7] performed deep sequencing of the domestic reindeer genome using the HiSeq2500 and HiSeq 4000 systems (Illumina). However, the complete nucleotide sequence of this assembly, which had a total size of 2.66 Gb and included 26,785 protein-coding genes, cannot be found in the National Center for Biotechnology Information database and other platforms. As a result, despite significant successes in decoding the reindeer genome, this species remains in the non-model category.

Negative anthropogenic factors and unpredictable climate changes in the reindeer habitat have led to irreversible critical changes in the biodiversity of this species [9, 10]. At present, there has been an active research for the search and implementation of various approaches for assessment of the genetic diversity, for the study of the population structure, and for the reliable differentiation of the intraspecific groups [11]. SNPs have been widely used as DNA markers for studying the genetic structure of populations [12]. The use of a DNA microarray enables simultaneous genotyping of myriad markers. However, the main task in most studies is to determine the pedigree, the group, or the individual origin of the animal [13]. In this case, it is reasonable to use only those SNPs that can most accurately differentiate relevant individuals at a lower cost using faster procedures compared to that in genome-wide genotyping approaches [13, 14]. In this study, SNPs that met the criteria as markers for creating a custom DNA microarray were selected for the reindeer using a minor allele frequency (MAF) above 0.3 to preserve the bioinformatics value and universal nature of this infor-

mation in population genetics studies of both the domestic and the wild forms of *R. tarandus*.

Our aim was to select the optimal number of SNPs that enabled population genetics studies of the reindeer without the loss of bioinformatics content and for performing a comparative analysis of the information of the selected and of all the polymorphic SNPs.

Techniques. The studies were performed on the wild and the domestic reindeer in 2019. The wild reindeer population (WLD, $n = 83$) included the reindeer inhabiting the western part of the Taimyr Peninsula and representatives of the Leno-Olenek and the Sundrun subpopulations from the Republic of Sakha (Yakutia) territory.

The domestic reindeer group consisted of animals of the Nenets breed, which are bred in the territory of the Nenets Autonomous district (NEN, $n = 100$ and the Murmansk Region (MUR, $n = 19$), as well as the Even and the Evenk breeds from the Republic of Sakha (Yakutia) (YAK, $n = 19$). Genomic DNA was extracted using the Extran2 DNA kits (Syntol CJSC, Moscow, Russia) according to the manufacturer's instructions. The quality and integrity of the DNA samples were checked by electrophoresis on a 1% agarose gel. DNA concentration was measured on a Qubit 2.0 fluorimeter (Invitrogen, Carlsbad, CA, USA). DNA purity was determined by evaluating the absorption ratio of A_{260}/A_{280} on a NanoDrop8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All the investigated reindeer individuals were genotyped using the Illumina BovineHD BeadChip, which contained 777,962 SNPs (Illumina). Genotypes were obtained using the Genome Studio 2 (Illumina). Reading quality (GenCall, GC) and the degree of SNP clustering (GenTrain, GT) were evaluated by establishing filters with limit values of 0.5 for both indicators [15]. In PLINK v. 1.90 [16], SNPs located on the sex chromosomes, characterized by MAF below 5% (-MAF 0.05), that deviated from the Hardy-Weinberg equilibrium at $p < 10^{-6}$ (-hwe $1e^{-6}$), those that were in linkage disequilibrium (--indep-pairwise 50 5 0.5), and those for which less than 90% of the animals were genotyped (- -geno0.1) were excluded from the analysis. After filtering, 4456 polymorphic SNPs were included for analysis.

The next step was selection of the most informative SNPs for inclusion in the user panel. Markers were selected in the TRES program (Toolbox for Ranking and Evaluation of SNPs) [13] according to the Delta method [17] in accordance with the equation:

$$\delta = |p^i_A - p^j_A|,$$

where p^i_A is the frequency of allele A in the i^{th} population and p^j_A is the frequency of the same allele in the j^{th} population.

Next, the loci were evaluated based on their δ values and ranked in a descending order from the highest values to the lowest value of 0.2. As a result, 368 SNPs were selected.

The graphs of the distribution of SNPs in the different groups depending on the MAF values were visualized using the R package [18]. Principal component analysis was performed in PLINK 1.9 and the results were visualized in the R package ggplot2 [19]. Discriminant analysis of the principal components for the investigated reindeer groups was performed using the R package adegenet 1.3-1 [20, 21], the cluster analysis — in Admixture 1.3 [22] with subsequent visualization with R package «pophelper» [23]. The pairwise fixation index (F_{ST}) values [24] were calculated with R package diveRsity [25]. All bioinformatics analyses were performed for the 4456 SNPs and 368 SNPs to evaluate the effectiveness of the proposed panel (368 SNPs) compared to the complete set of polymorphic SNPs (4456) identified using the Illumina BovineHD BeadChip for

the reindeer populations.

Results. The distribution of the selected SNPs among the groups were compared depending on the MAF values (Fig. 1), which revealed that the group with MAF of 0.1 contained the largest number of SNPs in the panel of 4456 markers (approximately 49.3%), whereas the number of markers in the remaining groups decreased from 800 in the group with MAF of 0.2 (17.9%) to 250 (5.6%) in the group with MAF of 0.5 (see Fig. 1, A). When using a panel of 368 markers, the distribution of SNPs was drastically different (see Fig. 1, B). Thus, the group with MAF of 0.1 accounted for only 4.1% of the total number of panel markers. The largest number of SNPs (57.1%) was represented by the groups with MAF values of 0.3 and 0.4. The group with MAF of 0.5 comprised approximately 15.8% of all panel markers.

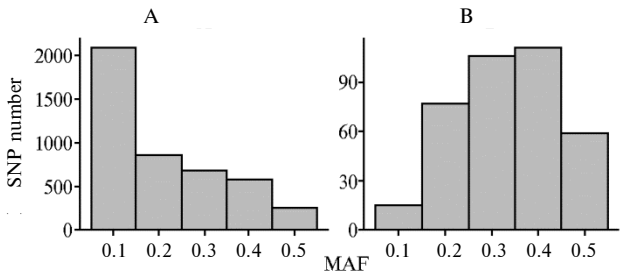

Fig. 1. Distribution of the SNPs by group (2019) **depending on the minor allele** (MAF) **frequency by comparison of two marker panels:** A — final data set comprised 4456 SNPs for the domestic and the wild reindeer (*Rangifer tarandus*) from the BovineHD BeadChip; B — 368 SNPs selected to create a custom DNA microarray.

In general, principal component analysis of the investigated reindeer groups based on 4456 SNPs and 368 SNPs revealed no significant differences in the character of the spatial formation of clusters (Fig. 2, A, B). Thus, the first main component (PC1) clearly separated the MUR and the NEN groups from the WLD group, whereas the YAK population, which was nearly located on PC1, was differentiated by the second main component (PC2) among the three reindeer clusters. However, the genetic variability magnitude explained by PC1 and PC2 when using a panel of 4465 SNPs (see Fig. 2, A) was several-fold lower compared to that in the second panel (see Fig. 2, B): 11.8% vs. 36.28% for PC1 and 3.21% vs. 10.32% for PC2.

The discriminant analysis of the principal components was used to examine the patterns in the genetic diversity of the investigated reindeer groups (Fig. 2, C, D). Linear discriminant 1 (LD 1) clearly differentiated the NEN, the MUR, and the YAK populations from the WLD group. The YAK group was the most distant for linear discriminant 2 (LD 2). In addition, the NEN, the MUR, and the WLD groups were close to the LD 2 axis. No significant differences were found in the information of the two investigated systems.

Comparison of the degree of genetic differences between the investigated reindeer groups (Table) showed the similarity of interpopulation relationships estimated using the 4456 SNPs and 368 SNPs. Thus, the highest genetic closeness was observed between the MUR and the NEN groups, and the maximum differentiation was found between the WLD and the NEN.

The results of the ADMIXTURE-analysis for the investigated reindeer groups based on the 4456 SNPs (see Fig. 3, A) and 368 SNPs (see Fig. 3, B) for the most probable number of clusters 2 and 3 have been shown in Figure 3. At K = 2, both panels showed similar clustering results. The NEN and the WLD populations were clearly differentiated and formed their own cluster (light grey for the WLD and black for the NEN), whereas the MUR and the YAK populations represented mixed clusters. At K = 3, the YAK group segregated into its own cluster, and presented a consolidation of individuals within which looked more identically by using a kit including all polymorphic SNPs (see Fig. 3, A).

However, differences in the pattern of distribution of the animal groups at K = 3 were insignificant.

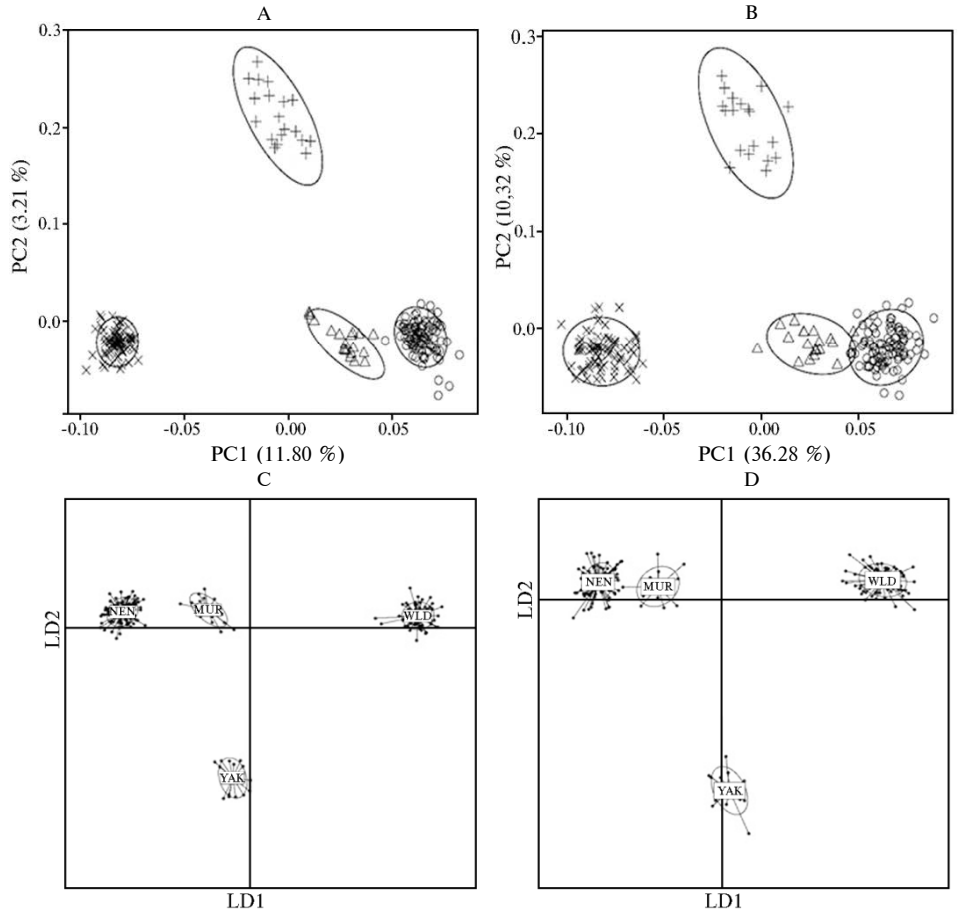

Fig. 2. Results of the principal component analysis (A, B) **and discriminant analysis of the principal components** (C, D)**, conducted for the four populations of reindeer (*Rangifer tarandus*) based on data from two SNP panels:** A, C — final data set comprised of 4456 SNPs detected with the BovineHD BeadChip; B, D — 368 SNPs selected to create a custom DNA microarray; WLD — wild population (the Taimyr Peninsula, the Republic of Sakha-Yakutia), NEN — domestic reindeer of the Nenets breed (the Nenets Autonomous Okrug), MUR — domestic reindeer of the Nenets breed (the Murmansk region), YAK — domestic reindeer of the Even and Evenk breeds (the Republic of Sakha-Yakutia) (2019).

The effectiveness of using a DNA microarray designed for cattle to evaluate the reindeer has been demonstrated in foreign [26, 27] and domestic studies [8, 28]. Nevertheless, among all the genotyped SNPs, only approximately 5% of the SNPs are polymorphic and have been directly used for analysis: 1068 for the black-tailed and the white-tailed deer [26], 1532 for the representatives of the genus Cervus [27], and 512 for the Yakut domestic reindeer [28] when using the Bovine SNP50 BeadChip and 8357 and 8145, respectively, for the domestic and the wild reindeer from among 777,962 SNPs on the BovineHD BeadChip [8].

Despite the success of using these DNA microarrays to characterise the genetic structure of the reindeer populations and assess their genetic diversity and relationships, there are several factors that prevent the use of this approach for routine testing. First, the DNA microarray is costly. Second, nearly 95% of the content of a DNA microarray is uninformative for the reindeer. In addition, detailed genomic studies are not always required for practical applications in the reindeer husbandry.

F_{ST} values calculated from 4456 and 368 SNPs in the four reindeer populations (*Rangifer tarandus*) (2019)

Population	NEN	MUR	YAK	WLD
NEN		0.025	0.184	0.218
MUR	0.012		0.142	0.138
YAK	0.043	0.040		0.215
WLD	0.060	0.042	0.048	

N o t e. WLD — wild population (the Taimyr Peninsula, the Republic of Sakha-Yakutia), NEN — domestic reindeer of the Nenets breed (the Nenets Autonomous Okrug), MUR — domestic reindeer of the Nenets breed (the Murmansk region), YAK — domestic reindeer of the Even and Evenk breeds (the Republic of Sakha-Yakutia); F_{ST} — fixation index. The values calculated for 368 SNPs are above the diagonal, the values calculated for the 4456 SNPs are below the diagonal.

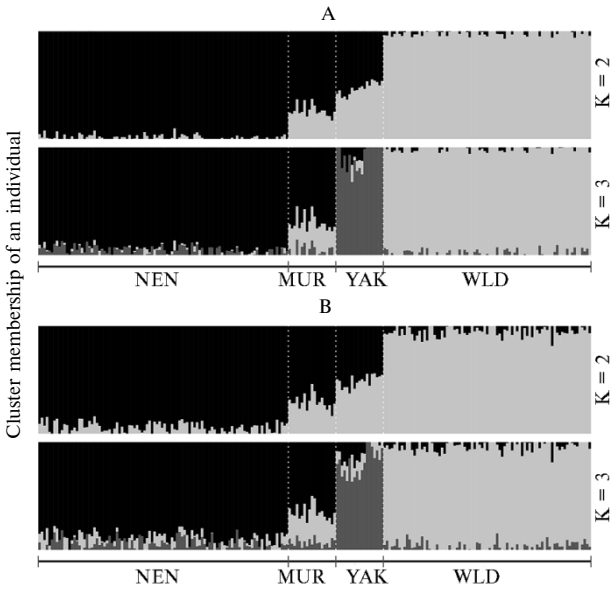

Fig. 3. Comparative ADMIXTURE analysis of the four reindeer populations (*Rangifer tarandus*) (2019) **for two SNP panels:** A — final data set comprised 4456 SNPs detected with the BovineHD BeadChip; B — 368 SNPs selected to create a custom DNA microarray; K — the number of most probable clusters; WLD — wild population (the Taimyr Peninsula, the Republic of Sakha-Yakutia), NEN — domestic reindeer of the Nenets breed (the Nenets Autonomous Okrug), MUR — domestic reindeer of the Nenets breed (the Murmansk region), YAK — domestic reindeer of the Even and the Evenk breeds (the Republic of Sakha-Yakutia).

In this case, the development of a custom DNA microarray may reduce the cost of analysis without significantly decreasing its biological significance [29, 30]. Similar SNP panels containing a limited number of informative markers have been designed for model farm animals, such as the sheep [31, 32], cattle [33], and horses [34].

Our study showed that 368 SNPs provided reproducible information for all detected polymorphic markers (4456), which was found to be consistent with previously obtained data for other mammalian species. According to Heaton et al. [33], 32 SNPs are sufficient to determine the origin with an accuracy of 99.99% in the beef cattle populations (even in cases of highly mixed herds). Kijas et al. [31] suggested using SNP panels of 88 markers for paternity confirmation in more than 80 sheep breeds, and using 101 markers in horse breeding for the same purposes [34]. In addition, a panel of 163 markers is sufficient for determining the breed and individual affiliation of a wide range of sheep breeds [32].

The MAF is an important parameter in selecting SNPs for the subsequent development of an informative custom DNA microarray. Using the TRES bioinformatics approach [13], we selected 368 SNPs from among 4456 polymorphic SNPs. The calculations showed bias in the selected SNPs towards increasing MAFs from 0.1 to 0.3-0.4. The observed trend was consistent with that obtained by Kijas et al. [31] and Heaton et al. [32] in the development of panels for determining the origin of sheep (panels consisted of 88 and 163 markers, respectively). According to Kijas et al. [31], choosing SNPs with a shift towards an MAF $\geq$ 0.3 for inclusion in the panel enhances the versatility of the panel, ena-

bling its application to diverse breeds and population compositions of the investigated species. The 368 markers selected in this study met this criterion.

We selected SNP markers from a DNA microarray developed for the cattle, in accordance with their localisation along the chromosomal characteristics of the cattle. As the alignment of the nucleotide sequences of the reindeer genome was performed using the cattle genome as a reference [6] (without breakdown into the reindeer chromosomes), the SNP panel can provide the same amount of information as those developed based on the reindeer genome assembly proposed by Taylor et al. [6].

Thus, the minimum required number of SNPs was selected, allowing for genetic studies in the reindeer populations without the loss of bioinformatics content. To assess the information of a panel of 368 SNPs, the data obtained were compared to the results calculated using 4456 SNPs identified by a high-density microarray. Among the markers included in the test panel, a clear bias towards SNPs with MAF of more than 0.3 was observed, corresponding to international criteria for creating panels for various types of agricultural animals. All bioinformatics approaches (the principal component analysis, the discriminant analysis of principal components, the cluster analysis, and calculation of pairwise genetic distances by F_{ST} values) showed no loss in efficiency of the 368 SNPs panel. Successful testing of the presented panel was performed in the three breeds of the domestic reindeer (the Nenets, the Even, the Evenk) and in the two populations of the wild reindeer (the Taimyr and the Yakut), demonstrating the universal nature of the selected SNPs. Selected SNPs may be recommended for inclusion during construction of a custom DNA microarray designed for genomic characterisation of the wild and the domestic reindeer.

REFERENCES

1. Baskin L.M. *Severnyi olen'. Upravlenie povedeniem i populyatsiyami. Olenevodstvo. Okhota* [Reindeer. Manage behavior and populations. Reindeer husbandry. Hunting]. Moscow, 2009 [in Russ.).
2. Laishev K.A., Yuzhakov A.A. *Nauchnyi vestnik Yamalo-Nenetskogo avtonomnogo okruga*, 2017, 1[94): 45-48 [in Russ.).
3. Zhang J., Chiodini R., Badr A., Zhang G. The impact of next-generation sequencing on genomics. *Journal of Genetics and Genomics,* 2011, 38[3): 95-109 [doi: 10.1016/j.jgg.2011.02.003).
4. Kchouk M., Gibrat J.F., Elloumi M. Generations of sequencing technologies: from first to next generation. *Biol. Med. [Aligarh)*, 2017, 9[3): 1000395 [doi: 10.4172/0974-8369.1000395).
5. Li Z., Lin Z., Ba H., Chen L., Yang Y., Wang K., Qiu Q., Wang W., Li G. Draft genome of the reindeer [*Rangifer tarandus*). *GigaScience*, 2017, 6[12): 1-5 [doi: 10.1093/gigascience/gix102).
6. Taylor R.S., Horn R.L., Zhang X., Golding G.B., Manseau M., Wilson P.J. The caribou [*Rangifer tarandus*) genome. *Genes*, 2019, 10[7): 540 [doi: 10.3390/genes10070540).
7. Weldenegodguad M., Pokharel K., Ming Y., Honkatukia M., Peippo J., Reilas T, Røed K.H., Kantanen J. OP149 sequencing of reindeer [*Rangifer tarandus*) genomes: insights into evolution, domestication, and adaptation. *Proc. 37th International Conference on Animal Genetics.* Lleida, Spain, 2019: 42.
8. Kharzinova V.R., Dotsev A.V., Deniskova T.E., Solovieva A.D., Fedorov V.I., Layshev K.A., Romanenko T.M., Okhlopkov I.M., Wimmers K., Reyer H., Brem G., Zinovieva N.A. Genetic diversity and population structure of domestic and wild reindeer [*Rangifer tarandus* L. 1758): a novel approach using BovineHD BeadChip. *PLoS ONE*, 2018, 13[11): e0207944 [doi: 10.1371/journal.pone.0207944).
9. Yannic G., Pellissier L., Ortego J., Lecomte N., Couturier S., Cuyler C., Dussault C., Hundertmark K.J., Irvine R.J., Jenkins D.A., Kolpashikov L., Mager K., Musiani M., Parker K.L., Røed K.H., Sipko T., Þórisson S.G., Weckworth B.V., Guisan A., Bernatchez L., Côté S.D. Genetic diversity in caribou linked to past and future climate change. *Nature Climate Change*, 2014, 4: 132-137 [doi: 10.1038/nclimate2074).
10. Yuzhakov A.A. *Materialy V Mezhdunarodnoi nauchnoi konferentsii «Ekologiya drevnikh i traditsionnykh obshchestv»* [Proc. V Int. Conf. «Ecology of ancient and traditional societies»]. Tyumen', 2016: 269-274 [in Russ.).
11. Ogden R. Unlocking the potential of genomic technologies for wildlife forensics. *Molecular Ecology Resources*, 2011, 11[Suppl. 1): 109-116 [doi: 10.1111/j.1755-0998.2010.02954.x).
12. Helyar S.J., Hemmer-Hansen J., Bekkevold D. Taylor M.I., Ogden R., Limborg M.T., Car-

iani A., Maes G.E., Diopere E., Carvalho G.R., Nielsen E.E. Application of SNPs for population genetics of nonmodel organisms: new opportunities and challenges. *Molecular Ecology Resources*. 2011, 11[Suppl. 1): 123-136 [doi: 10.1111/j.1755-0998.2010.02943.x).
13. Kavakiotis I., Triantafyllidis A., Ntelidou D., Alexandri P., Megens H.J., Crooijmans R.P.M.A., Groenen M.A.M., Tsoumakas G., Vlahavas I. TRES: identification of discriminatory and informative SNPs from population genomic data. *Journal of Heredity*, 2015, 106[5): 672-676 [doi: 10.1093/jhered/esv044).
14. Wilkinson S., Wiener P., Archibald A.L., Law A., Schnabel R.D., McKay S.D., Taylor J.F., Ogden R. Evaluation of approaches for identifying population informative markers from high density SNP chips. *BMC Genetics*, 2011, 12: 45 [doi: 10.1186/1471-2156-12-45).
15. Fan J.B., Oliphant A., Shen R., Kermani B.G., Garcia F., Gunderson K.L., Hansen M., Steemers F., Butler S.L., Deloukas P., Galver L., Hunt S., Mcbride C., Bibikova M., Rubano T., Chen J., Wickham E., Doucet D., Chang W., Campbell D., Zhang B., Kruglyak S., Bentley D., Haas J., Rigault P., Zhou L., Stuelpnagel J., Chee M.S. Highly parallel SNP genotyping. *Cold Spring Harb. Symp. Quant. Biol.*, 2003, 68: 69-78 [doi: 10.1101/sqb.2003.68.69).
16. Chang C.C., Chow C.C., Tellier L.C., Vattikuti S., Purcell S.M., Lee J.J. Second-generation PLINK: rising to the challenge of larger and richer datasets. *GigaScience*, 2015, 4[1): s13742−015−0047−8 [doi: 10.1186/s13742-015-0047-8).
17. Shriver M.D., Smith M.W., Jin L., Marcini A., Akey J.M., Deka R., Ferrell R.E. Ethnic-affiliation estimation by use of population-specific DNA markers. *American Journal of Human Genetics*, 1997, 60[4): 957-964.
18. R Core Team [2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available: https://www.R-project.org/. No date.
19. Wickham H. *ggplot2 — Elegant graphics for data analysis*. Springer-Verlag, NY, 2009 [doi: 10.1007/978-0-387-98141-3).
20. Jombart T. adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, 2008, 24[11): 1403-1405 [doi: 10.1093/bioinformatics/btn129).
21. Jombart T., Ahmed I. adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. *Bioinformatics*, 2011, 27[21): 3070-3071 [doi: 10.1093/bioinformatics/btr521).
22. Alexander D.H., Novembre J., Lange K. Fast model-based estimation of ancestry in unrelated individuals. *Genome Research*, 2009, 19[9): 1655-1664 [doi: 10.1101/gr.094052.109).
23. Francis R.M. POPHELPER: an R package and web app to analyse and visualize population structure. *Molecular Ecology Resources*, 2017, 17[1): 27-32 [doi: 10.1111/1755-0998.12509).
24. Weir B.S., Cockerham C.C. Estimating F-statistics for the analysis of population structure. *Evolution*, 1984, 38[6): 1358-1370 [doi: 10.2307/2408641).
25. Keenan K., McGinnity P., Cross T.F., Crozier W.W., Prodöhl P.A. diveRsity: an R package for the estimation of population genetics parameters and their associated errors. *Methods in Ecology and Evolution*, 2013, 4[8): 782-788 [doi: 10.1111/2041-210X.12067).
26. Haynes G.D., Latch E.K. Identification of novel single nucleotide polymorphisms [SNPs) in deer [*Odocoileus* spp.) using the BovineSNP50 BeadChip. *PLoS ONE*, 2012, 7[5): e36536 [doi: 10.1371/journal.pone.0036536).
27. Kasarda R., Moravčíková N., Trakovická A., Krupová Z., Ondrej K. Genomic variation across cervid species in respect to the estimation of red deer diversity. *Acta Veterinaria-Beograd*, 2017, 67[1): 43-56 [doi: 10.1515/acve-2017-0005).
28. Kharzinova V.R., Dotsev A.V., Solov'eva A.D., Fedorov V.I., Okhlopkov I.M., Vimmers K., Reier Kh., Brem G., Zinov'eva N.A. Population-genetic characteristics of domestic reindeer of yakutia based on whole-genome SNP analysis. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2017, 52[4): 669-678 [doi: 10.15389/agrobiology.2017.4.669eng).
29. Lorenz M.G.O., Cortes L.M., Lorenz J.J., Liu E.T. Strategy for the design of custom cDNA microarrays. *Biotechniques,* 2003, 34[6): 1264-1270 [doi: 10.2144/03346mt02).
30. Neerincx P.B.T., Rauwerda H., Nie H., Groenen M.A.M., Breit T.M., Leunissen J.A.M. OligoRAP — an Oligo Re-Annotation Pipeline to improve annotation and estimate target specificity. *BMC Proc.*, 2009, 3[Suppl. 4): S4 [doi: 10.1186/1753-6561-3-S4-S4).
31. Kijas J.W., McEwan J., Clarke S., Henry H., Maddox J., McCulloch R., Driver F., Ilic K., Heaton M. *Development of a SNP panel for parentage assignment in sheep*. International Sheep Genomics Consortium, 2012. Available: http://sheephapmap.org/news/PAG_2012_ParentagePoster.pdf. No date.
32. Heaton M.P., Leymaster K.A., Kalbfleisch T.S., Kijas J.W., Clarke S.M., McEwan J., Maddox J.F., Basnayake V., Petrik D.T., Simpson B., Smith T.P., Chitko-McKown C.G., International Sheep Genomics Consortium. SNPs for parentage testing and traceability in globally diverse breeds of sheep. *PLoS ONE*, 2014, 9[4): e94851 [doi: 10.1371/journal.pone.0094851).
33. Heaton M.P., Harhay G.P., Bennett G.L., Stone R.T., Grosse WM, Casas E., Keele J.W., Smith T.P.L., Chitko-McKown C.G., Laegreid W.W. Selection and use of SNP markers for animal identification and paternity analysis in U.S. beef cattle. *Mammalian Genome*, 2002, 13[5): 272-281 [doi: 10.1007/s00335-001-2146-3).
34. Holl H.M., Vanhnasy J., Everts R.E., Hoefs-Martin K., Cook D., Brooks S.A., Carpenter M.L., Bustamante C.D., Lafayette C. Single nucleotide polymorphisms for DNA typing in the domestic horse. *Animal Genetics*, 2017, 48[6): 669-676 [doi: 10.1111/age.12608).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1177-1187
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.294:636.086.2/.3:579

doi: 10.15389/agrobiology.2019.6.1177eng
doi: 10.15389/agrobiology.2019.6.1177rus

HABITAT AS A DETERMINING FACTOR FOR THE REINDEER RUMEN MICROBIOME FORMATION IN RUSSIAN ARCTIC

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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

Reindeer (*Rangifer tarandus*) is geographically isolated from other subspecies of the ruminant family *Cervidae*. It is known that belonging to certain environmental conditions can have a significant impact on the composition of the ruminant rumen microbiome. With the use of molecular-biological analysis, we studied for the first time the patterns of formation of the reindeer's rumen microbial communities for the *Rangifer tarandus* living in different natural and climatic zones of the Russian Federation. The purpose of the study is to assess the regional features of the reindeer's rumen microbiome into the different Arctic regions of Russia using T-RFLP analysis and quantitative PCR. It was made a comparative analysis of the influence of a number of factors on the composition of the reindeer rumen microbiome, incl. gender and age peculiarities, regional habitat conditions and feeding ration features. Samples of the rumen content were collected in the summer-autumn period of 2017 from 58 individuals ($n \geq 3$ from each age group) in the Yamalo-Nenets Autonomous District (AO), Nenets Autonomous District and the Murmansk region. The total number of bacteria, archaea, and fungi of the *Neocallimastigales* was analyzed by quantitative PCR, and the composition of the bacterial community by T-RFLP (terminal restriction fragment polymorphism) method. The main determinant of all components of the microbial community of the reindeer's rumen is regional habitat conditions, which, apparently, is due to differences in the composition of the pasture diet and the epizootic situation in the herd. The smallest similarity with other regions was found for samples from the Murmansk region, which is probably due to differences in reindeer pasture ration in this region, i.e. the differences in the composition of vegetation and lower nutritional values. Gender and age differences of animals were less significant though made a certain contribution to the ratio of microorganisms in the rumen. The clearest differences in the rumen microbiota were detected between groups of animals under 2 years of age and older than 2 years. In general, significant changes in the representation of a taxa number were noted in connection with the nutritional value of pasture ration. A statistically significant relationship was established between the level of fiber in grazing feed and members of the families *Veillonellaceae* ($r = -0.75$), *Nostocaceae* ($r = 0.52$), *Rivulariaceae* ($r = -0.88$), etc. in addition to traditionally associated with the processes of cellulose degradation bacteria. There is no significant correlation between the content of conditionally pathogenic microorganisms from the *Fusobacteria*, *Tenericutes* (*Mycoplasmataceae*), *Proteobacteria* (*Enterobacteri-*

aceae, *Campylobacteraceae*) and the nutritional value of feeds and other groups of microorganisms, which indicates the need for more research in this direction. The obtained data clarify aspects of the interaction and cohabitation of symbionts in the complex-component system of the reindeer rumen, which is characterized by the diversity of sources of plant polysaccharides and the variety of enzymes produced by microorganisms.

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

Reindeer (*Rangifer tarandus*) occupies a special place among herbivorous ruminants. The geographical isolation of this species from other subspecies of the *Cervidae* family [1] implies not only anatomical and morphological features in the structure of the digestive system but also the formation of specific rumen microbial communities [2-4]. Interest in the study of complex symbiotic communities of the reindeer rumen is also associated with the study of the adaptative features of the organism of these animals to adverse factors, in particular, the ability to effectively use the scarce plant resources of the tundra, forest-tundra and northern taiga areas [5]. The formation of microbial communities in the rumen of ruminant animals is influenced by a complex of interdependent factors: a variety of sources of plant fiber, interactions between microorganisms of various groups, taxonomic diversity of enzyme systems, the molecular structure of enzymes, physiology of microorganisms, as well as environmental aspects [6-8]. In this regard, the adaptive facilities of reindeer should be considered taking into account the conditions of their habitat, the availability of nutritional resources, and other factors [9].

Relation to certain environmental conditions affects the composition of the rumen microbiota in ruminants [10]. However, such studies focus mainly on the analysis of the representation of individual groups of microorganisms, for example, cellulolytic ones. Thus, a significant difference in the rumen microbial communities is shown for two geographically separated subspecies of the reindeer of Norway — *Rangifer tarandus* (Eurasian tundra reindeer, *R. tarandus tarandus*) of the continent part of the country and *R. tarandus platyrhynchus* (Svalbard reindeer, *R. tarandus platyrhynchus*) of the high Arctic Spitsbergen archipelago, located between Norway and the North Pole. At the same time, the representatives of *R. tarandus platyrhynchus*, which during the 8-10-month winter period are forced to eat food with a high lignin content, have a 6-14 times higher content of cellulolytic bacteria than *R. tarandus tarandus* [1]. However, the bacteria associated with plant feed fermentation processes were similar in species composition: *Peptostreptococcus anaerobius*, *Lachnospira multiparus*, *Butyrivibrio fibrisolvens*, *Eubacterium ruminantium*, *Selenomonas ruminantium*, *Fibrobacter succinogenes*, *Eubacterium pyruvovorans* and *Fusocillus* sp. Moreover, according to Aagnes et al. [11], the subspecies of *R. tarandus tarandus* has serious limitations in the digestion of fiber.

Molecular genetic technologies, such as T-RFLP analysis (terminal restriction fragment length polymorphism) and NGS sequencing (next-generation sequencing), provide new possibilities in studying the structure of microbial communities, which allows giving a deep characterization of their biodiversity, revealing not only dominant taxa but also other components, including uncultured microorganisms [12-15]. However, there are few reports to date of the reindeer rumen microbiome [16, 17].

In the work, using molecular analysis, it was for the first time shown that one of the key factors affecting the formation of the reindeer (*Rangifer tarandus*) rumen microbiome was the habitat, which is probably related to the characteristics of the feed base of animals.

The purpose of the work was to assess the regional and age-gender features of the rumen microbiome of reindeer living in various climatic zones of the Russian Federation using T-RFLP analysis and quantitative PCR.

Techniques. The object of the study was 58 reindeer animals (*Rangifer tarandus*) of the Nenets breed, calves (4-8-month old) and adult animals (males and females). The content of the reindeer rumen was samples in the summer-autumn period of 2017 ($n \geq 3$ from each age group) in the Yamalo-Nenets Autonomous District (Kharp village, forest-tundra natural and climatic zone), the Nenets Autonomous District (Nelmin-Nos village, tundra natural and climatic zone) and the Murmansk Province (station Loparskaya, tundra natural and climatic zone).

The total number of bacteria, archaea, and fungi of the *Neocallimastigales* class in the rumen content was analyzed by quantitative (real-time) polymerase chain reaction (qRT-PCR) using a kit for real-time PCR (RT-PCR) with intercalating colorant EVA Green (ZAO Syntol, Russia) and primers F — 5′-ACTCCTACGGGAGGCA-GCAG-3′, R — 5′-ATTACCGCGGCTGCTGG-3′ (bacteria), F — 5′-AG-GAATTGGCGGGGGAGCAC-3′, R − 5′-GCCATGC-ACCWCCTCT-3′ (archaea), F — 5′-GCACTTCATTGTGTGTACTG-3′, R — 5′-GGATGAAACTCGTTGACTTC-3′ (fungi) on the DT Lite-4 detection amplifier (NPO DNA-Technology, Russia) in the following mode: the first cycle − 3 min at 95 °C (1 repetition); second cycle − 13 sec at 95 °C, 13 sec at 57 °C, 30 sec at 72 °C (40 repetitions).

The rumen bacterial community was studied using the T-RFLP method [18]. DNA was extracted from the samples with the Genomic DNA Purification Kit (Fermentas, Lithuania) in accordance with the manufacturer's instructions. PCR (a Verity amplifier, Life Technologies, USA) was conducted with 63F bacterial primers (5′-CAGGCCTAACACATGCAAGTC-3′) with a fluorophore label at the 5′-end (WellRed D4 fluorophore, Beckman Coulter, USA) and 1492R (5′-TACGGHTACCTTGTTACGACTT-3′), amplifying the 16S pRNA gene fragment from the 63rd to the 1492th position, the first cycle — 3 min at 95 °C (1 repetition); the second cycle — 30 s at 95 °C, 40 s at 55 °C, 60 s at 72 °C (35 repetitions); the third cycle — 5 min at 72 °C. The final concentration of the total obtained DNA was determined by a Qubit 2 fluorimeter (Invitrogen, USA) using the Qubit dsDNA BR Assay Kits (Invitrogen, USA) according to the manufacturer's recommendations.

Amplicons of the 16S rRNA gene fragment, marked by a fluorescent label, were purified in accordance with the standard procedure [19]. Then, 30-50 ng of DNA were treated with restriction enzymes MspI, HaeIII, HhaI (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 using a CEQ 8000 sequencer (Beckman Coulter, USA); the error of CEQ 8000 is not more than 5 %. The height of the peaks and their area were measured with the Fragment Analysis software (Beckman Coulter, USA), as a result of which subtypes (phylotypes) were identified and their relative amount in the microbial community was calculated. The taxonomic affiliation of bacteria was determined using the database (http://mica.ibest.uidaho.edu/trflp.php).

The samples of pasture vegetation, which formed the basis of the diet of reindeer, were selected in each region and their botanical description was conducted. In addition, the ratio of various plant species in the diet and their nutritional value were determined.

The results were statistically processed by the method of variance analysis using the Microsoft Excel 2010 software. The mean values (*M*) and standard errors of the mean (±SEM) were determined, and the significance of the differences was evaluated by Student's *t*-test. The calculation of the Pearson correlation coefficients and the assessment of the similarity of bacterial communities by the Principal Component Analysis (PCA) method based on the Bray-Curtis coefficient, which accounts the number and relative abundance of certain taxa, were conducted using the Past program (http://folk.uio.no/ohammer/past/).

Results. Figure 1 shows the regions where the sampling was conducted. The average composition and nutritional value of the summer-autumn pasture diet of reindeer are presented in Table 1.

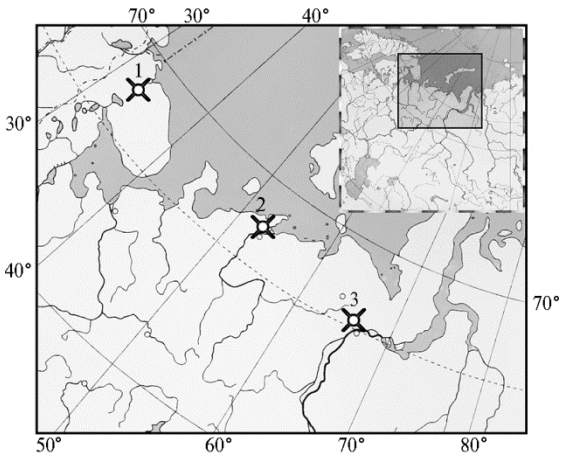

Fig. 1. Regions for selecting the rumen contents of reindeer (*Rangifer tarandus*) of the Nenets breed: 1 — Yamalo-Nenets Autonomous District, 2 — Murmansk Province, 3 — Nenets Autonomous District (2017).

The physiology of reindeer, including the state of rumen microflora, experiences a much lower anthropogenic load than domesticated ruminants have, in particular, bovine cattle. At the same time, the role of the microbial community of the rumen in ruminants in providing nutrition is especially significant, since their diet consists of extremely poor nutritionally valuable plant feeds [20]. It is known that the digestive system of reindeer has unique adaptations for microbiological fermentation of scarce plant resources in natural habitats, which can exert significant selection pressure on the structural and functional organization of the rumen microbiome.

1. Averaged composition and nutritional value of the summer pasture diet of Nenets reindeer (*Rangifer tarandus*) in three zones of Russian Arctic in the summer-autumn period (*M*±SEM, 2017)

Indicator	Yamalo-Nenets Autonomous District	Murmansk Province	Nenets Autonomous District
Nutritional value			
Soluble carbohydrates (sugars), g/kg	20.80±0.80	13.85±0.52	66.86±3.50
Mass fraction of dry matter, %	76.74±1.60	73.28±1.73	82.04±1.46
Crude fat, g/kg	16.90±0.61	12.39±0.84	15.46±0.54
Crude protein, g/kg	95.90±4.70	54.96±3.10	64.03±3.50
Crude ash, g/kg	36.40±1.10	24.99±2.20	23.95±1.80
Crude fiber, g/kg	142.80±7.30	134.62±6.50	160.55±8.60
Dietary components Компоненты рациона, %			
Cladonia	5	10	10
Nephroma	5	–	–
Betula pendula	5	20	20
Salix borealis	5	20	15
Salix polaris	15	–	–
Vaccinium uliginosum	10	–	5
Betula nana	25	20	20
Perennial grasses	30	30	30

Note. Dashes indicate the absence of a component in the diet.

The rumen of ruminants is inhabited by various groups of symbiotic microorganisms [4-7]. In our studies, in animals from the Yamalo-Nenets Autonomous District, a significantly larger ($p < 0.05$) number of bacteria and fungi

(Chytridiomycetes) was noted compared to animals from other regions (Table 2). In the rumen of reindeer from the Murmansk Region and the Nenets Autonomous District, a significantly larger ($p < 0.05$) counts of archaea was noted compared to animals from the Yamalo-Nenets Autonomous District.

The age and gender differences in animals in terms of the abundance of the main groups of microorganisms appeared to be less significant in comparison to regional features. However, the detected microbiota changes were not the same for animal units from different regions. Thus, in reindeer older than 2 years from the Murmansk Region and the Yamalo-Nenets Autonomous District, a significant increase in the number of bacteria ($p < 0.05$) was observed compared with animal units from the Nenets Autonomous District. Significant changes in the number of Chytridiomycetes in animals from the Murmansk Region and the Nenets Autonomous District were not observed with age, while they were noted in animals from the Yamalo-Nenets Autonomous District.

It can be assumed that the regularities that we found in the change in the number of microorganisms in the reindeer rumen community are interrelated with the structure of the summer pasture diet and its nutritional value, which is consistent with reports of foreign researchers. Thus, Olsen et al. [21] showed a decrease in the number of viable zoospores of *Chytridiomycetes* in winter, which have a wide range of multifunctional polysaccharide enzymes [22-23] in the rumen content of Norwegian reindeer living in natural pastures. Similar results were obtained for methanogenic archaea, the abundance of which decreased in the rumen of *R. tarandus* during the spring season compared to the autumn period [24]. At the same time, the reindeer *R. tarandus platyrhynchus* of the Spitsbergen archipelago showed the absence of significant changes in the rumen of the number of methanogens, bacteria and protozoa due to a change in the composition of the vegetation of natural pastures in the autumn and spring periods [25].

2. Abundance of microorganisms in the rumen community of Nenets reindeer (*Rangifer tarandus*) younger (I) **and older** (II) **than 2 years of age from Russian Arctic regions** (M±SEM, 2017)

Region, age	Bacteria	Archaea	Fungi *Neocallimastigales*
Yamalo-Nenets Autonomous District:			
I	$1.56\times10^{9}\pm5.38\times10^{7}$ [d]	$1.20\times10^{8}\pm3.94\times10^{6}$ [d]	$2.81\times10^{6}\pm8.24\times10^{4}$ [b]
II	$2.58\times10^{9}\pm1.84\times10^{6}$ [c]	$1.42\times10^{8}\pm6.72\times10^{6}$ [d]	$4.53\times10^{6}\pm1.95\times10^{5}$ [a]
Murmansk Province:			
I	$8.10\times10^{8}\pm4.01\times10^{3}$ [e]	$1.03\times10^{9}\pm5.25\times10^{4}$ [b]	$2.07\times10^{5}\pm8.09\times10^{2}$ [c]
II	$1.75\times10^{9}\pm2.87\times10^{8}$ [c. d]	$7.79\times10^{8}\pm2.87\times10^{6}$ [c]	$2.07\times10^{5}\pm8.09\times10^{2}$ [c]
Nenets Autonomous District:			
I	$6.37\times10^{9}\pm4.72\times10^{8}$ [a]	$8.74\times10^{7}\pm6.02\times10^{6}$ [e]	$3.24\times10^{5}\pm9.50\times10^{3}$ [c]
II	$4.41\times10^{9}\pm2.12\times10^{7}$ [b]	$2.15\times10^{9}\pm1.56\times10^{8}$ [a]	$3.39\times10^{5}\pm5.10\times10^{4}$ [c]

[a-e] Differences in indicators without a common upper index are statistically significant at $p < 0.05$.

The analysis of the similarity of the bacterial rumen communities in the examined animals based on the principal component method (Fig. 2) showed that all samples were divided into three main groups corresponding to individual regions, the Murmansk Region, the Yamalo-Nenets and the Nenets Autonomous Districts. The revealed patterns were presumably caused by differences in the composition of the pasture diet and the epizootic situation in the herd, since the meteorological parameters in the regions were similar for the summer-autumn period in which the study was conducted. Interestingly, the age and gender characteristics of animals, determined by the method of principal components, showed a less significant division of samples into subgroups. In general, the most significant differences were detected in the composition of the bacterial rumen community between groups of young animals (up to 2 years old) and adult animal units (over 2 years old) (see Fig. 2). It is worth noting that the au-

thors did not find any significant changes in the composition of the microbial rumen community in females and males.

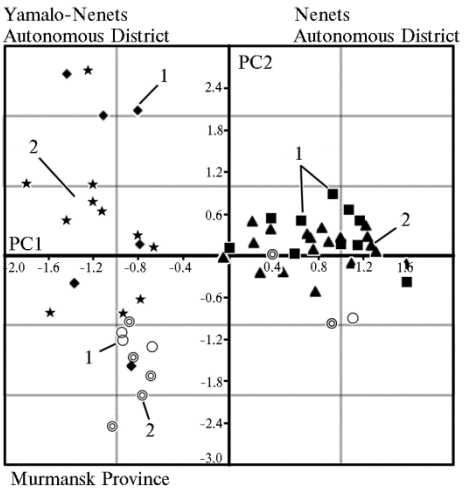

Fig. 2. T-RFLP-based Principal Component Analysis of bacterial rumen community pattern of Nenets reindeer (*Rangifer tarandus*) from Russian Arctic regions: 1 – young animals (up to 2 years), 2 – adult animals (over 2 years old) (2017 год).

Regardless of the region of sampling in the studied animal units of *R. tarandus*, the representatives of the phylum *Firmicutes* were dominant in the rumen content; bacteria of the phyla *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* were less detected, and the proportion of other identified taxa (*Tenericutes*, *Fusobacteria*, *Cyanobacteria*) in the community was minor; a significant number of bacterial sequences could not be identified up to the phylum level.

The greatest interest among researchers in the study of microbial rumen communities of ruminants is associated with microorganisms, the producers of enzymes that are not synthesized by the host organism, primarily cellulolytic ones. Due to the use of various enzymes (cellulases, hemicelluloses, xylanases, glycoside hydrolases, etc.), these microorganisms participate in the degradation of plant biomass with the formation of metabolites, including volatile fatty acids (VFAs): propionate, acetate, butyrate, isobutyrate, valerate, isovalerate and 2-methylbutyric, hexanoic and heptanoic acids [6, 7].

Representatives of the bacterial phyla *Firmicutes* and *Bacteroidetes* comprise the predominant part of the microbial rumen community of the ruminants [26, 27], which suggests an important ecological role of these microorganisms, probably due to their wide metabolic potential, including fermentation of plant polysaccharides. A high proportion of the *Firmicutes*/*Bacteroidetes* phyla, according to a number of authors, is characteristic of various ruminants [28, 29]. Nevertheless, only 33% of *Firmicutes* phylum bacteria were present in the rumen of the North American elk *Alces alces* [29].

It was reported that in reindeer, the bacteria representation of these taxa varies depending on the habitat. In particular, in the subspecies *R. tarandus tarandus* from the continent part of Norway, the representation of *Firmicutes* phylum bacteria (71%) was higher than in the animal units of *R. tarandus platyrhynchus* (55%) living on the Spitsbergen archipelago [1]. The analysis of the rumen microbiota in the studied animal units of *R. tarandus* showed that the smallest percentage of representatives of the phylum *Bacteroidetes* was present in animals from the Nenets Autonomous District, and the largest – in reindeer from the Yamalo-Nenets Autonomous District (Fig. 3). The total share of *Firmicutes* phylum bacteria did not significantly differ in adult animal units from different regions, while in young animals from the Murmansk Region, the number of these microorganisms was significantly lower ($p < 0.05$) than in animal units from the Nenets and the Yamalo-Nenets Autonomous Districts.

The data on the abundance of bacteria involved in fermentation of plant fiber in the rumen of reindeer are consistent with the estimates of nutritional

value of plants from pastures. The composition and nutritional value of multi-component samples repeating the composition of the average summer pasture ration from three regions of the Russian Arctic zone varied. In general, the feed of reindeer was characterized by a high content of crude fiber and a low content of other nutrients. According to the composition of the rumen microbiome, reindeer from the Murmansk Region had the least resemblance to animal units from other regions, which is probably due to the peculiarities of the pasture diet of animals in this region – the composition of vegetation and lower nutritional values (see Table 1).

It was reported that the proportion of the *Firmicutes* and *Bacteroidetes* phyla in the rumen of ruminants depends on the diet type. Thus, the representatives of the phylum *Bacteroidetes* are mainly associated with the presence of easily fermentable carbohydrates (such as starch) and proteins in the diet of animals, while a number of representatives of the phylum *Firmicutes* (bacteria of the genera *Ruminococcus*, *Butyrivibrio*, *Clostridium*, etc.) are associated with the fermentation of plant cellulose [7, 10, 30]. The difference in the enzymatic activity of these microorganisms can also affect the metabolism of the host through the production of VFAs as a result of fermentation of plant polysaccharides.

Interestingly, compared to young animals, adult animals had a wider taxonomic diversity of bacteria associated with fiber enzymes, including the families *Eubacteriaceae*, *Clostridiaceae*, *Lachnospiraceae* of the phylum *Firmicutes* (p 0.05), which indicates an increase in the ability of the microbial community to ferment plant polysaccharides.

We found a statistically significant correlation between the composition of *R. tarandus* rumen microorganisms, involved in the fermentation of plant polysaccharides and nutritional indicators of diets. In particular, the amount of fiber was significantly negatively associated with the presence in the rumen of animals of the families *Bacteroidaceae* ($r = -0.75$, $p < 0.05$), *Rivulariaceae* ($r = -0.88$, $p < 0.05$), *Veillonellaceae* ($r = -0.75$, $p < 0.05$) and significantly positively with the presence of the families *Lachnospiraceae* ($r = 0.89$, $p < 0.05$), *Clostridiaceae* ($r = 0.51$, $p < 0.05$), *Nostocaceae* ($r = 0.52$, $p < 0.05$), *Eubacteriaceae* ($r = 0.46$, $p < 0.05$), and *Prevotellaceae* ($r = 0.56$, $p < 0.05$).

Prevalence of some cellulolytic microorganisms (for example, the families *Clostridiaceae*, *Lachnospiraceae*, *Eubacteriaceae*) was positively interrelated ($p < 0.05$) with the proportion of bacteria from the families *Nostocaceae*, *Enterococcaceae*, *Lactobacillaceae* and negatively ($p < 0.05$) with the representatives of the families *Prevotellaceae*, *Bacteroidaceae*, *Veillonellaceae*, and *Streptococcaceae*. Interestingly, the reindeer, studied by the authors, showed an inverse pattern between the content in the rumen of bacteria utilizing acids of the *Veillonellaceae* family and cellulolytic microorganisms such as *Lachnospiraceae* ($r = -0.60$, $p < 0.05$), *Clostridiaceae* ($r = -0.55$, $p < 0.05$), and *Eubacteriaceae* ($r = -0.60$, $p < 0.05$). Bacteria utilizing acids belong to the physiologically important group of microorganisms for ruminants, since they allow maintaining the necessary acidity in the rumen due to their ability to metabolize acids (including acetic, propionic, butyric, and lactic acids), formed as a result of fermentation of monosaccharides, oligo- and polysaccharides [31].

The patterns we revealed confirm the opinion that during the evolution in the rumen of animals, certain relations were formed between microorganisms, which make it possible to clarify the relationship between the presence of a number of microorganisms in the rumen of reindeer.

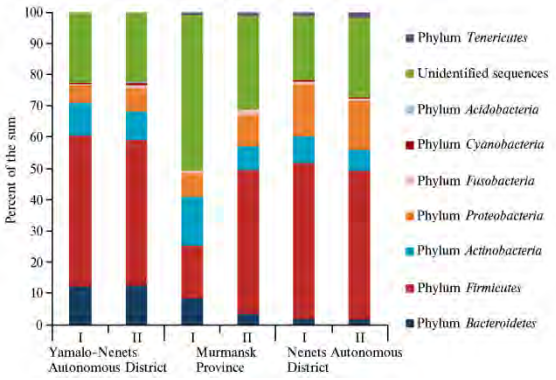

Fig. 3. Bacterial phyla pattern in the rumen of Nenets reindeer (*Rangifer tarandus*) from Russian Arctic regions: 1 — young animals (up to 2 years), 2 — adult animals (over 2 years old) (2017 год).

A significant relationship between the content of potentially pathogenic microorganisms from the phyla *Fusobacteri*a, *Tenericutes* (family *Mycoplasmataceae*), *Proteobacteria* (*Enterobacteriaceae*, *Campylobacteraceae* families) and other microorganisms was not found. However, more research isrequired to study deeply this problem. The authors also did not find a direct pattern characterizing age-related changes in the content of potentially pathogenic and pathogenic bacteria in the rumen of *R. tarandus*. Probably, the differences revealed in this case were associated with other factors, i.e. the particularities of the pasture diet and the epizootic situation in the herd.

Thus, our studies showed that in reindeer (*Rangifer tarandus*) of the Nenets breed older than 2 years from the Murmansk Region and the Yamalo-Nenets Autonomous District (but not the Nenets Autonomous District), the number of bacteria increases significantly ($p < 0.05$) compared to calves. Regardless of the region, the *Firmicutes* phylum dominates in the rumen contents; bacteria of the phyla *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* are less prevalent, and other detected taxa (*Tenericutes*, *Fusobacteria*, *Cyanobacteria*) are minor. Significant changes are identified for a number of taxa of microorganisms in connection with the nutritional value of the pasture diet, depending on the region. A statistically significant relationship is found between the fiber content in pasture feeds and the abundance of the members of families *Veillonellaceae* ($r = -0.75$), *Nostocaceae* ($r = 0.52$), *Rivulariaceae* ($r = -0.88$). For conditionally pathogenic microorganisms from the phyla *Fusobacteria*, *Tenericutes* (family *Mycoplasmataceae*) and *Proteobacteria* (families *Enterobacteriaceae*, *Campylobacteraceae*), such regularities are not observed. In general, the obtained results indicate that habitat conditions are the main factor influencing the microbial community of the reindeer rumen from various regions of the Russian Arctic. Most likely, this is due to the characteristics of feed rations of animals. Other factors (gender, age) have a lesser effect, although they make a certain contribution to the ratio of microorganisms in the rumen. The results of metagenomic analyses (due to their high resolution), allow discovering new patterns and clarifying aspects of the interaction and cohabitation of symbionts in the complex ecosystem of the reindeer rumen of *R. tarandus*.

REFERENCES

1. Sundset M.A., Præsteng K.E., Cann I.K.O., Mathiesen S.D., Mackie R.I. Novel rumen bacterial diversity in two geographically separated sub-species of reindeer. *Microb. Ecol.*, 2007, 54(3): 424-438 (doi: 10.1007/s00248-007-9254-x).
2. Malmuthuge N., Guan L.L. Gut microbiome and omics: a new definition to ruminant production and health. *Animal Frontiers*, 2016, 6(2): 8-12 (doi: 10.2527/af.2016-0017).
3. Matthews C., Crispie F., Lewis E., Reid M., O'Toole P.W., Cotter P.D. The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes*, 2019, 10(2): 115-132 (doi: 10.1080/19490976.2018.1505176).
4. Hackmann T.J., Spain J.N. Invited review: ruminant ecology and evolution: perspectives useful

to ruminant livestock research and production. *Journal of Dairy Science*, 2010, 93(4): 1320-1334 (doi: 10.3168/jds.2009-2071).
5. Morgavi D.P., Kelly W.J., Janssen P.H., Attwood G.T. Rumen microbial (meta)genomics and its application to ruminant production. *Animal*, 2013, 7(1): 184-201 (doi: 10.1017/S1751731112000419).
6. *The ruminant animal: Digestive physiology and nutrition*. D.C. Church (ed.). Prentice Hall, New Jersey, 1993.
7. Hungate R.E. *The rumen and its microbes*. Academic Press, NY, 1966.
8. Grilli D.J., Fliegerová K., Kopečný J., Lama S.P., Egea V., Sohaefer N., Pereyra C., Ruiz M.S., Sosa M.A., Arenas G.N., Mrázek J. Analysis of the rumen bacterial diversity of goats during shift from forage to concentrate diet. *Anaerobe*, 2016, 42: 17-26 (doi: 10.1016/j.anaerobe.2016.07.002).
9. Mathiesen S.D., Mackie R.I., Aschfalk A., Ringø E., Sundset M.A. Microbial ecology of the gastrointestinal tract in reindeer — changes through season. In: *Microbial ecology of the growing animal; Biology of the growing animals. V. 3*. W. Holzapfel, P. Naughton (eds.). Elsevier Press, Oxford: 73-100.
10. Henderson G., Cox F., Ganesh S., Jonker A., Young W., Global Rumen Census Collaborators, Janssen P.H. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.*, 2015, 5: 14567 (doi: 10.1038/srep14567).
11. Aagnes T.H., Sørmo W., Mathiesen S.D. Ruminal microbial digestion in free-living, in captive lichen-fed, and in starved reindeer (*Rangifer tarandus tarandus*) in winter. *Appl. Environ. Microbiol.*, 1995, 61(2): 583-591.
12. de la Fuente G., Belanche A., Girwood S.E., Pinloche E., Wilkinson T., Newbold C.J. Pros and cons of Ion-Torrent next generation sequencing versus Terminal Restriction Fragment Length Polymorphism T-RFLP for studying the rumen bacterial community. *PLoS ONE*, 2014, 9(7): e101435 (doi: 10.1371/journal.pone.0101435).
13. Wang L., Xu Q., Kong F., Yang Y., Wu D., Mishra S., Li Y. Exploring the goat rumen microbiome from seven days to two years. *PLoS ONE*, 2016, 11(5): e0154354 (doi: 10.1371/journal.pone.0154354).
14. Peng S., Yin J., Liu X., Jia B., Chang Z., Lu H., Jiang N., Chen Q. First insights into the microbial diversity in the omasum and reticulum of bovine using Illumina sequencing. *J. Appl. Genetics*, 2015, 56(3): 393-401 (doi: 10.1007/s13353-014-0258-1).
15. Elekwachi C.O., Wang Z., Wu X., Rabee A., Forster R.J. Total rRNA-Seq analysis gives insight into bacterial, fungal, protozoal and archaeal communities in the rumen using an optimized RNA isolation method. *Front. Microbiol.*, 2017, 8: 1814 (doi: 10.3389/fmicb.2017.01814).
16. Gruninger R.J., Sensen C.W., McAllister T.A., Forster R.J. Diversity of rumen bacteria in Canadian cervids. *PLoS ONE*, 2014, 9(2): e89682 (doi: 10.1371/journal.pone.0089682).
17. Zielińska S., Kidawa D., Stempniewicz L., Łoś M., Łoś J.M. New insights into the microbiota of the Svalbard Reindeer *Rangifer tarandus platyrhynchus*. *Front. Microbiol.*, 2016, 7: 170 (doi: 10.3389/fmicb.2016.00170).
18. Laptev G.Yu., Novikova N.I., Il'ina L.A., Iyldyrym E.A., Nagornova K.V., Dumova V.A., Soldatova V.V., Bol'shakov V.N., Gorfunkel' E.P., Dubrovina E.G., Sokolova O.N., Nikonov I.N., Lebedev A.A. *Normy soderzhaniya mikroflory v rubtse krupnogo rogatogo skota* [The standards of microflora in cattle rumen]. St. Petersburg, 2016 (in Russ.).
19. Maniatis T., Fritsch E.F., Sambrook J., *Molekulyarnoe klonirovanie* [Molecular cloning]. Moscow, 1984 (in Russ.).
20. Zabrodin V.A., Laishev K.A., Dubovik I.K. *Izvestiya Sankt-Peterburgskogo gosudarstvennogo agrarnogo universiteta*, 2015, 40: 108-112 (in Russ.).
21. Olsen M.A., Aagnes T.H., Mathiesen S.D. The effect of timothy silage on the bacterial population in rumen fluid of reindeer (*Rangifer tarandus tarandus*) from natural summer and winter pasture. *FEMS Microbiol Ecol.*, 1997, 24(2): 127-136 (doi: 10.1111/j.1574-6941.1997.tb00429.x).
22. Orpin C.G., Mathiesen S.D., Greenwood Y., Blix A.S. Seasonal changes in the ruminal microflora of the high-arctic Svalbard reindeer (*Rangifer tarandus platyrhynchus*). *Appl. Environ. Microbiol.*, 1985, 50(1): 144-151.
23. Wang T.Y., Chen H.L., Lu M.J., Chen Y.C., Sung H.M., Mao C.T., Cho H.Y., Ke H.M., Hwa T.Y., Ruan S.K., Hung K.Y., Chen C.K., Li J.Y., Wu Y.C., Chen Y.H., Chou S.P., Tsai Y.W., Chu T.C., Shih C.A., Li W.H., Shih M.C. Functional characterization of cellulases identified from the cow rumen fungus *Neocallimastix patriciarum* W5 by transcriptomic and secretomic analyses. *Biotechnol. Biofuels*, 2011, 4: 24 (doi: 10.1186/1754-6834-4-24).
24. Fonty G., Joblin K.N. Rumen anaerobic fungi: their role and interactions with other rumen microorganisms in relation to fiber digestion. In: *Physiological aspects of digestion and metabolism in ruminants*. T. Tsuda, Y. Sasaki, R. Kawashima (eds.). Academic Press, Toronto, ON, 1990: 665-680 (doi: 10.1016/B978-0-12-702290-1.50033-3).
25. Sundset M.A., Edwards J.E., Cheng Y.F., Senosiain R.S., Fraile M.N., Northwood K.S., Præsteng K.E., Glad T., Mathiesen S.D., Wright A.D.G. Molecular diversity of the rumen microbiome of Norwegian reindeer on natural summer pasture. *Microb. Ecol.*, 2009, 57(2): 335-348 (doi: 10.1007/s00248-008-9414-7).

26. Schofield B.J., Lachner N., Le O.T., McNeill D.M., Dart P., Ouwerkerk D., Hugenholtz P., Klieve A.V. Beneficial changes in rumen bacterial community profile in sheep and dairy calves as a result of feeding the probiotic *Bacillus amyloliquefaciens* H57. *J. Appl. Microbiol.*, 2018, 124(3): 855-866 (doi: 10.1111/jam.13688).
27. Weimer P.J. Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. *Front. Microbiol.*, 2015, 6: 296 (doi: 10.3389/fmicb.2015.00296).
28. Samsudin A.A., Evans P.N., Wright A.G., Jassim R.A. Molecular diversity of the foregut bacteria community in the dromedary camel (*Camelus dromedarius*). *Environmental Microbiology*, 2011, 13(11): 3024-3035 (doi: 10.1111/j.1462-2920.2011.02579.x).
29. Ishaq S.L, Wright A.D. Insight into the bacterial gut microbiome of the North American moose (*Alces alces*). *BMC Microbiol.*, 2012, 12: 212 (doi: 10.1186/1471-2180-12-212).
30. McCann J.C., Elolimy A.A., Loor J.J. Rumen microbiome, probiotics, and fermentation additives. *Veterinary Clinics of North America: Food Animal Practice*, 2017, 33(3): 539-553 (doi: 10.1016/j.cvfa.2017.06.009).
31. Nocek J.E. Bovine acidosis: implications on laminitis. *Journal of Dairy Science*, 1997, 80: 1005-1028 (doi: 10.3168/jds.S0022-0302(97)76026-0).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1188-1195
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.294:591.16:612.616.2

doi: 10.15389/agrobiology.2019.6.1188eng
doi: 10.15389/agrobiology.2019.6.1188rus

CHANGE OF PHYSIOLOGICAL AND MORPHOLOGICAL SPERM QUALITY TRAITS IN REINDEER (*Rangifer tarandus*) DURING CRYOPRESERVATION

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation, project No. 17-16-01023
Received August 22, 2019

Abstract

Assisted reproductive technologies allow effective preservation and use of endangered animal gene pool and creation of new breeding forms. In reindeer (*Rangifer tarandus*) herding, the technique of sperm cryopreservation is still under development. This is due to the difficulty of collecting reindeer sperm in the Arctic conditions. Besides, the rutting season of reindeer begins in the autumn and lasts about a month. Only during this period is it possible to collect sperm as spermatogenesis in reindeer stops after rutting season. The aim of the work was to study the effects of cooling and freezing-thawing on the physiological and morphological traits of reindeer sperm quality. Reindeer sperm was collected by electric ejaculator or by washing out of the epididymis. After assessing the quality of ejaculated and epididymal semen (volume, total and progressive motility and sperm concentration), the sperm was diluted with Steridyl medium to a final concentration of 100 million/ml, packed in 0.25 ml straws and cooled to 5 °C for 120 min. After cooling and balancing, the straws were kept in liquid nitrogen vapors on a float at –110 °C for 12 min and then lowered into liquid nitrogen. Semen was thawed at 37 °C. The initial assessment of ejaculated sperm showed that the average volume of reindeer ejaculate was 0.5±0.08 ml, with concentration of 0.520±0.069 billion/ml, total motility of 64.3±4.07 %, and progressive motility of 47.9±4.24 %. The epididymal sperm cell concentration was on average 0.260±0.078 billion/ml, total and progressive motility was 43.6±8.49 % and 20.8±5.25 %, respectively. There was a large variability between ejaculates on the extent of changes in sperm motility after cryopreservation. Thus, total motility decreased by 41.9±5.38 % on average with fluctuations from 1 % to 89 %, progressive — by 36.8±5.29 % with fluctuations from 0 % to 75 %. A total of 42 % of ejaculates lost more than 50 % total motility. In some cases, there was a complete loss of motility after freezing, and in some samples the changes were insignificant. Large variability in changing cell motility was observed both in ejaculated and in epididymal semen. Epididymal sperm cells had higher motility after freezing than ejaculated spermatozoa, but showed more pronounced disturbances in the motility character. Sperm morphology analysis showed that there is an increased percentage with wrinkled or missing acrosome as compared to other animal species, i.e. 6.9±0.76 % in both ejaculated and epididymal reindeer sperm cells. There was no significant increase in damages of the sperm tail, neck and acrosome. The number of cells with injuries in tail and neck increased by 4.2±1.05 % with a range from 0.01 % to 15.7 %, and acrosome — by 2.5±0.35 with a range from 0.6 % to 8.3 %. High variability in the increase of plasma membrane damages was observed, i.e. 10.9±5.02 % with fluctuations from 0.13 % to 45 %. Such a large variability is due to the peculiarities of the reindeer sperm cryoresistance and differ-

ences between individual ejaculates. Significant difference in physiological and morphological changes in semen quality after cryopreservation between ejaculated and epididymal sperm were not found. Thus, the greatest changes in the cryopreserved reindeer semen are in motility and membrane integrity. The obtained data on physiological and morphological changes in reindeer semen during freezing should be taken into account when optimizing the composition of diluents and cryopreservation protocol.

Keywords: *Rangifer tarandus,* reindeer, cryopreservation, sperm motility, acrosomes, cell membranes

In the Russian Federation, there are about 2 million wild and domestic reindeer (*Rangifer tarandus*) whose habitat occupies more than half of the country's territory. For the indigenous peoples of the Arctic, reindeer husbandry has become a major industry. Venison is a dietary product with a high content of important microelements and vitamins. Improving breeding methods aimed at improving meat productivity is an urgent problem for the breeding and selection of reindeer in Russia [1]. The existing methods do not provide the required growth rates for the production of reindeer-breeding products [1, 2]. In addition, the active development of the Arctic led to climate change, a reduction in the number of pastures and, as a consequence, a decrease in the number of domestic and wild reindeer [3-5].

Modern reproductive technologies make it possible to preserve and use the gene pool of endangered species indefinitely, to conduct intensive selection and choose the best animal units. However, these technologies have not yet found wide application in reindeer husbandry [6-8]. The high cost and complexity of transporting deer in the conditions of the North, as well as an unfavorable prognosis for male acclimatization, necessitate the development of methods for cryopreservation of reindeer semen and the creation of a sperm cryobank. The use of such sperm will allow the exchange of genetic material between remote regions of the Arctic [1, 5, 6].

There are references in the literature to only a few cases of artificial insemination in reindeer husbandry. Scottish researchers Dott and Utsi presented the data on artificial insemination of reindeer females with fresh sperm [9, 10]. Mkrtchyan and Deryazhentsev in the 1970s published the work on the assessment and cryopreservation of reindeer sperm [11-13]. In the 1990s and 2000s, there were reports of artificial insemination with cryopreserved sperm [14-17]. Mkrtchan et al. [18] were the first people in the world who managed to get reindeer calves after artificial insemination with frozen-thawed sperm. However, the technique of cryopreservation of reindeer sperm has not yet been developed [5, 19].

An important feature of the adaptation of the deer organism to the conditions of the Arctic is the cessation of spermatogenesis outside the rutting season. The harsh Arctic climate and the short breeding season complicate the procedure for obtaining the sperm of these animals [17, 20, 21].

To develop a method for deep freezing of reindeer sperm as part of the program for the preservation of the Arctic biological resources, a comprehensive study of the processes occurring in spermatozoa under the influence of low temperatures is required. However, there is no published data on the changes occurring with the sperm of these animals during cryopreservation.

In the present work, the quality of semen of reindeer before and after freezing was first characterized. It was shown that the largest changes during cryopreservation were undergone by the motor activity of germ cells, as well as membrane integrity. Reindeer have an increased proportion of sperm with a wrinkled or missing acrosome. After freezing, epididymal sperm cells retain

higher motility compared to the ejaculated ones, but more disturbances in the nature of their movement are noted.

The aim of the study was to assess changes in physiological and morphological indicators of reindeer sperm quality during cryopreservation.

Techniques. In the experiments, the sperm of reindeer (*Rangifer tarandus*) aged 1.5 to 7.5 years living on the Taimyr Peninsula was used. From 11 animal units, sperm was obtained by electro-ejaculation (DC100-240V ejaculator, Minitьb GmbH, Germany), at least three samples from each. Moreover, sperm was received from the epididymis from two males aged 2.5 and 3.5 years, who were castrated and left in the herd to work in harness, and post-mortal from six wild animals (age from 1.5 years) after the shoot-off. Sperm was obtained with automatic dispensers after cutting the epididymis and then transferred to 1.5-ml tubes with Steridyl diluent (Minitьb GmbH, Germany). Blood was avoided from getting into the sample.

After assessing the quality of ejaculated and epididymal sperm (volume, total and progressive motility, and sperm concentration), it was diluted in Steridyl medium to a final concentration of 100 million/ml, packed in 0.25-ml straws and cooled to 5 °C within 120 minutes. After cooling and equilibration, the straws were kept in liquid nitrogen vapor on a float at a temperature of –110 °C for 12 min and then lowered into liquid nitrogen. The samples were thawed at 37 °C.

The concentration, general and progressive sperm motility were determined using the CASA method (Computer-Assisted Semen Analysis) using the Argus-CASA program (OOO Argussoft, Russia), and the morphology and condition of acrosomes were evaluated by phase-contrast light microscopy. The damage level of the sperm plasma membrane was studied using a Sperm VitalStain stainer (Nidacon International AB, Sweden). Staining was conducted in Eppendorf tubes (50 μl of sperm was mixed with 50 μl of stain), the smears were prepared on glass slides. The preparations were scanned with ×1000 zoom with oil immersion, counting at least 200 cells in each sample, white cells are intact, red or pink are sperm cells with damaged membranes. For visualization, the Argus-CASA system and a BA410 microscope (Motic China Group Co., Ltd., China) were used.

The data were processed using the SigmaPlot 12.5 program (Systat Software Inc., USA) and Microsoft Excel. The mean values (*M*), standard errors of the mean (±SEM), minimum (min) and maximum (max) values of indicators are given in the article.

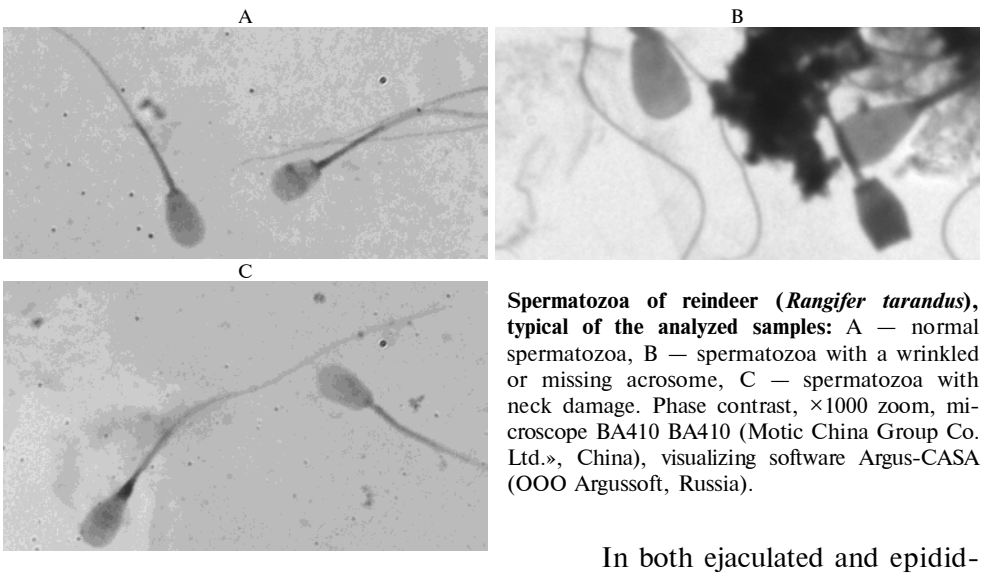
Results. Currently, there is a lot of data on the damage to spermatozoa of different animals during cryopreservation [22, 23]. For the most part, there are changes in the motility and nature of the movement of sperm, as well as damage to cellular structures – tails, acrosomes, and membranes. Cell damage occurs mainly due to the formation of ice crystals and osmotic stress [24]. For example, in bulls' sperm, even under optimal cooling and thawing conditions, 40-50% of cells cannot be frozen [25]. In horse sperm, the greatest damage after cryopreservation was observed in the acrosome area [26]. Boar sperm has the lowest level of cryoresistance [23].

According to the data, the average volume of reindeer ejaculate was 0.5±0.08 ml, the difference between ejaculates was from 0.02 to 2 ml, the concentration was 0.520±0.069 billion/ml (with a difference of 0.195 to 1.2 billion/ml), total motility 64.3±4.07 (10-84%), progressive motility 47.9±4.24% (3-79%). For epididymal sperm, the concentration, total and progressive sperm motility were 0.260±0.078 billion/ml (0.042-0.441 billion/ml), 43.6±8.49% (10-94%) and 20.8±5.25% (3-45%), respectively. These results are consistent with the data of

foreign [16, 17] and USSR researchers [13, 18], as well as with the data, previously obtained by the authors [20]. For cryopreservation, 25 samples of sperm were taken with total motility of at least 65%.

General and progressive sperm motility after thawing on average amounted to 23.2±5.25% (0-64%) and 16.3±3.46% (0-45%) in ejaculated sperm and 40.8±6.92% (21-63%) and 18.0±3.6% (8-32%) in epididymal sperm. For comparison, the total motility of thawed sperm of another *Cervidae* family member, the the Javan rusa (*Rusa timorensis*) was from 7.5 to 39.8%, the progressive one from 2.5 to 14.7% [27]. The authors froze the sperm diluted in a Tris-based extender, similar in composition to that we used (Steridyl). In our tests, the total motility of spermatozoa in thawed ejaculated reindeer sperm was lower than in the epididymal one, the progressive motility was almost the same, and the number of motile cells that had rectilinear-translational motion was 70 and 45%, respectively. Martínez et al. [28] note that although the cell motility of the epididymal and ejaculated deer sperm after cryopreservation does not differ much, the pattern of cell motion is better in ejaculated sperm.

An important criterion in sperm selection is determination of the number of normal (Fig., A) and pathological spermatozoa in the ejaculate [25]. Spermatozoa can be damaged by rapid cooling or low temperature [29]. Spermatozoa with abnormalities in the structure of the head, neck, and tail are considered pathological. There are several types of morphological changes in the acrosome. We noted such abnormalities as partial deformation of the outer membrane of the acrosome, ruptures and wrinkled outer membrane, as well as the absence of the acrosome.



Spermatozoa of reindeer (*Rangifer tarandus*), typical of the analyzed samples: A — normal spermatozoa, B — spermatozoa with a wrinkled or missing acrosome, C — spermatozoa with neck damage. Phase contrast, ×1000 zoom, microscope BA410 BA410 (Motic China Group Co. Ltd.», China), visualizing software Argus-CASA (OOO Argussoft, Russia).

In both ejaculated and epididymal sperm of reindeer, an increased proportion of spermatozoa with a wrinkled or missing acrosome was observed (see Fig., B) compared to other animal species, on average 6.9±0.76% with fluctuations from 1 to 15.2% (Table 2). Perhaps, this is a special feature of reindeer sperm. It should be noted that after freezing, there was no significant increase in the number of cells with acrosome damage, on average 10.1±0.78% with fluctuations from 3.6 to 18.3%. The number of cells with injuries in the tail area (see Fig. C) on average amounted to 11.9±1.31% (1.6-33.2%) before freezing and 15.5±1.15% (5.0-34.4%) after thawing.

Membrane integrity is a prerequisite for the functioning of cells. In fresh (ejaculated and epididymal) reindeer sperm, an average of 15.5±4.09% (1.78-

50.0%) of membrane damage was observed, with 21.8±4.44% (3.5-53.3%) for frozen-thawed sperm.

According to the change in sperm motility after cryopreservation, the ejaculate significantly differed. General motility decreased on average by 41.9±5.38% (the difference between ejaculates was from 1 to 89%), progressive motility by 36.8±5.29% (0 to 75%). Spermatozoa in 42% of ejaculates lost more than 50% of their total motility during cryopreservation, and in 25% of ejaculates, less than 20% of their motility. In some samples, a complete loss of motility after freezing was observed; in some cases, the changes were insignificant. Such great variability is due to both the characteristics of the cryoresistance of deer sperm and the differences between animal units. Significant individual variability in cell motility after thawing is also noted in red deer [30].

We did not observe a noticeable increase in the frequency of damage in the tail and neck of spermatozoa and acrosome. The number of cells with damage to the tail and neck increased on average by 4.2±1.05% (0.01-15.7%), with damage to the acrosome by 2.5±0.35% (0.6-8.3%). In other deer species, for example, in red deer, the number of damages in acrosomes by freezing and thawing increased on average by 20% [31]. In stallion sperm, the number of spermatozoa with acrosome hypoplasia and lack of internal contents after cryopreservation increased by 20.9%, and with acrosome degradation by 10.4% [32]. In the studies on the effect of cryopreservation and thawing on the sperm of red deer, the authors noted individual variability between males for the preservation of membrane integrity and acrosomes [33, 34].

We also did not reveal high variability between the samples in terms of their susceptibility to damage to the plasma membranes of spermatozoa. On average, the damage increased by 10.9±5.02% (0.13 to 45%). This confirms the theory that in reindeer sperm, membranes are most sensitive to low temperatures, which also affects cell motility. There was no significant difference in changes in morphological indicators of reindeer sperm quality after cryopreservation between ejaculated and epididymal sperm. It should be noted that sperm retains its fertilizing ability, despite a sharp decrease in sperm motility after thawing. Thus, as a result of in vitro fertilization of reindeer egg cells that we conducted using thawed epididymal sperm of a male which sperm motility reduced after cryopreservation by more than 50% (from 96 to 40%), embryos at the blastocyst stage were obtained (data are not presented).

Thus, the motor activity of the cells and the integrity of the membranes are subjected to the greatest changes during cryopreservation of reindeer sperm. The concentration of sperm cells in freshly obtained ejaculates is 0.520±0.069 billion/ml, total and progressive motility is 64.3±4.07 and 47.9±4.24%, in epididymal semen samples 0.260±0.078 billion/ml, 43.6±8.49 and 20.8±5.25%, respectively. After cryopreservation, the overall motility decreases on average by 41.9±5.38%, the progressive one by 36.8±5.29%. The frequency of damage to plasma membranes of spermatozoa on average increases by 10.9±5.02%. No significant differences in changes in physiological and morphological indicators of sperm quality during freezing and thawing between ejaculated and epididymal sperm were found. However, after cryopreservation, high individual variability is observed for all indicators of sperm quality. The data obtained on changes in physiological and morphological quality indicators of reindeer sperm after freezing and thawing expand the understanding of the reproductive biology of this species. The identified features should also be taken into account when optimizing the composition of diluents and the cryopreservation protocol to reduce damage in germ cells.

REFERENCES

1. Laishev K.A., Samandas A.M., Prokudin A.V., Romanenko T.M., Goncharov V.V., Mukhamadeeva T.V. *Dostizheniya nauki i tekhniki APK*, 2013, 11: 42-44 (in Russ.).
2. Goncharov V.V., Nikitkina E.V. *Genetika i razvedenie zhivotnykh*, 2016, 2: 3-7 (in Russ.).
3. Holand Ø., Røed K.H., Mysterud A., Kumpula J., Nieminen M., Smith M.E. Effect of male composition during rut on reproduction and reproductive parameters in reindeer (*Rangifer tarandus tarandus*). *Journal of Wildlife Management,* 2003, 67: 25-33.
4. Holand Ø., Gjøstein H., Losvar A., Kumpula J., Smith M.E., Røed K.H., Nieminen M., Weladji R.B. Social rank in female reindeer (*Rangifer tarandus*): effects of body mass, antler size and age. *Journal of Zoology*, 2004, 263(4): 365-372 (doi: 10.1017/S0952836904005382).
5. Ropstad E., Veiberg V., Säkkinen H., Dahl E., Kindahl H., Holand O., Beckers J.F., Eloranta E. Endocrinology of pregnancy and early pregnancy detection by reproductive hormones in reindeer (*Rangifer tarandus tarandus*). *Theriogenology*, 2005, 63(6): 1775-1788 (doi: 10.1016/j.theriogenology.2004.08.003).
6. Nikitkina E.V., Goncharov V.V., Krutikova A.A., Sergeeva O.K., Musidrai A.A., Plemyashov K.V. *Genetika i razvedenie zhivotnykh*, 2017, 1: 9-14 (in Russ.).
7. Garde J.J., Martínez-Pastor F., Gomendio M., Malo A.F., Soler A.J., Fernández-Santos M.R., Esteso M.C., García A.J., Anel L., Roldán E.R.. The application of reproductive technologies to natural populations of red deer. *Reproduction in Domestic Animals*, 2006, 41(Suppl. 2): 93-102 (doi: 10.1111/j.1439-0531.2006.00773.x).
8. Lindeberg H., Aalto J., Amstislavsky S., Järvinen M., Piltti K., Oksman M., Korhonen H., Vahtiala S., Eloranta E., Pudas T., Nieminen M., Helin A., Reinikainen E., Valtonen M. Development of assisted reproductive technology in the farmed European polecat (*Mustela putorius*) and in the semi-domesticated reindeer (*Rangifer tarandus tarandus*). *Extended Abstracts of the 28th Congress of the International Union of Game Biologist.* Hannover, Germany, 2005: 240-241.
9. Dott H.M., Utsi M.N.P. Artificial insemination in reindeer (*Rangifer tarandus*). *Journal of Zoology*, 1973, 170(4): 505-508 (doi: 10.1111/j.1469-7998.1973.tb05065.x).
10. Dott H.M., Utsi M.N.P. The collection and examination of semen of the reindeer (*Rangifer tarandus*). *Journal of Zoology*, 1971, 164(4): 419-424 (doi: 10.1111/j.1469-7998.1971.tb01325.x).
11. Deryazhentsev V.I. V sbornike: *Trudy Magadanskogo zonal'nogo nauchno-issledovatel'skogo instituta sel'skogo khozyaistva Severo-Vostoka* [In: Proceedings of the Magadan Zonal Research Institute of Agriculture of the North-East]. Magadan, 1974, tom 4: 30-35 (in Russ.).
12. Mkrtchyan M.E., Deryazhentsev V.I. V: *Sb. nauchnykh rabot Murmanskoi olenevodcheskoi opytnoi stantsii* [In: Collection of scientific papers of the Murmansk reindeer herding experimental station]. Murmansk, 1973, tom 2: 34-36 (in Russ.).
13. Mkrtchan M.E., Deryazhentsev V.I. V: *Sb. nauchnykh rabot Murmanskoi olenevodcheskoi opytnoi stantsii* [In: Collection of scientific papers of the Murmansk reindeer herding experimental station]. Murmansk, 1973, tom 2: 31-33 (in Russ.).
14. Lindeberg H., Valtonen M. Ex-situ preservation of endangered species as frozen gametes and embryos. Programme and abstracts of the 10th Nordic Conference on Reindeer Research, 13.-15.3.1998, Kautokeino, Norway. R.E. Haugerud (ed.). *Rangifer*, 1998, 18(6): 97-98 (doi: 10.7557/2.18.6.1372).
15. Vahtiala S., Lindeberg H., Eloranta E., Säkkinen H. Intrauterine artificial insemination in the reindeer. Programme and abstracts — 11th Arctic Ungulate Conference, 24-28 August 2003, Saariselkä, Finland.R.E. Haugerud (ed.). *Rangifer*, 2003, 23(3): 67 (doi: 10.7557/2.23.3.1645).
16. Lindeberg H., Oksman M., Järvinen M., Eloranta E., Vahtiala S., Valtonen M. Semen collection in the reindeer (*Rangifer tarandus tarandus*). In: *Book of abstracts for the XII Post Graduate Symposium of Animal Physiology, Research Station of Oulanka, Oulanka, Finland, 7.-9. September 2000.* S. Saarela, E. Hohtola (eds.). University of Oulu, 2000, 6: 12.
17. Shipka M.P., Rowell J.E., Bychawski S. Artificial insemination in reindeer using frozen-thawed semen. *Animal Science*, 2010, 88(E-Suppl. 2): 124. Abstract M327.
18. Mkrtchyan M.E., Rombe S.M. *Zhivotnovodstvo*, 1973: 72-74 (in Russ.).
19. Plemyashov K., Nikitkina E., Krutikova A., Timofeeva S., Shiryaev G., Musidray A., Goncharov V. Semen collection, evaluation and freezing in reindeer (*Rangifer tarandus*). *Animal Reproduction Science*, 2018, 194: e13-e14 (doi: 10.1016/j.anireprosci.2018.04.032).
20. Plemyashov K., Nikitkina E., Lindeberg H., Holand O., Krutikova A., Timofeeva S., Shiryaev G., Musidrai A. Basic semen evaluation in reindeer (*Rangifer tarandus*). Poster Presentations. *Reproduction in Domestic Animals*, 2018, 53(S2): 183 (doi: 10.1111/rda.13272).
21. Musidrai A., Plemyashov K., Nechaev A., Nikitkina E., Krutikova A., Timofeeva S., Shiryaev G., Kudinov A., Polteva E., Goncharov V. Analysis of fixing methods of reindeer (*Rangifer tarandus*) for semen collection. Poster Presentations. *Reproduction in Domestic Animals*, 2018, 53(S2): 170 (doi: 10.1111/rda.13272).
22. Büyükleblebici S., Tuncerb P.B., Bucak M.N., Ekend A., Sarıözkan S., Taşdemir U., Endirlik B.Ü. Cryopreservation of bull sperm: Effects of extender supplemented with different cryo-

protectants andantioxidants on sperm motility, antioxidant capacity and fertility results. *Animal Reproduction Science*, 2014, 150(3-4): 77-83 (doi: 10.1016/j.anireprosci.2014.09.006).
23. Pezo F., Romero F., Zambrano F., Sánchez R.S. Preservation of boar semen: an update. *Reproduction of Domestic Animals*, 2019, 54(3): 423-434 (doi.org/10.1111/rda.13389).
24. Rodriguez-Martinez H., Wallgren M. Advances in boar semen cryopreservation. *Veterinary Medicine International*, 2011, 2011: Article ID 396181 (doi: 10.4061/2011/396181).
25. Grötter L.G., Cattaneo L., Marini P.E., Kjelland M.E., Ferré L.B. Recent advances in bovine sperm cryopreservation techniques with a focus on sperm post-thaw quality optimization. *Reproduction in Domestic Animals*, 2019, 54(4): 655-665 (doi: 10.1111/rda.13409).
26. Atroshchenko M.M., Kalashnikov V.V., Bragina E.E., Zaitsev A.M. Comparative study of the structural integrity of spermatozoa in epididymal, ejaculated and cryopreserved semen of stallions. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2017, 52(2): 274-281 (doi: 10.15389/agrobiology.2017.2.274eng).
27. Monaco D., Zappia E., Apichaya S., Lacalandra G.M., Thongtip N. Post-thawing effects of three cryopreservation diluents on Rusa deer (*Rusa timorensis*) spermatozoa. *Reproduction in Domestic Animals*, 2019, 54(3): 635-638 (doi: 10.1111/rda.13380).
28. Martínez A.F., Martínez-Pastor F., Álvarez M., Fernández-Santos M.R., Esteso M.C., de Paz P., Garde J.J., Anel L. Sperm parameters on Iberian red deer: electroejaculation and post-mortem collection. *Theriogenology*, 2008, 70(2): 216-226 (doi: 10.1016/j.theriogenology.2008.04.001).
29. Gao D.Y., Critser J.K. Mechanisms of cryoinjury in living cells. *ILAR Journal*, 2000, 41(4): 187-196 (doi: 10.1093/ilar.41.4.187).
30. García-Álvarez O., Soler A.J., Maulen Z., Maroto-Morales A., Iniesta-Cuerda M., Martín-Maestro A., Fernández-Santos M.R., Garde J.J. Selection of red deer spermatozoa with different cryoresistance using density gradients. *Reproduction in Domestic Animals*, 2016, 51: 895-900 (doi: 10.1111/rda.12755).
31. Esteso M.C., Fernández-Santos M.R., Soler A.J., Montoro V., Quintero-Moreno A., Garde J.J. The effects of cryopreservation on the morphometric dimensions of Iberian red deer (*Cervus elaphus hispanicus*) epididymal sperm heads. *Reproduction in Domestic Animals*, 2006, 41(3): 241-246 (doi: 10.1111/j.1439-0531.2006.00676.x).
32. Atroshchenko M.M., Bragina E.E., Zaitsev A.M., Kalashnikov V.V., Naumenkova V.A., Kudlaeva A.M., Nikitkina E.V. Conservation of genetic resources in horse breeding and major structural damages of sperm during semen cryopreservation in stallion. *Nature Conservation Research*, 2019, 4(Suppl. 2): 78-82 (doi: 10.24189/ncr.2019.024).
33. Soler A.J., García A.J., Fernández-Santos M.R., Esteso M.C., Garde J.J. Effects of thawing procedure on postthawed in vitro viability and in vivo fertility of red deer epididymal spermatozoa cryopreserved at –196 °C. *Journal of Andrology*, 2003, 24(5): 746-756 (doi: 10.1002/j.1939-4640.2003.tb02737.x).
34. Zomborszky Z., Nagy S., Nánássy L., Szabari M., Body S. Experiences in deer sperm cryopreservation under practical conditions — a pilot study. *Animal Reproduction Science*, 2005, 90(1-2): 185-190 (doi: 10.1016/j.anireprosci.2005.01.014).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1196-1205
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Aspects of reproduction

УДК 636.2:591.16:612.616.2

doi: 10.15389/agrobiology.2019.6.1196eng
doi: 10.15389/agrobiology.2019.6.1196rus

QUANTITATIVE AND QUALITY INDICATORS OF SPERM PRODUCTION IN HOLSTEIN BULLS DEPENDING ON GEOMAGNETIC ACTIVITY

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Foundation for Basic Research, grant No. 18-016-00128 A
Received July 13, 2019

Abstract

A magnetic storm is one of the most important factors affecting the biological objects. The magnetic storms have profound effects at the molecular level, affecting certain cell structures. Spermatozoa are affected by various biotic and abiotic factors inside and outside the body. During the period of geomagnetic activity, the frequency of exacerbations of chronic diseases increases, the functions of a number of systems are disrupted. However, the reports on the influence of geomagnetic activity on the reproductive function, in particular on the quantity and quality of sperm, are objectively quite limited, and for agricultural species of animals such information was not found in the available literature. The objective of the work was to study the relationships between the geomagnetic activity and the sperm production quality and quantity parameters. The subject to the survey was the sperm collected from the Holstein-Friesian bull sires (n = 10, Joint-Stock Company "Head Center for the Reproduction of Farm Animals", Moscow Province, 2018). Monitoring the K-index reflecting the geomagnetic conditions was carried out according to the data from the Pushkov Institute of Terrestrial Magnetism, Ionosphere and Radio Wave Propagation RAS. The statistical analysis was performed with SPSS v.15.0 for a one-way analysis of variance. The variables between the groups for $K \leq 1.0$ (no geomagnetic disturbance) and $K \geq 5.0$ (geomagnetic storm) were compared with the Scheffe's test. The assessment of the sperm quality was performed with the Argus-CASA software (ArgusSoft, Russia) with Nikon Eclipse Ni microscope (Nikon, Japan). Our data indicate that during the survey, the K-index was a factor affecting the biological adequacy of the bull sires. The results prove that the magnetic storm has a significant impact on the qualitative and quantitative parameters of the bull sire sperm production. We have established the statistically valid criteria for the ejaculate volumes ($F = 6.49$; $p < 0.05$) and the progressively motile sperm number ($F = 8.36$; $p < 0.05$) per ejaculate. The volume of the ejaculates of the bull sires tends to a 28.2 % decrease ($p < 0.001$) during the magnetic storm period (K-index value ≥ 5.0 vs. K-index ≤ 1.0). The geomagnetic activity causes a 11.3 % decline ($p < 0.001$) in the sperm activity.

Keywords: magnetic storm, spermatozoa, sperm motility, morphology, chromatin, nDNA, DNA fragmentation index, bull sires, CASA

Magnetic storms are one of the natural abiotic factors affecting living organisms [1, 2]. The details of the dependence of biological processes on the state of the geomagnetic field have not been fully studied yet but it is recognized that most physiological rhythms are synchronized with solar and geomagnetic activity [3-6]. At the same time, both in individual cells and in the body as a whole, the reaction to external influences depends not only on their nature and strength but

also on the properties of the biological objects themselves [7].

During magnetic storms, chronic diseases of the cardiovascular and central nervous systems become more acute, blood flow is disrupted [8-10], the adrenal secretion of adrenaline increases markedly, and the state of the autonomic nervous system changes, which regulates the work of internal organs and glands of internal secretion [11]. The change in geomagnetic activity is associated with the production of melatonin [12, 13], which is involved in the regulation of circadian rhythms, improves the work of the endocrine [14], immune [15], and reproductive [16, 17] systems.

The influence of geomagnetic activity at the cellular level is explained by changes in the state and functions of cell membranes, disruption of transmembrane transport, the formation of free radical lipid oxidation products, and a decrease in the buffer capacity of the antioxidant system [18]. At the same time, data on magnetic field effects on cells are contradictory. Thus, the static magnetic field did not cause oxidative stress in mouse fibroblasts and even led to some increase in antioxidant activity [19]. Some cell compartments, such as mitochondria and endoplasmic reticulum, are more sensitive to magnetic fields [20].

Spermatozoa are affected by various biotic and abiotic factors inside and outside the body [21-23]. Most of these factors have an indirect and direct impact on the reproductive qualities of bull sires. The influence degree of external factors on spermatozoa depends on the nature of these factors and the state of the spermatozoa themselves. External factors may cause changes in morphology, nucleus, acrosome, mitochondria, and other structures. Electromagnetic fields have a significant influence on sperm motility and morphology [24, 25].

The most important indicator that characterizes the biological full-value of spermatozoa is the state of nuclear DNA in chromatin, on which male fertility depends largely. Numerous biotic and abiotic factors influence the degree of sperm nuclear DNA fragmentation [26].

Thus, numerous, mostly medical, observations indicate the dependence of the physiological state, functional activity and risks of exacerbation of some pathologies on the impact of magnetic storms on the body, but reports on the impact of geomagnetic activity on reproductive function, in particular on the quantity and quality of sperm, are objectively quite limited. We have not found such information in the scientific literature for agricultural species of animals, although in modern livestock breeding, based on the large-scale use of cryopreserved sperm and artificial insemination, the problem of a sufficient number of biologically full-fledged sperm of sires is very acute. An in-depth study of this topic in agricultural species is also important for understanding the effect of magnetic storms on male germ cells in vivo (on the body level), in addition to identifying the mechanisms of cellular responses to magnetic and electromagnetic fields in vitro.

In this paper, we show that a magnetic storm has a statistically significant effect on the volume of ejaculate ($F = 6.49$, $p < 0.05$) and the progressively motile sperm number ($F = 8.36$, $p < 0.05$) in Holstein bulls. During a magnetic storm, the volume of ejaculate decreases by 28.2% ($p < 0.001$), and sperm activity decreases by 11.3% ($p < 0.001$).

The purpose of the study was to determine whether the quantitative and qualitative indicators of sperm production in cattle change under the influence of geomagnetic activity.

Techniques. A group of 10 Holstein bulls (JSC Head Center for the Reproduction of Farm Animals, Moscow Region, 2018) aged 3-5 years with a live weight of 900-1100 kg was tested. The conditions for feeding and keeping animals were similar and corresponded to the developed standards (Ernst Federal Science Center for Animal Husbandry). Sperm collection was carried out by the

operators of JSC Head Center for the Reproduction of Farm Animals following the national technology of freezing and using the sperm of bull sires [27]. Two ejaculates were taken from each bull with an interval of 10-15 minutes. Depending on the concentration, the ejaculates were diluted with OPTIXcell™ synthetic medium (IMV Technologies, France). The volume of ejaculates was taken into account and quality indicators of sperm were determined on freshly collected samples. Three samples of each ejaculate were examined (the total sample size was 360 samples).

To determine the concentration and motility of spermatozoa, the authors used Makler's chamber (sperm counting chamber) (Sefi Medical Instruments, Israel) and a computerized analysis system, a Nikon Eclipse Ni microscope equipped with a Nikon DS-Qi2 camera with high resolution (4908×3264) (Nikon, Japan) (Argus-CASA — Computerized Assisted Semen Analysis software, ArgusSoft, Russia). For motility analysis, data were obtained for at least 300 spermatozoa. The following indicators were evaluated: VAP (average path velocity, mm/s) is the average velocity of head movement average path, rm/s; VSL (straight-line velocity, rm/s) is the speed of rectilinear movement of the head, μm/s (average velocity of a sperm head movement along the straight line segment between the start and end point of the trajectory); VCL (curvilinear velocity, μm/s) is the actual speed of the spermatozoa movement in a curvilinear path, μm/s, ALH (amplitude of lateral head displacement, rm) is the average deviation of the head, μm (amplitude of the lateral displacement of the spermatozoa head relative to the path of motion); BCF (beat-cross frequency, Hz), the averaged vibrational movements frequency, Hz (average frequency of intersection of the curved trajectory of the sperm cell with its average trajectory per unit of time); STR (straightness, VSL/VAP) is a measure of the straightness of spermatozoa directional movement (average of the trajectory, %; LIN (linearity, VSL/VCL, %), the degree of the tracks undulation (oscillations of the true motion path to the average trajectory), %.

When preparing smears for morphological studies, a drop of sperm was applied to a defatted glass slide, distributed with a plastic spatula in a thin layer and dried at room temperature, and the resulting preparations were stained (Quick-Diff kit, Abris+, St. Petersburg) following the attached protocol. The preparations were analyzed automatically using the Argus-CASA software following strict Krueger's criteria. The results were expressed in absolute values (the number of spermatozoa with abnormal morphology), as well as a percentage (the ratio of spermatozoa with abnormal morphology to the total number of spermatozoa, expressed as a percentage).

DNA fragmentation in spermatozoa (Sperm Chromatin Dispersion Test, SCD-test) was studied using the Halosperm® kit (Laboratories INDAS S.A.U., Spain) according to the attached instructions. Microscopy was performed at a magnification of ×40 using an Altami LUM-2 microscope equipped with a UCMOS14000KPA digital camera (Russia).

Depending on the halo size, spermatozoa were divided into five classes: without DNA fragmentation for large halo, medium halo; with fragmented DNA for small halo, no halo; degenerate for the nucleus of an unusual shape or poorly colored. Classification according to the degree of fragmentation was performed using the Argus-CASA software. The percentage of spermatozoa with different degrees of DNA fragmentation was calculated automatically.

Statistical analysis was performed with IBM SPSS Statistics 15.0 software (IBM Corp., USA). The mean values (M) and standard errors of means (±SEM)

were calculated. To assess the significance of the influence of the geomagnetic activity factor on spermatozoa parameters, a single-factor dispersion analysis (ANOVA) was performed, using F-test and determining p-significance. To identify the difference between groups, the Scheffe method of multiple comparisons was used. The differences were deemed statistically highly reliable at $p < 0.001$, , at $p < 0.01$ and $p < 0.05$ – reliable.

Results. For 8 months of observation (from January to October 2018), according to the Pushkov Institute of Terrestrial Magnetism, Ionosphere and Radio Wave Propagation RAS (Moscow) (http://geodata.izmiran.ru/), the magnetic storms with a K-index ≥ 5.0 (the K-index of the geomagnetic situation characterizes the deviation of the Earth's magnetic field from the norm during a 3-hour interval; it has values from 0 to 9) were recorded in the study area 4 times (February 27, April 20, June 25, and August 27).

Table 1 shows the average seminogram data of the studied bull sires in the dynamics depending on the geomagnetic activity.

1. Average seminogram values of Holstein bulls depending on geomagnetic activity K-index (*M*±SEM, JSC Head Center for the Reproduction of Farm Animals, Moscow Province, January-October, 2018)

Bull No.	Volume, ml	PR, %	NP, %	IM, %	VAP, μm/s	VSL, μm/s	VCL, μm/s	ALH, μm	BCF, Hz	STR, %	LIN, %
1	5.1±0.12	88.3±2.6	7.2±0.9	4.5±0.3	89.5±1.9	77.0±1.5	127.5±3.7	5.3±0.9	28.5±1.1	80.0±3.2	62.5±0.9
	4.2±0.11	77.5±1.9	17.5±1.1	5.0±0.1	78.5±2.4	66.5±2.3	110.5±2.9	4.5±0.8	22.0±0.8	70.5±1.9	61.5±1.7
2	4.8±0.11	82.3±2.1	5.3±0.6	12.4±0.2	80.3±1.5	65.3±1.9	111.3±2.7	4.5±0.3	26.3±1.3	75.3±2.7	58.7±0.9
	3.8±0.08	71.3±1.8	15.6±1.2	13.1±0.2	69.5±1.8	59.7±3.5	100.4±3.5	3.9±0.7	20.5±0.8	72.5±1.5	60.3±1.1
3	5.1±0.12	86.2±2.2	6.8±1.1	7.0±0.1	88.2±2.7	72.4±1.8	125.5±4.7	5.2±0.9	28.1±1.1	80.0±2.1	61.8±0.5
	4.0±0.08	73.2±1.9	19.5±1.6	7.3±0.2	74.2±1.6	60.4±3.4	105.0±1.9	4.4±0.8	22.0±0.9	74.5±3.4	57.5±1.7
4	5.2±0.09	84.1±1.9	6.7±0.9	9.2±0.3	81.2±1.9	69.0±1.9	109.4±2.4	4.7±0.2	26.8±0.7	76.0±1.9	63.5±2.5
	4.2±0.16	70.3±1.9	19.9±1.8	9.8±0.1	74.3±2.7	59.5±2.5	104.0±3.5	4.2±0.3	22.6±1.1	68.5±0.8	58.1±1.9
5	4.2±0.11	82.3±2.3	5.3±0.9	12.4±0.2	81.8±1.6	73.5±1.5	120.0±2.5	5.1±0.3	29.2±0.9	78.0±1.5	62.8±0.9
	3.4±0.12	72.2±1.9	14.6±1.1	13.2±0.2	73.5±2.5	64.7±3.7	107.8±2.6	4.5±0.1	21.3±1.2	74.2±2.4	61.0±2.5
6	4.3±0.13	85.2±1.4	6.4±0.9	8.4±0.1	86.6±1.9	75.6±1.9	119.7±1.8	5.0±0.2	27.5±0.8	81.0±2.8	63.8±1.9
	3.4±0.11	74.2±1.9	16.8±1.5	9.0±0.1	72.2±1.7	62.0±2.8	109.5±2.9	4.3±0.2	20.5±0.9	73.1±3.6	57.2±2.5
7	4.9±0.12	88.2±2.6	7.8±1.1	4.0±0.2	88.3±2.4	75.0±3.6	123.0±3.8	5.2±0.1	28.0±1.6	80.0±1.9	62.5±1.5
	4.0±0.11	77.5±1.9	18.0±0.8	4.5±0.1	77.3±1.6	60.4±1.9	104.5±1.5	4.4±0.2	21.1±0.9	73.0±0.8	58.1±2.7
8	5.2±0.12	87.2±2.3	6.9±0.6	5.9±0.2	88.5±1.7	70.3±2.5	117.0±2.3	4.6±0.2	26.4±1.5	74.0±1.1	60.1±0.8
	4.1±0.11	76.5±2.1	17.0±1.1	6.5±0.1	77.5±3.5	64.5±1.9	108.4±4.9	4.1±0.1	21.0±1.7	73.2±2.7	60.1±0.9
9	4.6±0.12	87.5±3.6	7.6±0.9	4.9±0.2	88.0±2.8	70.0±1.8	116.0±3.8	4.8±0.2	26.5±0.8	75.0±0.9	60.4±1.8
	3.2±0.15	76.2±1.9	18.4±1.4	5.4±0.1	77.5±1.6	65.0±3.1	107.0±1.3	3.9±0.2	20.6±1.1	72.0±2.6	60.3±2.7
10	4.8±0.16	85.1±2.5	7.2±0.9	7.7±0.1	85.0±1.9	67.6±2.5	115.0±2.5	4.4±0.2	26.1±2.1	76.5±1.5	58.8±0.9
	3.3±0.08	74.5±1.8	17.5±1.8	8.0±0.2	73.0±3.8	62.0±3.4	104.0±3.5	3.8±0.2	20.4±0.9	72.3±2.4	58.1±1.7

N o t e. PR — progressive motile; NP — non-progressive motile; IM — stationary; VAP — the average speed of head movement along the average trajectory, VSL — the speed of rectilinear movement of the head, VCL — actual speed of sperm movement along the real trajectory, ALH — average deviation of the head, BCF (beat-cross frequency) — frequency of oscillatory averaged movements, STR — degree of straightness of the directed movement of sperm, LIN — degree of undulation tracks. Above the line indicators at K ≤ 1.0, below the line at K ≥ 5.0.

The ANOVA analysis showed that the magnetic storm had a reliable significant effect on the volume of ejaculate ($F = 6.49$, $p < 0.05$) and the progressively motile sperm number ($F = 8.36$, $p < 0.05$) in the ejaculate of bull sires (Table 2). During the magnetic storm, the volume of ejaculate received decreased by 28.2% ($p < 0.001$, the Scheffe test, see Table 2) compared to that obtained during the period when the magnetic situation was normal and the K-index ≤ 1.0. Under the influence of a magnetic storm, when the geomagnetic activity index was 5 points or higher, sperm motility decreased by 11.3% ($p < 0.001$). Increased geomagnetic activity is accompanied by an increase in the proportion of spermatozoa with affected and oscillatory movement. On days with disturbed geomagnetic conditions, the parameters characterizing the activity of spermatozoa reduced significantly: for VAP by 10.9 μm/s ($p < 0.01$), for VSL by 9.1 μm/s

($p < 0.01$), and for ALH by 0.7 μm/s ($p < 0.01$) (see Table 1).

2. The volume of ejaculates and sperm motility in ejaculates of Holstein bulls during periods of different geomagnetic activity (*M*±SEM, *n* = 360, JSC Head Center for the Reproduction of Farm Animals, Moscow Province, 2018)

Indicator	Geomagnetic activity index	
	K≤ 1.0 (quiet geomagnetic environment)	K≥ 5.0 (magnetic storm)
Volume, ml	4.82±0.14	3.76±0.15***
PR, %	85.60±1.20	74.30±1.50***
NP, %	6.70±1.30	17.50±1.90**
IM, %	7.70±1.80	8.20±2.10
VAP, μm/s	85.70±1.60	74.80±2.10**
VSL, μm/s	71.50±2.30	62.40±1.70**
VCL, μm/s	118.40±3.20	106.10±2.10**
ALH, μm	4.90±0.10	4.20±0.20**
BCF, Hz	27.30±0.90	21.20±1.20**
STR, %	77.70±3.10	72.40±2.40**
LIN, %	61.50±2.50	59.20±1.70*

N o t e. PR — progressive motile; NP — non-progressive motile; IM — stationary; VAP — the average speed of head movement along the average trajectory, VSL — the speed of rectilinear movement of the head, VCL — actual speed of sperm movement along the real trajectory, ALH — average deviation of the head, BCF (beat-cross frequency) — frequency of oscillatory averaged movements, STR — degree of straightness of the directed movement of sperm, LIN — degree of undulation tracks. Above the line indicators at K ≤ 1.0, below the line at K ≥ 5.0.
*, **, *** Differences with indicators at K ≤ 1.0 are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

3. Single-factor variance analysis of frequency (%) of sperm morphology abnormalities in ejaculates of Holstein bulls as per K-index groups in periods of different geomagnetic activity (*M*±SEM, *n* = 360, JSC Head Center for the Reproduction of Farm Animals, Moscow Province, 2018)

Pathology type	Comparison	SS	df	MS	F	p
Spermatozoa pathological forms	Between groups	84.535	2	42.267	3.509	0.039
	Within groups	493.897	34	12.046		
	Total	578.432	36			
Head pathology	Between groups	0.200	2	0.100	0.385	0.699
	Within groups	1.300	34	0.260		
	Total	1.500	36			
Neck pathology	Between groups	26.449	2	13.224	1.515	0.234
	Within groups	296.794	34	8.729		
	Total	323.243	36			
Tail pathology	Between groups	6.451	2	3.225	2.324	0.034
	Within groups	19.432	34	1.388		
	Total	25.882	36			

N o t e. SS — the sum of squares, df — number of degrees of freedom, MS — mean square, F — Fisher test, p — statistical significance; Group I for K ≤ 1, Group II for K ≥ 5.

One-factor analysis of variance of morphological changes in spermatozoa depending on geomagnetic activity showed that the differences between the groups by average values for the content of all pathological forms and pathologies of the spermatozoa filament were reliable, and the levels of statistical significance were $p = 0.039$ and $p = 0.034$, respectively (Table 3). The statistical significance of the F-criterion for the incidence of the pathology of a head $p = 0.699$ and a midpiece $p = 0.234 > 0.05$; therefore, for these indicators, the zero hypothesis of the difference between the groups is not refuted.

On days with increased geomagnetic activity, the number of spermatozoa with pathology increased. At K ≥ 5.0 (magnetic storms), their share was 8.14%, which is 59.9% more than at K ≤ 1.0 (calm geomagnetic situation). Statistically significant differences in the frequency of filament pathologies were revealed (Fig. 1) depending on the geomagnetic activity. During a magnetic storm, the number of such sperms increased by 40.7% ($p < 0.05$), which led to an increase in the number of cells with affected oscillatory movement (non-progressive mo-

tile cells) (Table 4).

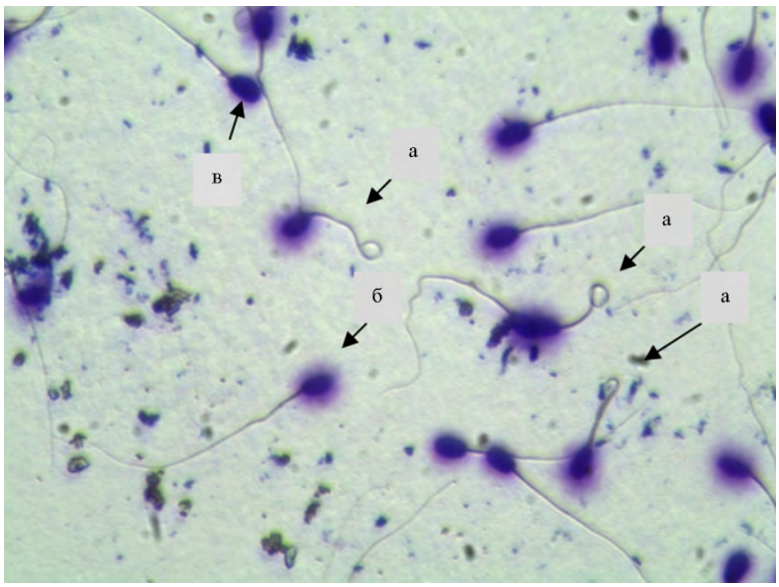

Fig. 1. Sperm Chromatin Dispersion Test (SCD-test) **in Holstein bulls:** a — tail pathology, b — spermatozoid without DNA fragmentation, c — spermatozoid with fragmented DNA (JSC Head Center for the Reproduction of Farm Animals, Moscow Province, 2018). Microscope Altami LUM-2 with a digital camera UCMOS14000KPA (Russia), ×40 zoom).

4. The proportion of sperm abnormalities in ejaculates of Holstein bulls as per K-index groups in periods of different geomagnetic activity (M±SEM, n =360, JSC Head Center for the Reproduction of Farm Animals, Moscow Province, 2018)

Indicator, %	Geomagnetic activity index	
	K ≤ 1.0	K ≥ 5.0
Spermatozoa pathological forms	4.88±0.55	8.14±0.36*
Head pathology	1.25±0.16	1.28±0.19
Neck pathology	1.64±0.30	2.30±0.24
Tail pathology	4.51±0.49	6.35±0.35*

* Differences with indicators at K ≤ 1.0 are statistically significant at p < 0.01.

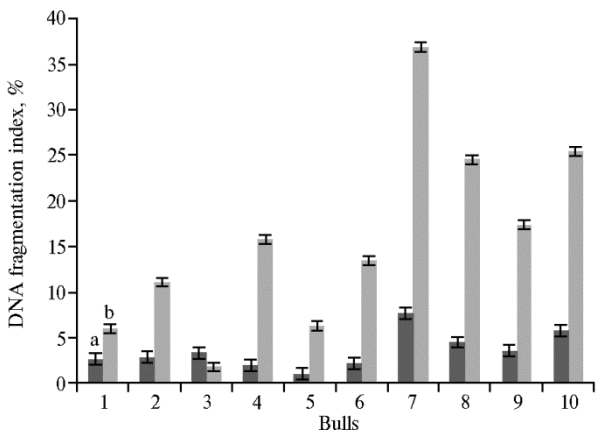

Fig. 2. Sperm DNA fragmentation index in ejaculates of Holstein bulls in periods of different geomagnetic activity: a — at K ≤ 1.0; b — at K ≥ 5.0 (JSC Head Center for the Reproduction of Farm Animals, Moscow Province, 2018).

In samples obtained from bull sires on days when magnetic storms were recorded, the fragmentation index of nuclear DNA exceeded the values at K ≤ 1.0 significantly (Fig. 2).

Studies of the impact of the magnetic storm on biological objects indicate the existence of a close dependence of physiological rhythms on geomagnetic activity [4-6]. Our investigation of the connection between the biological integrity of sperm in ejaculates and geomagnetic activity shows that magnetic storms negatively affect the quantitative (decrease in the volume of ejaculate by

an average of 22%) ($p < 0.001$) and qualitative indicators of ejaculate. The proportion of progressively motile sperm decreased by 11.3% at $p < 0.001$, of non-progressive motile sperm increased by 10.8% at $p < 0.01$. Sperm motility is one of the main parameters that characterize male fertility. Bull sires are used for reproduction depending on the spermatozoa motility [28]. Ejaculates with sperm motility values below 70% are rejected. The progressive movement of spermatozoa is necessary for them to reach the ovum. The absence or low content of sperm in the ejaculate with a straight-forward movement causes infertility [26, 28, 29].

The effect of geomagnetic disturbances is complex and depends both on their strength and the state of the biological objects themselves and their systems [24]. Analysis of the seminogram of bull sires in dynamics shows that the studied animals had decreased sperm production indicators during the days of geomagnetic activity. It should be noted that the reaction of bull sires to the geomagnetic situation was individual. The decrease in the number of progressively motile spermatozoa in individuals ranged from 11 to 16% ($p < 0.05$).

Numerous studies confirm the influence of abiotic factors on spermatozoa both when exposed to such agents on the paternal body, and in ejaculates or when found in the female tract [21-23]. Changes occur in the general morphology, the state of the nucleus and acrosome, in the mitochondria, filament, and other structures of spermatozoa [24, 25]. We observed an increase (by 40%, $p < 0.05$) in the proportion of sperm with abnormal morphology during the period of geomagnetic activity. At the same time, the share of spermatozoa with filament pathology increased the most (by 40.1%, $p < 0.05$).

The integrity of nDNA in the sperm chromatin is the most important characteristic of the biological full-value of spermatozoa [30, 31], it also determines fertility, the effectiveness of ovum fertilization and embryo development [32]. This study confirmed an increase in the index of DNA fragmentation in spermatozoa from ejaculates collected during the days of geomagnetic disturbances. The fragmentation degree varied from 1.80 to 38.84%, also indicating that the response to geomagnetic activity depends on the individual characteristics of the organism to a certain extent. It is known that one of the factors that cause DNA fragmentation in spermatozoa chromatin is oxidative stress [32, 33]. The plasma membrane of the spermatozoa contains a large amount of polyunsaturated fatty acids, which makes these germ cells more sensitive to such stress.

Thus, the data obtained in the present study indicate the influence of geomagnetic activity on the qualitative and quantitative characteristics of bull sperm obtained during the days of the magnetic storm. Comparative analysis of sperm biological full-value indicates an increase in the frequency of morphological anomalies and an increase in the index of nuclear DNA fragmentation in sperm from such ejaculates. These data obtained in vivo are important for the practice of using reproductive technologies. The observed effects should be considered as a result of the magnetic field influence on the organism level. A more detailed understanding of the processes occurring, in this case, can be obtained by an in-depth study of the physiological status of animals and the functional activity of the body systems, primarily reproductive and neurohumoral. Experiments with ejaculated cattle spermatozoa in vitro will be continued to fundamentally study the molecular mechanisms involved in these interactions and provide a cellular response to the action of geomagnetic fields.

REFERENCES

1. Liboff A.R. The electromagnetic field as a biological variable. In: *On the nature of electromagnetic*

field interactions with biological systems. A.N. Frey, R.J. Langes (eds.). Austin, 1994: 59-72.
2. Binhi V.N., Prato F.S. Biological effects of the hypomagnetic field: an analytical review of experiments and theories. *PLoS ONE*, 2017, 12(6): e0179340 (doi: 10.1371/journal.pone.0179340).
3. McCraty R., Atkinson M., Stolc V., Alabdulgader A.A., Vainoras A., Ragulskis M. Synchronization of human autonomic nervous system rhythms with geomagnetic activity in human subjects. *Int. J. Environ. Res. Public Health*, 2017, 14(7): 770 (doi: 10.3390/ijerph14070770).
4. Halberg F., Cornelissen G., McCraty R., Al-Abdulgader A.A. Time structures (chronomes) of the blood circulation, populations' health, human affairs and space weather. *World Heart Journal*, 2011, 3(1): 1-42.
5. Halberg F., Cornelissen G., Otsuka K., Watanabe Y., Katinas G.S., Burioka N., Delyukov A., Gorgo Y., Zhao Z., Weydahl A., Sothern R.B., Siegelova J., Fiser B., Dusek J., Syutkina E.V., Perfetto F., Tarquini R., Singh R.B., Rhees B., Lofstrom D., Lofstrom P., Johnson P.W., Schwartzkopff O., the International BIOCOS Study Group. Cross-spectrally coherent ~ 10.5- and 21-year biological and physical cycles, magnetic storms and myocardial infarctions. *Neuro Endocrinology Letters*, 2000, 21(3): 233-258.
6. Khabarova O., Dimitrova S. On the nature of people's reaction to space weather and meteorological weather changes. *Sun and Geosphere*, 2009, 4(2): 60-71.
7. Foster K.R. Mechanisms of interaction of extremely low frequency electric fields and biological systems. *Radiation Protection Dosimetry*, 2003, 106(4): 301-310 (doi: 10.1093/oxfordjournals.rpd.a006364).
8. Cornélissen G., Halberg F., Breus T., Syutkina E.V., Baevsky R., Weydahl A., Watanabe Y., Otsuka K., Siegelova J., Fiser B., Bakken E.E. Non-photic solar associations of heart rate variability and myocardial infarction. *Journal of Atmospheric and Solar-Terrestrial Physics*, 2002, 64(5-6): 707-720 (doi: 10.1016/S1364-6826(02)00032-9).
9. Giannaropoulou E., Papailiou M., Mavromichalaki H., Gigolashvili M., Tvildiani L., Janashia K., Preka-Papadema P., Papadima T. A study on the various types of arrhythmias in relation to the polarity reversal of the solar magnetic field. *Nat. Hazards*, 2014, 70: 1575-1587 (doi: 10.1007/s11069-013-0890-9).
10. Caswell J.M., Carniello T.N., Murugan N.J. Annual incidence of mortality related to hypertensive disease in Canada and associations with heliophysical parameters. *Int. J. Biometeorol.*, 2016, 60: 9-20 (doi: 10.1007/s00484-015-1000-3).
11. Panov G.A., Chizov A.Ya., Kotova I.N. Magnet storms influence on students' functional systems. *Vestnik Rossiiskogo universiteta druzhby narodov. Seriya: Ekologiya i bezopasnost' zhiznedeyatel'nosti*, 2008, 1: 60-67 (in Russ.).
12. Burch J.B., Reif J.S., Yost M.G. Geomagnetic disturbances are associated with reduced nocturnal excretion of a melatonin metabolite in humans. *Neuroscience Letters*, 1999, 266(3): 209-212 (doi: 10.1016/s0304-3940(99)00308-0).
13. Bergiannaki J.-D., Paparrigopoulos T.J., Stefanis C.N. Seasonal pattern of melatonin excretion in humans: relationship to day length variation rate and geomagnetic field fluctuations. *Experientia*, 1996, 52(3): 253-258 (doi: 10.1007/BF01920718).
14. Cipolla-Neto J., Amaral F.G.D. Melatonin as a hormone: new physiological and clinical insights. *Endocrine Reviews*, 2018, 39(6): 990-1028 (doi: 10.1210/er.2018-00084).
15. Pohanka M. Impact of melatonin on immunity: a review. *Open Medicine*, 2013, 8(4): 369-376 (doi: 10.2478/s11536-013-0177-2).
16. Rocha C.S., Rato L., Martins A.D., Alves M.G., Oliveira P.F. Melatonin and male reproductive health: relevance of darkness and antioxidant properties. *Current Molecular Medicine*, 2015, 15(4): 299-311 (doi: 10.2174/1566524015666150505155530).
17. Lampiao F., Du Plessis S.S. New developments of the effect of melatonin on reproduction. *World J. Obstet. Gynecol.*, 2013, 2(2): 8-15 (doi: 10.5317/wjog.v2.i2.8).
18. Agadzhanyan N.A., Makarova I.I. *Ekologiya cheloveka*, 2005, 9: 3-9 (in Russ.).
19. Glinka M., Gawron S., Siero A., Pawłowska-Gyral K., Cieślar G., Sieroń K. Impact of static magnetic field on the antioxidant defense system of mice fibroblasts. *BioMed Research International*, 2018, 2018: Article ID 5053608 (doi: 10.1155/2018/5053608).
20. Zablotskii V., Polyakova T., Lunov O., Dejneka A. How a high-gradient magnetic field could affect cell life. *Sci. Rep.,* 2016, 6: 37407 (doi: 10.1038/srep37407).
21. Marshall D.J. Environmentally induced (co)variance in sperm and offspring phenotypes as a source of epigenetic effects. *Journal of Experimental Biology*, 2015, 218: 107-113 (doi: 10.1242/jeb.106427).
22. Toraco E.G., García M.G., Fernández-Morera J.L., Nico-García P., Fernández A.F. The impact of external factors on the epigenome: in utero and over lifetime. *BioMed Research International*, 2016, 2016: Article ID 2568635 (doi: 10.1155/2016/2568635).
23. Jensen N., Allen R.M., Marshall D.J. Adaptive maternal and paternal effects: gamete plasticity in response to parental stress. *Funct. Ecol.*, 2014, 28(3): 724-733 (doi: 10.1111/1365-2435.12195).
24. Li D.K., Yan B., Li Z., Gao E., Miao M., Gong D., Weng X., Ferber J.R., Yuan W. Exposure to magnetic fields and the risk of poor sperm quality. *Reproductive Toxicology*, 2010, 29(1): 86-92 (doi: 10.1016/j.reprotox.2009.09.004).

25. Kumari K., Capstick M., Cassara M., Herrala M., Koivisto H., Naarala J., Tanila H., Vilukselaa M., Juutilainen J. Effects of intermediate frequency magnetic fields on male fertility indicators in mice. *Environmental Research*, 2017, 157: 64-70 (doi: 10.1016/j.envres.2017.05.014).
26. Borunova S.M., Iolchiev B.S., Badmaev O.E., Tumilovich YA.I., Iolchiev P.B., Ribchenko A.S. *Veterinariya, zootekhniya i biotekhnologiya*, 2017, 11: 57-64 (in Russ.).
27. Vinogradov V.N., Strekozov N.I., Abilov A.I et al. *Natsional'naya tekhnologiya zamorazhivaniya i ispol'zovaniya spermy plemennykh bykov-proizvoditelei* [National technology for freezing and using sperm of bulls sires]. Moscow, 2008 (in Russ.).
28. Wang X., Yang C., Guo F., Zhang Y., Ju Z., Jiang Q., Zhao X., Liu Y., Zhao H., Wang J., Sun Y., Wang C., Zhu H., Huang J. Integrated analysis of mRNAs and long noncoding RNAs in the semen from Holstein bulls with high and low sperm motility. *Scientific Reports*, 2019, 9: Article number 2092 (doi: 10.1038/s41598-018-38462-x).
29. Brody S.A. *Andrologiya i genital'naya khirurgiya*, 2014, 3: 33-41 (doi: 10.17650/2070-9781-2014-3-33-41) (in Russ.).
30. Gonzalez-Marin C., Gosalvez J., Roy R. Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. *International Journal of Molecular Sciences*, 2012, 13(11): 14026-14052 (doi: 10.3390/ijms131114026).
31. Kim G.Y. What should be done for men with sperm DNA fragmentation? *Clin. Exp. Reprod. Med.*, 2018, 45(3): 101-109 (doi: 10.5653/cerm.2018.45.3.101).
32. Agarwal A., Said T.M. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Human Reproduction Update*, 2003, 9(4): 331-345 (doi: 10.1093/humupd/dmg027).
33. Esteves S.C., Sanchez-Martin F., Sanchez-Martin P., Schneider D.T., Gosálvez J. Comparison of reproductive outcome in oligozoospermic men with high sperm DNA fragmentation undergoing intracytoplasmic sperm injection with ejaculated and testicular sperm. *Fertility and Sterility*, 2015, 104(6): 1398-1405 (doi: 10.1016/j.fertnstert.2015.08.028).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1206-1213
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Cell cultures

UDC 636.2:591.39

doi: 10.15389/agrobiology.2019.6.1206eng
doi: 10.15389/agrobiology.2019.6.1206rus

FINAL MATURATION OF BOVINE OOCYTES IN A FERT-TALP MEDIUM INCREASED THEIR QUALITY AND COMPETENCE TO IN VITRO EMBRYO DEVELOPMENT

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The authors declare no conflict of interests
Acknowledgements:
Supported by grant from Russian Foundation for Basic Research (project No. 17-29-08035) and the Ministry of Science and Higher Education of the Russian Federation
Received September 17, 2019

Abstract

In vitro maturation (IVM) of the oocytes is an important step for in vitro embryo production (IVP). In vitro culture during maturation decreases oocyte quality and therefore, IVM conditions need to be improved. Routinely, IVM of bovine oocytes is performed using one-step medium, the TC-199 complemented with fetal bovine serum (FBS) and gonadotropins. However, the asynchrony of oocyte nuclear and cytoplasm maturation processes may require the differential hormonal environment during IVM. In the present work, for the first time we have compared the efficiency of the two-step IVM protocols, which include the first step in standard conditions and final maturation in hormone-free mediums TC-199 or Fert-TALP; the last one is a routine medium for in vitro fertilization (IVF). The objective was to study the effects of two-step IVM protocols to chromosome modifications and apoptotic events in mature oocytes and a quality of in vitro produced embryos. Oocyte-cumulus cells complexes (OCC) were in vitro matured during 16 h in medium TC-199 complemented with 10 % FBS, 10 μg/ml follicle-stimulating hormone and 10 μg/ml luteinizing hormone, and then transferred to either medium TC-199 without gonadotropins (System 1) or Fert-TALP medium (System 2) for additional 8 h of IVM. After final IVM, nuclear state and apoptosis were checked in a part of the mature oocytes. IVF was performed on remaining OCC to analyze in vitro embryo development. No difference was observed in nuclear maturation rates between the systems 1 and 2 after 24 h IVM: 83.3 % and 84.7 % of the oocytes reached metaphase-II. Apoptosis rate were significantly lower ($p < 0.05$) in the oocytes matured in System 2 (11.7±0.7 %) compared to System 1 (19.4±1.1 %). In addition, blastocyst rate after 7 days of in vitro embryo development was significantly higher ($p < 0.05$) in System 2 (30.0±2.9 %), than in System 1(17.4±0.4 %). Oocyte cleavage rate, cell number and apoptosis rate in the blastocysts were similar in both IVM systems. In conclusion, two-step IVM system using final 8-hour maturation in Fert-TALP medium is more advantageous compared to a system including hormone-free TC-199 medium at the end of IVM. Fert-TALP IVM system decreased apoptosis rate in the oocytes and increased their competence to in vitro embryo development after IVF.

Keywords: bovine oocytes, in vitro maturation, maturation media, apoptosis, embryo development

In bovine cattle, reproductive cellular technologies, including in vitro embryo production (IVP), have extensive scope of application in scientific research, as well as in supporting reproduction, maintaining genetic diversity and breeding of animals possessing the preset properties [1-3]. By now, there has been a significant advancement in development of IVP technology; the usefulness of embryos developed in vitro, however, is still considerably lower than of those developed in vivo. Moreover, many issues relating to the vitality of the new breed are still unsolved [4, 5].

Standard IVP technology involves several phases, the first of which is in vitro ovum maturation. In immature oocytes, in response to extraction from a follicle and placement in culture medium, meiosis restarts and nuclear transfor-

mations from diplotene stage to metaphase II stage (MII) commence. Oocytes also undergo various structural and molecular transformations at cytoplasm level (cytoplasmic maturation) which are necessary to prepare an oocyte for fertilization and further embryonic development [6]. As is commonly known, when the oocytes restart meiosis in vitro, the same processes of nucleus maturation as in oocytes in vivo occur in them, while the level of cytoplasm maturity remains insufficient, affecting the quality of ova and, after their fertilization, the quality of IVP embryos. The attempts to resolve that issue, despite persistent effort of the researches, have still not yielded a desirable result. The conditions in which oocytes mature remain suboptimal and require further simulations [4, 7, 8].

Most times, one-step culture in TC-199 medium supplemented with fetal bovine serum (FBS) and gonadotropic hormones is used for in vitro maturation of cow oocytes. It was demonstrated that in such conditions, the average of 80% of immature oocytes reach metaphase II of the second meiotic division [9] but only 15-40% develop to the blastocyst stage [10, 11]. Low production of embryos may be caused by a combination of factors. First, one-step culture omits the temporal mismatch (asynchrony) between nuclear and cytoplasmic transformations. Second, TC-199 is a complex medium developed for culturing somatic cells, and although the serum contained in it serves as a source of growth factors, amino acids and endotoxins [12], which is important for maturity level, it is a variable component that can be the cause of reduction in the number of IVP embryos [13].

Earlier, for the first time we demonstrated that two-step culture of cow oocytes, when the standard system is used at the first step and is followed by oocytes being moved to a fresh medium free of gonadotropic hormones, can increase the number of nuclei in embryos at the blastocyst stage [14]. Such improvement could be caused by corresponding cytoplasmic transformations occurring during the final stage of maturing, on which the oocytes' capacity for further embryonic development ultimately depends. We have assumed that two-step culture in media differing in composition at the initial and final stages of oocyte maturation may be an alternative (differentiated) approach in the methodology of in vitro embryo production, and that further research may be required to improve its efficacy [14].

In this paper we have for the first time assessed the expediency of use during the second maturation stage of Fert-TALP [15], which, unlike TC-199, is a less complex serum-free solution. In addition, Fert-TALP medium is further used to fertilize the mature ova, thus its use during the period of maturing may reduce the stress arising in oocytes upon forced change in culture conditions when transitioning from maturing stage to in vitro fertilization.

The purpose of this paper is studying the state of chromosomes and degree of apoptotic generation in oocytes maturing in two different two-step culture systems and the assessment of effect of these conditions on development and quality of IVP embryos.

Techniques. In all experiments, except for the expressly stated cases, the Sigma-Aldrich (USA) reagents were used.

Cow ovaries (*Bos taurus taurus*) collected after slaughtering, were delivered to the laboratory within 3-5 hours at 30-35 °C, were freed of adjacent tissue and washed multiple times in a sterile physiological solution containing antibiotics (100 MU/ml penicillin, 50 μg/ml streptomycin). Oocyte-cumulus cells complexes (OCC) were isolated from the ovaries by dissecting the follicle walls with a blade, flushed 3 times in TC-199 medium containing 5% of fetal bovine serum (FBS), heparin (10 μg/ml), sodium pyruvate (0.2 mM) and gentamicin (50 μg/ml), and morphologically studied. For further culture, orbed oocytes with

homogenous cytoplasm, regular-width pellucid zone surrounded by multilayer compact cumulus were selected. All oocyte manipulations were performed under SMZ stereomicroscope (Nikon, Japan) at 37 °C.

After selection, OCC were cultured in groups, 20-30 each, in 500 μl medium at 38.5 °C in the atmosphere containing 5% CO_2 at 90% humidity. To produce mature oocytes, a two-step system was used. For the first 16 hours, OCC were cultured in TC-199 medium containing 10% FBS, sodium pyruvate (1 mM), gentamicin (50 μg/ml), follicle-stimulating hormones (FSH) (10 μg/ml) and luteinizing hormones (LH) (10 μg/ml) (standard maturation medium), whereafter they were moved to a fresh medium for another 8 hours. During the second maturation stage, TC-199 containing 10% FBS, sodium pyruvate (1 mM) and gentamicin (50 μg/ml), or a medium for further fertilizing Fert-TALP [15] that contained 114 mM NaCl, 3.2 mM KCl, 0.4 mM NaH_2PO_4, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 10 mM sodium lactate, 0.25 mM sodium pyruvate, bovine serum albumin (BSA, 6 mg/ml) and gentamicin (50 μg/ml).

After 24 hours of maturing, a portion of oocytes was freed from cumulus cells, fixed and used for cytological analysis of maturity and apoptosis as per the protocol described [16]. Another portion was moved to Fert-TALP medium supplemented with BSA (6 mg/ml), heparin (10 μg/ml), penicillamine (20 μM), hypotaurine (10 μM) and epinephrine (1 μM) for in vitro fertilization and assessment of capacity for further embryonic development.

Then, 1.5 hours prior to fertilization of oocytes, the straws containing frozen semen were unfrozen, active spermatozoa were produced by "swim-up" method in Sperm-TALP medium [17] containing sodium pyruvate (1mM) and BSA (6 mg/ml). For this purpose, the contents of straws were underlaid, in 220 μl portions, to the 1.8 ml test tubes (Nunc, Denmark) with 1 ml of Sperm-TALP medium and were incubated (a MCO-18AIC, Sanyo, Japan) for 50 minutes. In the end of incubation, 750 μl of the upper layer was collected, diluted with fresh medium and centrifuged at 300 g for 10 minutes. The resulting pellet containing motile sperm were added to the fertilization medium containing OCC (spermatozoa concentration: 1.5×10^6/ml).

Gamets were incubated jointly for 18-20 hours, whereafter the prospective zygotes were moved to the embryo-development medium [14] and cultured for 7 days. On day 2 after fertilization of oocytes cleaved zygotes were morphologically analyzed, and in the end of culturing the number of embryos that have developed to the stage of blastocyst was counted.

The resulting blastocysts were fixed with 4% paraformaldehyde solution and subjected to permeabilization in Triton X-100 solution. The extent of apoptotic changes of nuclear material in embryos was determined by TUNEL method using the In Situ Cell Death Detection Kit, fluorescein (Roche Diagnostics, Switzerland) in accordance with the instructions of the manufacturer. The embryos were then stained with DAPI solution (1 μg/ml) for localizing the nuclei, and transferred to the glass slide for analysis. Microphotography and evaluation of preparations was carried out under Axio Imager.M2 fluorescent microscope equipped with 65 HE filter (for TUNEL, excitation at λ = 445-470 nm) and 49 (for DAPI, excitation at λ = 365 nm) using digital camera Axiocam 506 and ZEN 2 pro application (Carl Zeiss, Germany). The apoptosis rate in the embryos was evaluated by the TUNEL-positive nuclei percentage of the total number of nuclei.

In statistical processing, one-way analysis of variance was applied (SigmaStat application by Systat Software, Inc., USA). The results are given as means (M) and standard errors of the means (±SEM). To evaluate the significance of

differences between the compared means, Tukey's test was used

Results. The medium surrounding oocytes in vitro critically affect the quality of ova, which makes these conditions a subject of targeting. In the paper presented, in order to induce the maturation of cow oocytes, instead of standard one-step IVM protocol we used two-step oocyte cultivation that implied their maturation for the first 16 hours in TC-199 medium containing 10% FBS and gonadotropic hormones and for the next 8 hours either in TC-199 free of hormones (system 1) or in Fert-TALP medium [15] (system 2). The time of changing the medium of 16 hours was selected on the basis of previous data on the initial shows of deterioration of oocyte quality occurring by that period of their in vitro maturing [18].

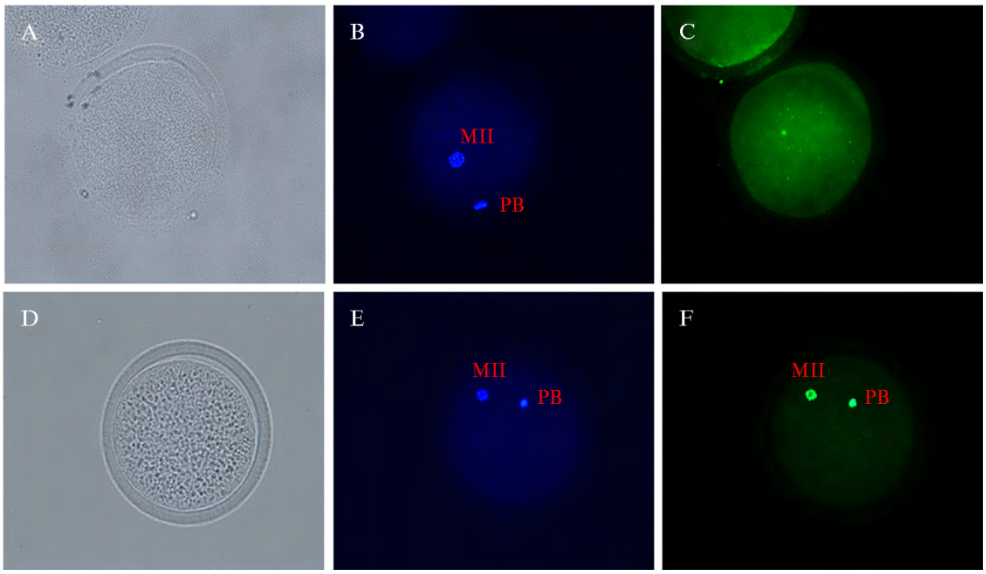

Fig. 1. Microphotographs of cytological preparations of mature cow oocytes (*Bos taurus taurus*) after 24 hrs of in vitro maturing: A, D — oocyte morphology, B, E — staining nuclear material using DAPI (blue color), MII — chromosomes at the stage of metaphase of the second meiotic division, PB — polar body; C, F — staining nuclear material using TUNEL method, upper section (C) — oocytes without any shows of apoptosis, lower section (F) — with shows of apoptosis (TUNEL-positive MII chromosomes and PB are stain green). ×400 zoom, fluorescent microscope Axio Imager.M2 equipped with 65 HE filter (for TUNEL, excitation at λ = 445-470 nm) and 49 (for DAPI, excitation at λ = 365 nm) with color digital camera Axiocam 506 (Carl Zeiss, Germany).

1. State of chromosomes and frequency of apoptotic degeneration in cow oocytes (*Bos taurus taurus*) after maturing for 8 hours in different culture systems (*M*±SEM)

Test group (system)	Count		Percentage	
	experiments	total oocytes	oocytes at MII stage	MII oocytes with shows of apoptosis
System 1	4	85	83.3±5.6	19.4±1.1
System 2	4	83	84.7±2.4	11.7±0.7*

Note. MII — metaphase II; system 1: oocyte maturing for the first 16 hours in TC-199 medium containing 10 % fetal bovine serum (FBS) and follicle-stimulating hormones (FSH) and luteinizing hormones (LH), and for the next 8 hours in TC-199 medium free of hormones; system 2: oocyte maturing for 16 hours in conditions similar to those of system 1 and for the next 8 hours in Fert-TALP fertilization medium free of heparin, hypotaurine and epinephrine.

* Differences between the compared groups are statistically significant at $p < 0.05$.

Cytological analysis did not reveal any impact of the culture system on completion of nuclear maturation. Percentage of oocytes at MII meiosis stage (Fig. 1, B-E) after 24 hours of maturing (16 + 8 hrs) was similar in both groups and made 83.3 and 84.7 % (Table 1). At the same time, the percentage of oocytes with the shows of apoptosis (see Fig. 1, F) was lower (11.7±0.7 %) when

maturing in Fert-TALP medium, while for those cultured in TC-199 containing FBS this value increased by 7.7% ($p < 0.05$) (Table 1).

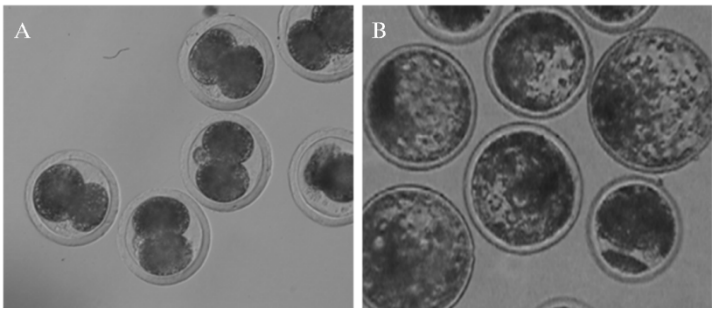

Fig. 2. Microphotographs of cow oocytes (*Bos taurus taurus*) cleaved after in vitro fertilization and of embryos developed to the blastocyst stage (B) (×200 zoom, Eclipse Ti-U microscope by Nikon, Japan).

Capacity of mature oocytes for development after in vitro fertilization that serves a criterion of the level of cytoplasmic maturity was evaluated by the oocytes' capability to the first cleavage division (Fig. 2, A) and reach blastocyst stage (see Fig. 2, B) (Table 2).

2. Cow oocyte (*Bos taurus taurus*) embryonic developmental capability in different systems for in vitro maturing (*M*±SEM)

Test group (system)	Count		Oocyte cleavage rate, %	Development to blastocyst stage, %	
	experiments	total oocytes		of number of oocytes	of number of embryos
System 1	5	150	65.4±1.1	17.4±0.4	26.6±0.9
System 2	5	123	71.7±1.7	30.0±2.9*	42.1±4.9**

N o t e. For experiment design (media and systems) see Table 1.
*, ** Differences between the compared groups are statistically significant at $p < 0.05$ and $p < 0.01$.

The percentage of cleaved oocytes determined on the 2nd day of cultivating did not vary between the experimental groups and made 65.4±1.1 and 71.7±1.7 % for system 1 and 2, respectively. Nevertheless, the impact of conditions of the second step of oocyte maturing (8 hours) on their development to blastocyst stage was found. In case of transfer of OCC after 16 hours of cultivating to TC-199 medium, the production of blastocysts was 17.4±0.4 %. Use during the relevant period of cultivation of Fert-TALP medium increased this value up to 30.0±2.9 % ($p < 0.05$) (see Table 2).

Use of two compared protocols of two-step oocyte culture did not significantly change the quality of IVP embryos that were assessed by the nuclei count on day 7 after fertilization (Fig. 3, C), however when culturing during the second stage of maturing in Fert-TALP medium, the tendency towards increase of this value was observed (Table 3). The percentage of embryonic nuclei with shows of apoptosis (see Fig. 3, C) did not also change between experimental groups and made 5.1±0.9 and 4.6±0.4 % for system 1 and system 2 respectively (see Table 3).

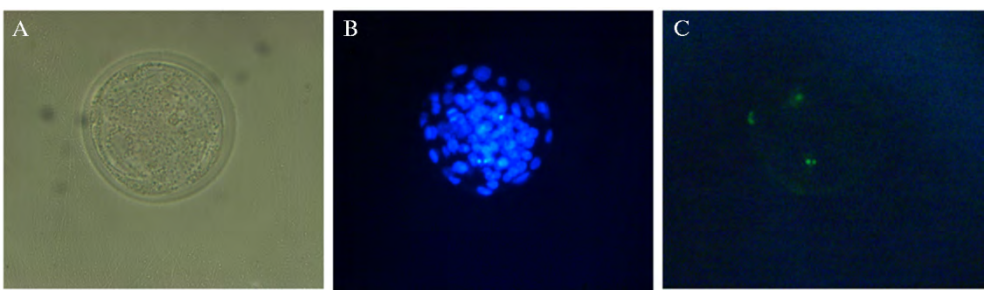

Fig. 3. Microphotographs of cytological preparations of bovine embryos (*Bos taurus taurus*) on day 7 of culture: A — blastocyst morphology; B — staining blastocyst nuclei using DAPI (blue color); C — staining apoptotic nuclei in blastocyst using TUNEL method (TUNEL-positive nuclei are stained green). ×200 zoom, Axio Imager.M2 fluorescent microscope by Carl Zeiss, Germany.

3. Quality of IVP (in vitro embryo production) **embryos after 8 hours maturing of cow oocytes (*Bos taurus taurus*) in different culture system followed by in vitro fertilization** (*M*±SEM)

Maturing medium (system)	Experiments	Total blastocyst count	Blastocysts nuclei count	
			total	apoptotic, %
TCM + 10 % FBS (1)	5	26	62.9±5,2	5,1±0,9
Fert-TALP (2)	5	37	72,2±5,1	4,6±0,4

N o t e. For experiment design (media and systems) see Table 1.

The expediency of application of two-step bovine IVM protocol has been actively studied over the past few years. In order to resolve the problem of asynchrony between nuclear and cytoplasmic maturation, OCC extracted from follicles are for some time cultured in maturation medium supplemented by meiosis inhibitors, and then without them [19]. However, cytoplasmic transformations, on which the oocytes developmental capability ultimately depends, occurs not on the initial but on the final stage of IVM, which demonstrates the relevancy of research of the specific needs of female gametes during this very period [4].

Therefore, this work for the first time compares two-step IVM protocols that include cow oocyte maturation during the first step in the standard medium and maturation in one of two hormone-free media (TC-199 and Fert-TALP) during the final step. Although TC-199 medium containing FBS is commonly used in the majority of one-step IVM protocols and is capable of sustaining the high ova capability for further development [15], in our two-step system during the final maturation stage it turned out to be less effective in terms of embryo production than Fert-TALP medium. Moreover, its use in the similar conditions has adversely affected the apoptotic degradation in ova. The cause of such effect could be not only the fact that TC-199 medium, unlike Fert-TALP, is a complex solution, but also the fact that it is blood serum instead of BSA that is used there as a source of protein. It is believed that adding the serum results in uncertain and changing conditions of cell cultivation [11, 13, 20, 21]. In addition, it is shown that adding the serum to the embryo cultivation medium negatively affects their development at early cleavage stages and alters the ultrastructure and the nature of gene expression during the later stages of in vitro embryo development [22-24].

Thus, in two-step in vitro maturation (IVM), culturing oocytes during the second-step maturation (8 hrs) in Fert-TALP medium is more preferable than in TC-199 medium containing fetal bovine serum. Such conditions improve the quality of mature oocytes and their embryonic developmental capability after in vitro fertilization.

REFERENCES

1. Lonergan P., Fair T. In vitro-produced bovine embryos: dealing with the warts. *Theriogenology*, 2008, 69: 17-22 (doi: 10.1016/j.theriogenology.2007.09.007).
2. Galli C., Duchi R., Crotti G., Turini P., Ponderato N., Colleoni S., Lagutina I., Lazzari G. Bovine embryo technologies. *Theriogenology*, 2003, 59(2): 599-616 (doi: 10.1016/S0093-691X(02)01243-8).
3. Ponsart S., Le Bourhis D., Knijn H., Fritz S., Guyader-Joly C., Ottern T., Lacaze S., Charreaux F., Schibler L., Dupassieux D., Mullaart E. Reproductive technologies and genomic selection in dairy cattle. *Reproduction, Fertility and Development*, 2013, 26(1): 12-21 (doi: 10.1071/RD13328).
4. Blanco M.R., Demyda Peyrõs S., Moreno M.M., Genero E. Developmental competence of in vivo and in vitro matured oocytes: a review. *Biotechnology and Molecular Biology Review*, 2011, 6: 155-165.
5. Nagano M. Acquisition of developmental competence and in vitro growth culture of bovine oocytes. *Journal of Reproduction and Development*, 2019, 65(3): 195-201 (doi: 10.1262/jrd.2019-022).
6. Hyttel P., Xu K.P., Smith S., Greve T. Ultrastructure of in-vitro oocyte maturation in cattle. *J. Reprod. Fertil.*, 1986, 78: 615-625 (doi: 10.1530/jrf.0.0780615).
7. Abd El-Aziz A.H., Mahrous U.E., Kamel S.Z., Sabek A.A. Factors influencing in vitro production of bovine embryos: a review. *Asian Journal of Animal and Veterinary Advances*, 2016, 11: 737-756 (doi: 10.3923/ajava.2016.737.756).
8. Niemann H., Wrenzycki C. Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent de-

velopment. *Theriogenology*, 2000, 53: 21-34 (doi: 10.1016/s0093-691x(99)00237-x).
9. Dominko T., First N.L. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. *Molecular Reproduction and Development*, 1997, 47: 456-467 (doi: 10.1002/(SICI)1098-2795(199708)47:4<456::AID-MRD13>3.0.CO;2-U).
10. Ward F., Enright B., Rizos D., Boland M., Lonergan P. Optimization of in vitro bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. *Theriogenology*, 2002, 57(8): 2105-2117 (doi: 10.1016/s0093-691x(02)00696-9).
11. Russell D.F., Baqir S., Bordignon J., Betts D.H. The impact of oocyte maturation media on early bovine embryonic development. *Molecular Reproduction and Development*, 2006, 73(10): 1255-1270 (doi: 10.1002/mrd.20553).
12. McKiernan S.H., Bavister B.D. Different lots of bovine serum albumin inhibit or stimulate in vitro development of hamster embryos. *In Vitro Cell Dev. Biol. — Animal*, 1992, 28A: 154-156 (doi: 10.1007/bf02631084).
13. Sagirkaya H., Misirlioglu M., Kaya A., First N.L., Parrish J.J., Memili E. Developmental potential of bovine oocytes cultured in different maturation and culture conditions. *Animal Reproduction Science*, 2007, 101: 225-240 (doi: 10.1016/j.anireprosci.2006.09.016).
14. Singina G.N., Lebedeva I.Yu., Shedova E.N., Taradajnic T.E., Mityashova O.S., Tsyndrina E.V., Danch S.S. Bovine oocyte ability to embryonic development when maturing in different two-phase culture systems. *Agricultural Biology* [*Sel'skokhozzyaistvannaya Biologiya*], 2017, 52(4): 776-784 (doi: 10.15389/agrobiology.2017.4.776eng).
15. Parrish J.J. Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. *Theriogenology*, 2014, 81(1): 67-73 (doi: 10.1016/j.theriogenology.2013.08.005).
16. Lebedeva I.Yu., Singina G.N., Shedova E.N., Lopukhov A.V., Mityashova O.S. Resistence of bovine oocytes to age-realated changes after exposure to luteotropic factors during the second phase of culture till the metaphase II stage. *Agricultural Biology* [*Sel'skokhozzyaistvannaya Biologiya*], 2018, 53(6): 1202-1211 (doi: 10.15389/agrobiology.2018.6.1202eng).
17. Singina G.N., Bagirov V.A., Danch S.S., Taradainik T.E., Dotsev A.V., Zinovieva N.A. In vitro production of interspecies hybrid embryos of cattle (*Bos taurus*) and wisent (*Bison bonasus*). *Agricultural Biology* [*Sel'skokhozzyaistvannaya Biologiya*], 2016, 51(6): 824-829 (doi:10.15389/agrobiology.2016.6.824eng).
18. Lebedeva I.Yu., Singina G.N., Lopukhov A.V., Zinovieva N.A. Dynamics of morphofunctional changes in aging bovine ova during prolonged culture in vitro. *Cell and Tissue Biology*, 2014, 8(3): 258-266 (doi: 10.1134/S1990519X14030080).
19. Guemra S., da Silva Santo E., Zanin R., Monzani P.S., Sovernigo T.C., Ohashi O.M., Verde Leal C.L., Adona P.R. Effect of temporary meiosis block during prematuration of bovine cumulus-oocyte complexes on pregnancy rates in a commercial setting for in vitro embryo production. *Theriogenology*, 2014, 81(7): 982-987 (doi: 10.1016/j.theriogenology.2014.01.026).
20. Salhab M., Tosca L., Cabau C., Papillier P., Perreau C., Dupont J, Mermillod P., Uzbekova S. Kinetics of gene expression and signaling in bovine cumulus cells throughout IVM in different mediums in relation to oocyte developmental competence, cumulus apoptosis and progesterone secretion. *Theriogenology*, 2011, 75(1): 90-104 (doi: 10.1016/j.theriogenology.2010.07.014).
21. Mastromonaco G.F., Semple E., Robert C., Rho G.J., Betts D.H., King W.A. Different culture media requirements of IVF and nuclear transfer bovine embryos. *Reproduction in Domestic Animals*, 2004, 39: 462-467 (doi: 10.1111/j.1439-0531.2004.00548.x).
22. Lonergan P., Kearney-Flynn M.O., Boland M.P. Effect of protein supplementation and presence of an antioxidant on the development of bovine zygotes in synthetic oviduct fluid medium under high or low oxygen tension. *Theriogenology*, 1999, 51: 1565-1576 (doi: 10.1016/s0093-691x(99)00099-0).
23. Abe H., Yamashita S., Itoh T., Satoh T., Hoshi H. Ultrastructure of bovine embryos developed from in vitro-matured and fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. *Molecular Reproduction and Development*, 1999, 53: 325-335 (doi: 10.1002/(SICI)1098-2795(199907)53:3<325::AID-MRD8>3.0.CO;2-T).
24. Wrenzycki C., Herrmann D., Carnwath J.W., Niemann H. Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. *Molecular Reproduction and Development*, 1999, 53: 8-18 (doi: 10.1002/(SICI)1098-2795(199905)53:1<8::AID-MRD2>3.0.CO;2-K).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1214-1224
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 619+616.5]:57.085.23

doi: 10.15389/agrobiology.2019.6.1214eng
doi: 10.15389/agrobiology.2019.6.1214rus

MAINTENANCE OF MULTIPOTENT MESENCHYMAL STEM CELLS OF FARM ANIMALS IN CRYOGELS BASED ON NATURALLY-DERIVED POLYMERS

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The authors declare no conflict of interests
Acknowledgements:
This work was performed as part of research work No. 0578-2018-0006 "Creation of new cell systems with desired properties based on mammalian stem cells, including farm animals for veterinary medicine, virology and biotechnology".
Received August 2, 2019

Abstract

Multipotent mesenchymal stem cells (MMSCs) of farm animals, whose growth in culture is determined by attachment to a solid substrate, are promising cellular material for veterinary medicine and biotechnology, as well as virology. One of the methods to overcome cell adhesion in suspension bioreactors in order to obtain a large number of cells with permanent properties of acceptable quality is the use of porous carriers formed from polymers of natural origin. Thus, for the first time we obtained data that allow us to make a scientific substantiation of the parameters for the cultivation of adhesive cultures of animal MMSCs using spatial protein-based cryogel carriers for subsequent suspension cultivation of the obtained constructs. The purpose of the work is to study the possibility to culture MMSC of farm animals in three-dimensional matrix sponges, i.e. the cryogels based on gelatin, blood plasma total protein and fetal bovine serum (FBS). MMSCs isolated from bovine bone marrow (BM) and adipose tissue (AT) and ovine BM, as well as mouse fibroblast STO cell line were used. We found that the optimal cell concentration for the settlement of the cell suspension by the method of natural absorption with swelling of squeezed sponges (0.24 cm^3 in volume) is 1.0×10^6 cells per 100 μl of medium for 2 hrs of saturation. The loading efficiency of MMSCs in sponge scaffolds is 98 %. The analysis of histological slices (at least 10 per sample) of three cryogels demonstrated the ability of all three-dimensional porous scaffolds to maintain cell culture for 14 days. Sponges were filled with cells that preserved morphology and proliferated in places of attachment to the polymer surface. The results of experiments on the effect of the matrix material on cell migration showed that all cells migrate from the monolayer in the volume of cryogel from the bottom and are not detected on the upper sides of the cryogels under study. On day 10 of culture, fibroblast STO cell line were detected in the volume of sponge scaffolds based on gelatin, blood plasma and FBS protein at a distance of 2990, 2871 and 1930 μm, respectively. MMSCs isolated from bovine AT migrated into the porous structure of matrix sponges to a depth of 607, 1364 and 657 μm, respectively. Expansion of MMSCs isolated from bovine and ovine BM in cryogels on the basis of different materials did not differ significantly from the migration of AT-MMSCs. The ability of farm animals' MMSCs on the early passages (2 to 3) and late passages (9 to 10) to attach to macroporous cryogels was not significantly different. Comparative analysis of the results of the experiments obtained in three replicates showed that the macroporous matrices based on gelatin, bovine blood plasma proteins and FBS support the viability of MMSCs during short-term culture, promote cellular adhesion, proliferation and migration. The obtained data allow us to predict the use of these cryogels as matrices for MMSCs of farm animals for research and practical use.

Keywords: multipotent mesenchymal stem cells, adipose tissue, bone marrow, farm animals, adhesion, migration, viability, cryogels, gelatin, bovine protein of blood plasma and serum-

derived scaffolds

The multipotent mesenchymal stem cells (MMSCs) capable of self-renewal and having the potential for adipogenic, osteogenic and chondrogenic differentiation in vitro are promising for veterinary medicine, cell and tissue engineering, virology and drug screening [1, 2]. The fundamental criterion for using the MMSCs' potential is reproducible and low-cost production of a sufficient amount of cells of consistently high quality. Common way of MMSCs production is adhesive culture in the presence of serum. However, current adhesive methods cannot ensure a suitable culture because of the dissimilar conditions that leads to changing the cells quality between batches. Besides, the methodology is laborious and cell yield is limited by substrate area for their growing that leads to limitations in scalability [3-5]. Microcarriers used in suspension bioreactors allow cell adhesion problem to be overcome [6-8]. Such systems need the improvement of the matrixes and adaptation of the cells culture parameters [9].

Porous gels are a promising substrate for matrixes in cell culture owing to their ability to imitate the main properties of most soft tissues. The cross-linked polymer chains detain a large amount of water, they facilitate the transportation of oxygen, nutrients, metabolites and soluble factors. Many of gels can be formed under conditions favorable for living cells, and can easily be modified for bestowing the desired physical and mechanical properties and the rate of degradation. Using natural biopolymers in bioengineered scaffolds makes it possible to most exactly imitate the structure, properties of tissues and organs, as well as to reproduce the microenvironment with the structure similar to that of natural cell niches. This provides optimal artificial niches for MMSCs population and contributes to their almost complete differentiation into the desired cell types [10].

In bioengineered structures, the pores inside the three-dimensional carrier must be interconnected, have the dimensions optimal for cells and sufficient area for their growth. The macroporous cryogels, formed in slightly frozen medium, meet these requirements [11]. They have some specific features compared to ordinary gels formed at the temperatures above the crystallization point of the solvent. Cryogels are characterized by macroporosity, and their macropores are interconnected [12, 13]. Depending on the initial concentration of components, their properties and regimes of cryogenic treatment, it is possible to obtain the macroporous matrixes with the pores with the cross section from tenths to 10 μm and supermacroporous (sponge) systems with the pores of tens and hundreds of micrometers. The cryogels based on materials of natural origin, which are the components of the extracellular matrix (ECM), for example, collagen, gelatin, etc., are of particular interest [14-17].

In a series of experiments, it was demonstrated that the protein-based cryogels (serum albumin, total blood serum protein) owing to their macroporous morphology can be used as porous substrates for cells cultivation [18, 19]. The main criteria of a biocompatible matrix must be the absence of cytotoxicity, maintenance of adhesion, migration, proliferation or the differentiation of the cells on its surface [20-22], as well as the mechanical strength and bioresorbability optimal for further use [23].

In this report, for the first time we present the data substantiating parameters of animal MMSCs adhesive cultures with bulky protein-based cryogel carriers for suspension culturing the obtained structures in a bioreactor.

Our objective was to assess suitability of cryogels based on gelatin, total blood plasma protein and blood serum of cattle fetuses culture as three-dimensional sponge matrixes for cell culture of mesenchymal stem cells of farm

animals.

Techniques. The experiments were carried out (2016-2019) with MMSCs from cattle bone marrow (BM) and adipose tissue (AT) isolated and characterized by us previously [24], from sheep BM [25], and with mouse fibroblasts of the STO line have been used.

For cells culture, the materials and reagents produced by the PanEco company (Russia) were used. The MMSCs were cultured in DMEM with the low glucose concentration (1 g/l), 10% fetal bovine serum (FBS) (GE Healthcare, USA) and 1× nonessential amino acids and antibiotics. The fibroblasts of the STO line were cultured in DMEM with 4 g/l glucose, 10% FBS, 2 mM L-glutamine; 50 μg/ml streptomycin, 50 U/ml penicillin (final concentrations). The cells were cultured at 37 °C in humidified atmosphere with 5% CO_2. For the long-time culture the MMSCs (5×10^3 cells/cm^2) were subcultured.

The porous matrixes for cells culturing were gelatin-based cryogels produced as previously published [26], and cryogels based on cattle total plasma protein or blood serum protein [27, 28].

Matrix sterilization procedure was as follows. The samples were placed in the wells of the sterile 24-well plate (Nunc, Denmark) and covered with ethyl alcohol for 1 hour. The alcohol was decanted, and the samples were washed three times with the Hanks' solution by adding 2 ml into each well. Then 1.5 ml of DMEM was added into wells and the plate was allowed overnight in an incubator for the complete removal of the ethyl alcohol. Before adding the cells, the cryogel was squeezed out with tweezers, and 100 μl of the cell suspension containing 1×10^6 cells was applied to the upper surface of the squeezed out matrix. Due to the rapid swelling of the sponge material, the cell suspension was pulled by capillary forces into the pore inner space and filled the carrier. The sponges were saturated with the cells for 2 hours in the Petri dish preventing the cells adhesion to the plastic at 37 °C in the CO_2 incubator, followed by transfer into 50 ml tubes with untreated surface and gas-tight covers, which simulated a minibioreactor.

Cattle and sheep MMSCs immobilization on the polymer matrixe pore surface was assessed by counting of the non-adsorbed cells. The efficiency of cell seeding was calculated as the difference from subtraction (cell number in the initial suspension minus cell number after immobilization) divided by the initial cell number.

The express analysis of the localization of the viable cells in the three-dimensional matrixes цфы performed using the fluorescein diacetate stain (FD) (Thermo Fisher Scientific, USA). FD was added to the culture medium at a final concentration of 25 μg/ml and incubated for 5 min at 37 °C. Then the sponges with cells were washed with the serum-free medium and examined (a fluorescence microscope, Carl Zeiss, Germany) at the fluorescence excitation wavelength of $\lambda = 450$ nm with closing filter G 247.

For studying cells migration ability, the MMSCs and STOs were seeded into the 24-well plates and grown up to a monolayer. The square 10×10 mm pieces of the cryogel cut out aseptically and washed with DMEM were placed on the surface of the cells monolayer with slightly pressing and cultured under the standard CO_2 conditions in the incubator for 10 days. Then the cryogels were washed with the Dulbecco's phosphate-buffered saline with the Ca^{2+} and Mg^{2+} ions (PBS-1) and fixed in the solution of 4% paraformaldehyde in PBS-1. At least 10 areas from the lower and upper sides of the cryogel, as well as the cross sections of the investigated matrixes were analyzed.

The cell analysis in the three-dimensional matrixes was performed using standard techniques for histological studies [29]. To prepare sections (a Mikrom

HM 525 cryotome, Thermo Scientific, Germany; fixation on the cryotome specimen stage with Neg-50 mounting medium, Thermo Fisher Scientific, USA), specimens were frozen at –15...–22 °C for 30-40 minutes, and 10-20 μm slices were made with one-off blades Microm Sec35e (Thermo Fisher Scientific, USA). The slices were mounted on Surgipath X-tra Adhesive slides with the adhesive coating (Leica Biosystems, Germany). Immediately after drying, they were fixed with methyl alcohol, washed and stained with hematoxylin and eosin or Giemsa stain (PanEco, Russia) according to the manufacturer's instructions. After the clarification in xylene, the stained preparations were embedded into the mounting medium. The morphological analysis was performed visually using the Axio Observer D.1 phase-contrast microscope (Carl Zeiss, Germany) with AxioVision Rel. 4.8 software (Carl Zeiss, Germany) for measurements.

The statistical processing was performed using the GraphPad Prism software (GraphPad Software, USA). The tables show the mean arithmetic values (*M*) and their standard errors (±SEM). The significance of the differences was assessed according to the Student's *t*-test at $p < 0.05$.

Results. The macroporous cryogels (sponges) based on the A type commercial gelatin were white-transparent 0.2-0.7 cm thick round disks 2.2-3.5 cm in a diameter (see Fig. 1, A) with the pore sizes from 50 to 100 microns. The cryogels from bovine blood plasma total protein and FBS had the similar cylindrical shape with the diameter of 1 cm and thickness of 0.6-1 cm (see Fig. 1, B, C).

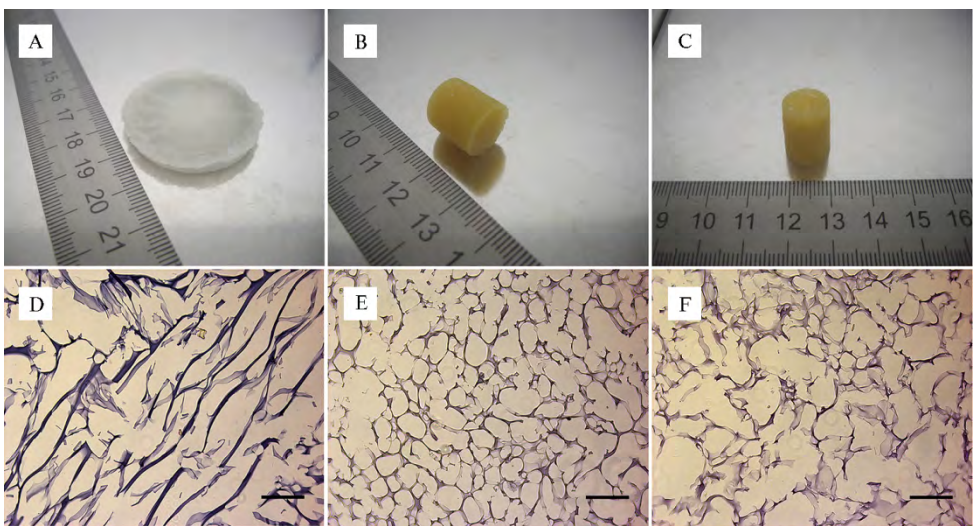

Fig. 1. Macro- and microstructure of the spongy cryogels based on gelatin (A, D)**, proteins of bovine blood plasma** (B, E) **and serum of the blood of cows' fetuses** (C, F). Giemsa staining, scale 500 μm (Axio Observer D.1, Carl Zeiss, Germany).

Cryogels have a system of branched interconnected pores ranging from 20 to 200 μm, which are resulted from formation of ice crystals in a frozen medium [11]. In our work, we found the 10 μm thick slices obtained at –20...–22 °C to be more suitable for histological analysis of cell distribution in cryogels. If a sponge has the greater porosity, the thickness of 20 microns is suitable for better visualization.

Histological analysis showed a macroporous structure of the matrixes, their strength and long-time stability during incubation in CO_2 (see Fig. 1, D-F). The gelatin-based sponges had interconnected large pores, 80-130 microns. The distinctive feature of such matrixes was the gradient changing of a pore size from the periphery toward the interior part as the result of the formation of ice

crystals while freezing of the initial solution of the biopolymer. The matrixes based on the total protein of bovine plasma and of FBS also had a macroporous structure with pores 70-170 microns in a diameter.

Within 24 hours after seeding MMSCs, the protein substrate had no cytotoxic effect on the cells. Our observations are consistent with the data obtained by other authors [13, 28, 30].

1. Efficiency of seeding multipotent mesenchymal stem cells into the gelatin cryogels (0.24 cm^3 matrix, $^1/_4$ part of the cryogel)

Cell counts	Growth medium, μl	Seeding efficiency, %
5×10^5	100	87
5×10^5	500	76
1×10^6	100	98
1×10^6	500	86

Experimental estimates of proper cells concentration and volume of the medium for seeding cells into cryogels have been made using the gelatin matrixes as an example (Table 1). The medium volume of 100 μl turned out to be optimal for saturation of gelatin matrixes. The optimum cells concentration for seeding by natural swelling of squeezed out 0.24 cm^3 sponges was 1.0×10^6 cells/100 μl of the medium during 2-hour saturation. The efficiency of MMSCs seeding into the matrix was 98%. The increase of the medium volume resulted in the loss of cellular material, probably due to an excess amount of the liquid phase. Previously, the dynamic (perfusion) seeding method was used for gelatin cryogels with high efficiency [30].

Cell seeding was performed with a special device which consists of two vessels connected by the flexible plastic tube. The porous carrier was placed into one of the vessels, wherein the porous matrix diameter matched the vessel's inner diameter, and about 100-200 μl of the cell suspension (1.3×10^6 cells/ml) was placed into the second vessel, after that the matrix was slowly saturated with the cells containing the growth medium by force of the gentle back-and-forth motion of the pistons in cylinders. The saturated matrix was allowed for 3 hours in the vessel placed into the CO_2 incubator, thereafter it was moved into the plate wells containing about 1 ml of the growth medium. To seed the cryogels with the cells, the technique of the sponge squeezing after the sterilization was applied. After the squeezing out for the removal of the medium residuals, the carriers quickly and easily restored their shape due to the elasticity of their material, while absorbing the cell suspension applied on their upper surface. Such method of loading the cells into the macroporous spongy matrixes by its efficiency was not inferior than the perfusion method and also was simple in performance.

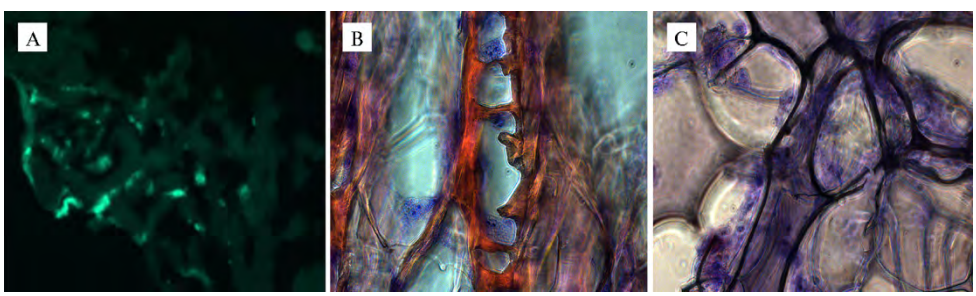

Fig. 2. Large-porous matrixes derived from gelatin (A, B) **and bovine plasma proteins** (C) **with multipotent mesenchymal stem cells from cattle bone marrow attached to the macropore walls.** MMSCs are stained with the fluorescein diacetate (A) and with the Giemsa stain (B, C) (Axio Observer D.1, Carl Zeiss, Germany, zoom ×200 (A) and ×400 (B, C).

The cell viability inside the spongy matrixed in dynamics, on day 7 and day 14 of culture has been studied. The results of the analysis of histological

staining of the slices (at least 10 for each sample) demonstrated the ability of three-dimensional porous matrix to maintain cells in culture for 14 days. These data were confirmed by staining cells with FDA (fluorescein diacetate), a hydrophobic non-fluorescent compound easily penetrating through the cell membrane into cells, where it is metabolized by cell esterases to fluorescein stain. The FDA having the green fluorescence appears only in the cytoplasm of the viable cells which have an intact cytoplasmic membrane, since FDA cannot penetrate through damaged cell membranes.

The histological comparison of slices of three cryogels showed the suitability of both gelatin matrixes and the matrixes based on total protein of bovine blood and of FBS for culturing MMSCs of farm animals. The sponges were filled with the cells which retained their morphology and proligerated at the places of their attachment to the macropore walls (Fig. 2).

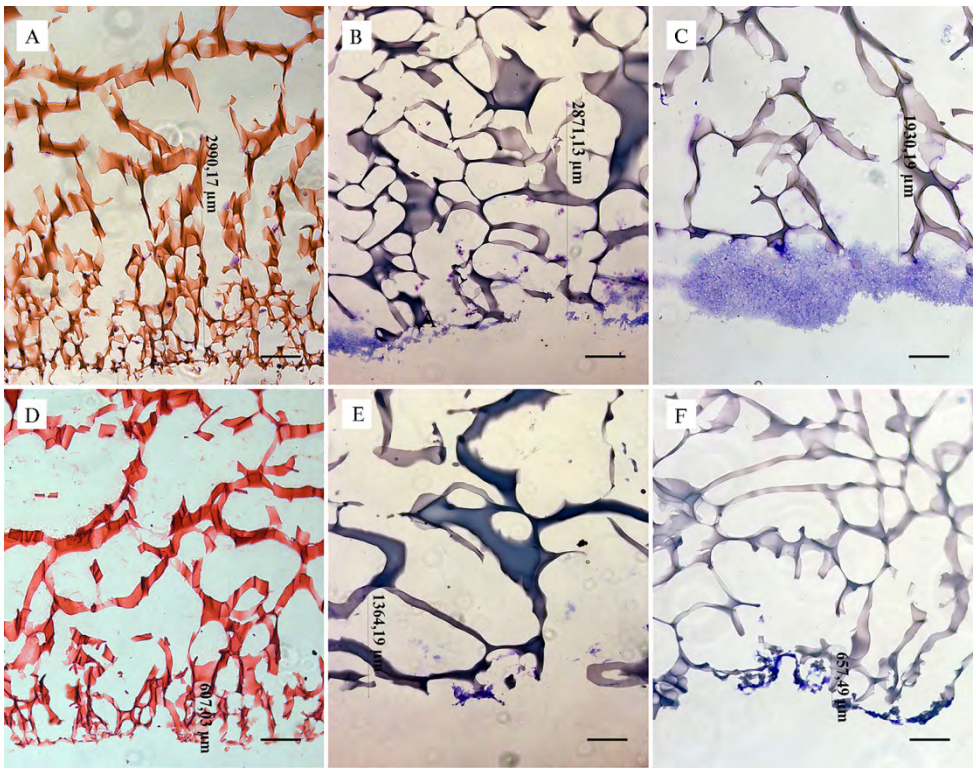

Fig. 3. The depth of cells spreading inside the matrixes on day 10 of culturing: the STO fibroblasts in the matrixes based on gelatin (A), protein of bovine blood plasma (B), FBS (C); the multipotent mesenchymal stem cells from adipose tissue in the matrixes based on gelatin (D), protein of bovine blood plasma (E), FBS (F). Staining with hematoxylin and eosin (A, D), and Giemsa staining (B, C, E, F), scale 500 μm (Axio Observer D.1, Carl Zeiss, Germany).

Histological examination of the effect of the matrix material on the cells migration (Fig. 3) revealed that all cells migrated from the monolayer's lower side into the cryogel depths and were absent on the upper sides of the sponges. The number of cells inside the cryogel was increasing when culturing. Thus, on the day 10, the STO fibroblasts penetrated into the matrixes based on gelatin, blood plasma protein and FDS to the distance of 2,990, 2,871 and 1,930 μm respectively (see Fig. 3, A, B, C). The MMSCs from cattle adipose tissue migrated to a depth of 607, 1,364, and 657 μm (see Fig. 3, D, E, F). MMSCs from bone marrow of cattle and sheep in the studied cryogels did not not differ significantly from migration of the AT MMSCs. The comparative analysis of the results obtained in three repeated experiments showed that the spongy cryogel ma-

tric based on gelatin, proteins of bovine plasma and FBS provided the MMSCs viability during the short-term culturing and promoted the cell adhesion, proliferation and migration. The structure of these cryogels turned out to be biocompatible for both mouse fibroblasts and MMSCs of farm animals. However, in case of this method, the immortalized mouse fibroblasts showed the significantly higher ability of adhesion and migration in all the cryogels.

Earlier, we showed the effect of the long-term culturing of the MMSCs from human AT on the efficiency of cells adhesion to the extracellular matrix proteins. The cells on the passages 2 and 17 of culturing differed in their ability to attach to the surface with previously applied ECM proteins, the fibronectin, collagen, and laminin. The cells attached to fibronectin and collagen on the passage 2 exceeded those on the passage 17 2 and 5 times respectively. At the same time, the number of cells adhering to laminin on the passage 17was 2 times more compared the passage 2 [31].

It was of interest to compare the bovine and sheep MMSCs on the early and late passages by their ability of adhesion to the investigated matrixes. For this, 1×10^6 cells/100 μl medium was layered on the cryogel surfaces. The cells adhesion on the early and late passages of culturing in the macroporous cryogels did not differ significantly (Table 2). This indicates that the bovine and sheep MMSCs retained their adhesive properties during culturing

2. Effect of early and late passages of the bovine and sheep multipotent mesenchymal stem cells on the adhesion to matrixes ($M\pm$SEM, $n = 3$)

Source of the MMSCs	Passage	Counts of cells attached to matrix, $\times10^5$	
		gelatin-based	based on bovine blood plasma protein
Bovine adipose tissue	2	8.0±0.10	7.8±0.13
	9	7.5±0.30	7.9±0.10
Bovine bone marrow	2	9.0±0.20	8.5±0.70
	10	8.7±0.01	8.5±0.23
Sheep bone marrow	3	7.2±0.17	7.0±0.50
	10	6.9±0.70	7.1±0.12

The analysis of the scientific literature showed that gelatin cryogels are used for culturing primary keratinocytes and human fibroblasts [31]. They formed the continuous layer of epithelium on the surface of the obtained in vitro spongy substrates. It was found out that fibroblasts are able to easily migrate deep into the porous structure of these carriers. The preclinical trials on pigs demonstrated the biocompatibility of gelatin cryogels and their nontoxicity to animals' organisms. There is the data on the addition to gelatin of other components which increase the strength of gelatin cryogels, for example, on the introduction into the initial composition of chitosan which contributes to maintaining the cells adhesion and proliferation [32-35].

MMSCs are considered a promising cellular material for the regeneration of animal joints and ligaments [1, 2]. The implantation of cellular preparations based on porous biodegradable matrixes is a method of introducing cells into the organism. Protein-based cryogels are used as porous substrates for tridimensional culturing of MMSCs and subsequent implantation of the obtained samples of certain tissue owing to their macroporous morphology and biodegradability [4, 9]. There is the data on using different cryogels based on gelatin [17], serum albumin [21] and total protein of blood serum [18-20] for this purpose. It was of interest to test cryogels in relation to the development in our laboratory of the method of creating "cultured meat in vitro" [10]. For this, we used a number of protein cryogels which served as the substrates for three-dimensional culturing the MMSCs from bovine and sheep bone marrow and adipose tissue. The re-

sults we got allow us to assume that the serum (plasma) of bovine blood includes the factors enhancing the adhesion and proliferation of MMSCs. As a result of cryotropic formation of gels based on blood plasma proteins, such components are embedded into the matrix of the formed cryogels, which create more favorable conditions for culturing stem cells compared to albumin-based carriers [19].

So, to date, the methods of cell biology including the three-dimensional culturing of mammalian cells is gaining research and practical priority importance in various fields of science and technology. We showed that using the cryogels based on gelatin and proteins of bovine blood plasma and fetal bovine serum makes it possible to adapt the adhesive culture of bovine and sheep multipotent mesenchymal stem cells (MMSCs) to the suspension culturing in a bioreactor. The data we got allow us to forecast the using of these cryogels as the matrixes for large-scale technologies of growing the MMSCs of farm animals.

REFERENCES

1. Savchenkova I.P., Gulyukin M.I. *Veterinariya*, 2011, 7: 3-5 (in Russ.).
2. Rogovaya O.C., Krasnov M.S., Kosovskaya E.V., Kosovskii G.Yu. Mesenchymal stem cells (MSC) as agents, reducing immunogenicity of the graft(review). *Sel'skokhozyaistvennaya Biologiya*, 2011, 2: 15-20 (in Russ.).
3. Panchalingam K.M., Jung S., Rosenberg L., Behie L.A. Bioprocessing strategies for the large-scale production of human mesenchymal stem cells: a review. *Stem Cell Res. Ther.*, 2015, 6: 225 (doi: 10.1186/s13287-015-0228-5).
4. Tavassoli H., Alhosseini S.N., Tay A., Chan P.P.Y., Weng Oh S.K., Warkiani M.E. Large-scale production of stem cells utilizing microcarriers: A biomaterials engineering perspective from academic research to commercialized products. *Biomaterials*, 2018, 181: 333-346 (doi: 10.1016/j.biomaterials.2018.07.016).
5. Grein T.A., Leber J., Blumenstock M., Petry F., Weidner T., Salzig D., Czermak P. Multiphase mixing characteristics in a microcarrier-based stirred tank bioreactor suitable for human mesenchymal stem cell expansion. *Process Biochemistry*, 2016, 51(9): 1109-1119 (doi: 10.1016/j.procbio.2016.05.010).
6. Moloudi R., Oh S., Yang C., Teo K.L., Lam A.T-L., Warkiani M.E., Naing M.W. Inertial-based filtration method for removal of microcarriers from mesenchymal stem cell suspensions. *Scientific Reports*, 2018, 8: 12481 (doi: 10.1038/s41598-018-31019-y).
7. Rafiq Q.A., Ruck S., Hanga M.P., Heathman T.R.J., Coopman K., Nienow A.W., Williams D.J., Hewitt C.J. Qualitative and quantitative demonstration of bead-to-bead transfer with bone marrow-derived human mesenchymal stem cells on microcarriers: utilising the phenomenon to improve culture performance. *Biochemical Engineering Journal*, 2018, 135: 11-21 (doi: 10.1016/j.bej.2017.11.005).
8. Nienow A.W., Hewitt C.J., Heathman T.R.J., Glyn V.A.M., Fonte G.N., Hanga M.P., Coopman K., Rafiq Q.A. Agitation conditions for the culture and detachment of hMSCs from microcarriers in multiple bioreactor platforms. *Biochemical Engineering Journal*, 2016, 108: 24-29 (doi: 10.1016/j.bej.2015.08.003).
9. Li B., Wang X., Wang Y., Gou W., Yuan X., Peng J., Guo Q., Lu S. Past, present, and future of microcarrier-based tissue engineering. *Journal of Orthopaedic Translation*, 2015, 3(2): 51-57 (doi: 10.1016/j.jot.2015.02.003).
10. Volkova I.M., Korovina D.G. Three-dimensional matrixes of natural and synthesic origin for cell biotechnology. *Applied Biochemistry and Microbiology*, 2015, 51(9): 841-856 (doi: 10.1134/S0003683815090082).
11. Lozinskii V.I. *Uspekhi khimii*, 2002, 71(6): 559-585 (doi: 10.1070/RC2002v071n06ABEH000720) (in Russ.).
12. Lozinsky V.I. Polymeric cryogels as a new family of macroporous and supermacroporous materials for biotechnological purposes. *Russ. Chem. Bull.*, 2008, 57(5): 1015-1032 (doi: 10.1007/s11172-008-0131-7).
13. Tikhvinskaya O.A., Rogul'skaya E.Yu., Volkova N.A., Grishchuk V.P., Revenko E.B., Mazur S.P., Lozinskii V.I., Petrenko Yu.A., Petrenko A.Yu. *Problemi kriobiologiï i kriomeditsini*, 2018, 28(1): 044-048 (doi: 10.15407/cryo28.01.044) (in Russ.).
14. Geckil H., Xu F., Zhang X., Moon S., Demirci U. Engineering hydrogels as extracellular matrix mimics. *Nanomedicine*, 2010, 5(3): 469-484 (doi: 10.2217/nnm.10.12).
15. Katsen-Globa F., Meiser I., Petrenko Y.A., Ivanov R.V., Lozinsky V.I., Zimmermann H., Petrenko A.Y. Towards ready-to-use 3-D scaffolds for regenerative medicine: adhesion-based cryopreservation of human mesenchymal stem cells attached and spread within alginate–gelatin cryogel scaffolds. *J. Mater. Sci.: Mater. Med.*, 2014, 25(3): 857-871 (doi: 10.1007/s10856-013-5108-x).

16. Afewerki S., Sheikhi A., Kannan S., Ahadian S., Khademhosseini A. Gelatin-polysaccharide composite scaffolds for 3D cell culture and tissue engineering: towards natural therapeutics. *Bioengineering & Translational Medicine*, 2019, 4(1): 96-115 (doi: 10.1002/btm2.10124).
17. Ghaderi Gandomani M., Sahebghadam Lotfi A., Kordi Tamandani D., Arjmand S., Alizadeh S. The enhancement of differentiating adipose derived mesenchymal stem cells toward hepatocyte like cells using gelatin cryogel scaffold. *Biochemical and Biophysical Research Communications*, 2017, 491(4): 1000-1006 (doi: 10.1016/j.bbrc.2017.07.167).
18. Ferrero-Gutierrez A., Menendez-Menendez Y., Alvarez-Viejo M., Meana A., Otero J. New serum-derived albumin scaffold seeded with adipose-derived stem cells and olfactory ensheathing cells used to treat spinal cord injured rats. *Histol. Histopathol.*, 2013, 28(1): 89-100 (doi: 10.14670/HH-28.89).
19. Gallego L., Junquera L., Meana A., Alvarez-Viejo M., Fresno M. Ectopic bone formation from mandibular osteoblasts cultured in a novel human serum-derived albumin scaffold. *Journal of Biomaterials Applications*, 2010, 25(4): 367-381 (doi: 10.1177/0885328209353643).
20. Peña G.I., Álvarez-Viejo M., Alonso-Montes C., Menéndez-Menéndez Y., Gutiérrez Á.F., de Vicente Rodríguez J.C., Otero Hernández J., Meana Infiestae Á. Regeneration of mandibular defects using adipose tissue mesenchymal stromal cells in combination with human serum-derived scaffolds. *Journal of Cranio-Maxillofacial Surgery*, 2016, 44(9): 1356-1365 (doi: 10.1016/j.jcms.2016.06.012).
21. Gallego L., Junquera L., García E., García V., Álvarez-Viejo M., Costilla S., Fresno M.F., Meana Á. Repair of rat mandibular bone defects by alveolar osteoblasts in a novel plasma-derived albumin scaffold. *Tissue Engineering Part A*, 2010, 16(4): 1179-1187 (doi: 10.1089/ten.TEA.2009.0517).
22. Elowsson L., Kirsebom H., Carmignac V., Mattiasson B., Durbeej M. Evaluation of macroporous blood and plasma scaffolds for skeletal muscle tissue engineering. *Biomaterials Science*, 2013, 4: 402-410 (doi: 10.1039/C2BM00054G).
23. Tibbitt M.W., Anseth K.S. Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol. Bioeng.*, 2009, 103(4): 655-663 (doi: 10.1002/bit.22361).
24. Volkova I.M., Viktorova E.V., Savchenkova I.P., Gulyukin M.I. Characteristic of mesenchymal stem cells, isolated from bone marrow and fatty tissue of cattle. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2012, 2: 32-38 (doi: 10.15389/agrobiology.2012.2.32eng).
25. Korovina D.G., Volkova I.M., Vasil'eva S.A., Gulyukin M.I., Savchenkova I.P. *Tsitologiya*, 2019, 61(1): 35-44 (doi: 10.1134/S0041377119010036) (in Russ.).
26. Lozinsky V.I., Kulakova V.K., Ivanov R.V., Petrenko A.Y., Rogulska O.Y., Petrenko Y.A. Cryostructuring of polymer systems. 47. Preparation of wide porous gelatin-based cryostructurates in sterilizing organic media and assessment of the suitability of thus formed matrices as spongy scaffolds for 3D cell culturing. *e-Polymers*, 2018, 18(2): 175-186 (doi: 10.1515/epoly-2017-0151).
27. Lozinskii V.I., Konstantinova N.R., Solov'eva N.I. *Sposob polucheniya poristogo belkovogo gelya. MPK A23J 3/00. Institut pishchevykh veshchestv RAN (RF). № 2058083. Zayavl. 24.02.1994. Opubl. 20.04.1996. Byul. № 11* [A method of obtaining a porous protein gel. MPK A23J 3/00. Institut pishchevykh veshchestv RAN (RF). № 2058083. Appl. 24.02.1994. Publ. 20.04.1996. Bul. № 11] (in Russ.).
28. Rodionov I.A. *Kriogeli na osnove syvorotochnogo al'bumina: sintez, svoistva, struktura i vozmozhnosti biomeditsinskogo primeneniya. Kandidatskaya dissertatsiya* [Serum albumin-based cryogels: synthesis, properties, structure and biomedical use. PhD Thesis]. Moscow, 2017 (in Russ.).
29. Pirs E. *Gistokhimiya: Teoreticheskaya i prikladnaya* [Theoretical and applied histochemistry]. Moscow, 1962 (in Russ.).
30. Petrenko Yu.A., Ivanov R.V., Lozinskii V.I., Petrenko A.Yu. *Kletochnye tekhnologii v biologii i meditsine*, 2010, 4: 225-228 (in Russ.).
31. Savchenkova I.P., Savchenkova E.A., Gulyukin M.I. *Tsitologiya,* 2017, 59(5): 307-314 (doi: 10.1134/S1990519X17050066) (in Russ.).
32. Allan I.U., Tolhurst B.A., Shevchenko R.V., Dainiak M.B., Illsley M., Ivanov A., Jungvid H., Galaev I.Y., James S.L., Mikhalovsky S.V., James S.E. An in vitro evaluation of fibrinogen and gelatin containing cryogels as dermal regeneration scaffolds. *Biomaterials Science*, 2016, 4(6): 1007-1014 (doi: 10.1039/c6bm00133e).
33. Chen C.H., Kuo C.Y., Wang Y.J., Chen J.P. Dual function of glucosamine in gelatin/hyaluronic acid cryogel to modulate scaffold mechanical properties and to maintain chondrogenic phenotype for cartilage tissue engineering. *Int. J. Mol. Sci.*, 2016, 17(11): 1957 (doi: 10.3390/ijms17111957).
34. Wahl E.A., Fierro F.A., Peavy T.R., Hopfner U., Dye J.F., Machens H.G., Egaña J.T., Schenck T.L. In vitro evaluation of scaffolds for the delivery of mesenchymal stem cells to wounds. *BioMed Research International*, 2015, 2015: 108571 (doi: 10.1155/2015/108571).
35. Kang B.J., Kim Y., Lee S.H., Kim W.H., Woo H.M., Kweon O.K. Collagen I gel promotes homogenous osteogenic differentiation of adipose tissue-derived mesenchymal stem cells in serum-derived albumin scaffold. *Journal of Biomaterials Science, Polymer Edition*, 2013, 24(10): 1233-1243 (doi: 10.1080/09205063.2012.745717).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1225-1235
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Veterinary virology, microbiology

UDC 636.3:619:616.98:578:577.2

doi: 10.15389/agrobiology.2019.6.1225eng
doi: 10.15389/agrobiology.2019.6.1225rus

DEVELOPMENT OF TEST SYSTEMS USING A RECOMBINANT NUCLEOCAPSID VIRAL PROTEIN FOR SERODIAGNOSIS OF PESTE DES PETITS RUMINANTS

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The authors declare no conflict of interests

Acknowledgements:

Supported financially by a subsidy of the Ministry of Science and Higher Education of the Russian Federation No. 0451-2019-0005

Received July 2, 2019

Abstract

Peste des petits ruminants (PPR) is an acute febrile viral disease of small ruminants. In severe cases of PPR, when animals manifest clinical signs, virus-specific antigens can be detected in blood and/or tissue samples. In subclinically infected animals, PPR can only be diagnosed using serological testing. Taking into account their simplicity, high sensitivity and cost-effectiveness, the test systems for carrying out indirect or competitive enzyme-linked immunosorbent assay (c-ELISA) are considered most suitable to be used both for the disease diagnosis and seroepidemiological surveillance. The up-to-date techniques for PPR serodiagnosis are developed on the basis of a recombinant nucleocapsid (N) protein. Various options are being worked out to create test systems for PPR serodiagnosis, in particular, using polyclonal sera against N-protein for c-ELISA or some modifications of indirect ELISA. This work was aimed at studying the characteristics of the components of some ELISA experimental test systems for PPR diagnosis using a recombinant N-protein in indirect ELISA with a protein A peroxidase conjugate or in competitive ELISA using a peroxidase IgG conjugate obtained from polyclonal rabbit sera against the nucleocapsid protein. Owing to immunization of rabbits with the purified recombinant N-protein, sera with titers of 1:512 to 1:1024 in c-ELISA were obtained. The potential of constructing a test system for PPR diagnosis through indirect ELISA, in which the peroxidase protein A conjugate was used to identify the PPR-specific antibodies bound to the antigen, has been demonstrated in experiments with sera from convalescent goats, as well as from rabbits immunized with the N-protein. It is important that the protein A peroxidase conjugate reacts with goat sera antibodies in immune complexes. The antibodies obtained from the blood serum of a rabbit immunized with the purified recombinant N-protein have been shown to react with the same epitopes as the positive goat serum antibodies. To construct c-ELISA test system for PPR serodiagnostics, a peroxidase conjugate was prepared using the IgG isolated from an N-protein specific rabbit serum. The sera from pigs immunized with the purified PPR virus and vaccinated against caprine PPR with the titers ≥ 1:64 as observed in the neutralization test (NT) were positive in c-ELISA in which the components of the experimental test system were used. The obtained results make it possible to positively evaluate the prospect of the developed test systems for PPR diagnostics.

Keywords: peste des petits ruminants, serodiagnosis, ELISA, recombinant nucleocapsid protein

Peste des petits ruminants (PPR) is an acute, contagious and economically significant viral infection of small ruminants (goats, sheep, gazelles, oryxes, white-tailed deer) manifesting the morbidity and mortality rate respectively 100

and 90% [1]. Clinically, the disease resembles the cattle plague and is characterized by severe hyperthermia, necrotic and erosive stomatitis, enteritis and pneumonia [2]. The transmission of the virus occurs through the secretions and excretions of infected animals when the close contacting to them of susceptible animals. Cattle, buffalos and pigs may be infected with the PPR virus through the natural way or experimentally, but in both cases, they become dead-end hosts because the virus cannot transmit from them to other animals [3, 4]. The immune defense of young animals for the period up to 4 months is acquired with the intake of colostral milk [5].

The PPR virus belongs to the *Morbillivirus* genus of the *Paramyxoviridae* family [6-8]. The spread of one (Line 4) of the four known PPR virus lines is limited to Asia, and the other ones are spread in Africa (9].

In the laboratory diagnosing of the disease, the virus neutralization tests (NT) [10], the diffusion precipitation [11], counter immunoelectroosmophoresis, indirect immunofluorescence [12, 13] reactions, direct and indirect enzyme-linked immunosorbent assay (ELISA) [14] as well as the competitive ELISA (c-ELISA) based on monoclonal antibodies [15-18] are used for the detection of virus-specific antigens and antibodies.

In severe cases of PPR lesion, when the clinical signs appear in animals, the viral antigens are detected in blood and tissue samples. In subclinically infected animals, the PPR can only be diagnosed by serological testing. The neutralization test for detecting the antibodies to the PPR virus is laborious, expensive and requires using the infectious virus. It implies working with the cell cultures and availability of qualified personnel. For these reasons, using NT for large-scale routine investigations is problematic. The alternative to NT is quick, inexpensive and sensitive serological tests based on various ELISA variants which are successfully used in the diagnosing of many diseases.

In terms of simplicity, high sensitivity and cost-effectiveness, the test systems for indirect and competitive ELISA are considered the most suitable for diagnosing and seroepidemiological surveillance of PPR. They can be oriented for detecting the presence of hemagglutinin (H) [15, 16, 19] or nucleocapsid (N) protein [20-25].

It should be noted that in most of single-stranded RNA viruses including the PPR virus, the N protein is a highly conservative and the most immunogenic protein. This is conditioned by the fact that the N proteingene is located close to the 3′-end of the virus genome and therefore is translated in the amounts exceeding any other structural proteins of the PPR virus [26]. The antibodies to N-protein do not protect animals from the disease, but, given its immunogenicity and high production level, the N protein is considered the most acceptable antigen for the development of the means for diagnosing PPR [27]. In addition, the N-protein apparently has both type-specific and cross-reactive epitopes which are preserved in the PPR virus from the lines originated from different geographical regions. Given these, modern means of the PPR serodiagnostics are developed basing on the recombinant N protein [28]. The peroxidase conjugates for the test systems based on various ELISA variants are prepared using the IgG of anti-species sera or monoclonal antibodies (MABs) to animal immunoglobulins, as well as the MABs to N protein. However, the accidental loss of MAB-producing hybrid clones due to a laboratory accident or inappropriate storage conditions may cause problems in the test systems production [29]. Therefore, different test systems for the PPR serodiagnosis, in particular, those with using the polyclonal sera to N protein for c-ELISA or the modifications of indirect ELISA are studied.

In this paper, we present the results of study of two modifications of the

test systems for PPR serodiagnosis, which we have created based on the recombinant N protein in comparison with the commercial ID Screen® PPR Competition kit (IDvet, France). The verification of the components of these test systems for the indirect and competitive ELISA based on the PPR-positive antisera of goats and pigs allow us to positively assess the perspective of using the test systems recommended by us for the PPR serodiagnosis.

The objective of this research was the construction of test systems for diagnosing PPR basing on the recombinant N protein in the indirect ELISA with the peroxidase conjugate of protein A and in the competitive ELISA with the peroxidase conjugate of IgG of the polyclonal rabbit serum to nucleocapsid protein.

Techniques. The animals (goats, pigs, rabbits) taken from the animal preparation sector (Federal Research Center for Virology and Microbiology, FRCVM) were kept under standard conditions and used in accordance with the requirements of GOST R 53434-2009 dated 02.12.2009 "Principles of Good Laboratory Practice (GLP)". During the period of acclimatization and experiment, the animals were placed in individual quarters and cages in accordance with the GLP requirements [30]. The briquetted compound feed and purified water were given ad libitum into feeders and drinking bowls.

The goats were vaccinated with the dry cell-derived virus-vaccine against the Peste des petits ruminants (FRCVM) according to the vaccine administration manual.

The vaccine strain 45G37/35-K of the PPR virus was taken from the FRCVM State Collection of Microorganisms. PPR virus proliferation and the determination of its infectious activity were performed respectively in polystyrene mattresses and 48-well plates (Costar, France). We used the Vero cell culture (collection of the Federal Research Center of Virology and Microbiology) in the Eagle MEM maintenance medium (PanEco, Russia) with 2.5% fetal bovine serum. To assess titers, virus-containing material was sequentially diluted 10-fold in 4 replicates. The infected and control Vero cells cultures were kept at the temperature of 37 °C in an air atmosphere with 5% CO_2 with the replacement of the maintenance medium every 2-3 days. The results were estimated by the cytopathic effect during 10 days. The titer of the virus was calculated according to the Kerber's method in the modification of Ashmarin and expressed in lg TCD_{50}/cm^3 [31].

Two female goats (Nos. 1 and 2) of the Russian White breed were vaccinated 1 time subcutaneously at the age of 1 year. The blood was sampled from the goatlet (No. 3) born from the vaccinated female goat No. 1 at the age of 1 month. The blood samples for investigation were collected from the adult female goats on the day 28 after the vaccination.

Four pigs (Nos. 1-4) of the Large White breed weighing 25-30 kg were immunized 1 time with the purified concentrated PPR virus (strain 45G37/35-K) by 2.5 cm^3 injections intramuscularly and intranasally, the titer 10^5 TCD_{50}/cm^3. The virions were purified by differential centrifugation method. For this, the virus cultural 30 cm^3 suspension with the titer of 10^5 TCD_{50}/cm^3 was centrifuged at 5,000 g for 40 minutes (J68, Beckman Coulter, USA) to remove cell debris, the supernatant fraction was re-centrifuged through the sucrose cushion (20%, weight/volume) at 45,000 g for 4.5 hours (Avanti JXN-30, Beckman Coulter, USA). The supernatant fraction was decanted, and the precipitate was resuspended in 20 cm^3 phosphate buffer (PBS, pH 7.2). The serum samples were obtained from the blood taken from the pigs on days 0 and 28.

Four rabbits (Nos. 1-4) of the Chinchilla breed weighing 1.5-2.0 kg were immunized with the purified recombinant N protein according to the following

scheme: No. 1 — on day 0 by intracutaneous injection into the foot pads of all four legs, 200 μg of N protein with the Complete Freund's Adjuvant (CFA), and second time on day 25 by the intramuscular injection into the upper part of the thigh, then on days 50 and 57 by intravenous injection into the ear, 200 μg of N protein without adjuvant; No. 2 — injection of 40 μg of N protein to each leg similarly to No. 1; No. 3 − similarly to No. 1, but with the Incomplete Freund's Adjuvant (IFA) No. 4 — similarly to No. 2, but with IFA. On day 64, the rabbits were dehematized post mortem. The IgG from the antisera was purified according to the description [32]. The recombinant N protein of the PPR virus from the cell lysates of the pET32a/N/10 clone of *E. coli* obtained by us previously was purified by the metal chelate chromatography (Ni Sepharose HIS-Select Nickel Affinity Gel, Sigma-Aldrich, USA) under native conditions. The protein was eluted stepwise with the aqueous solutions of imidazole (50, 100, 250 and 500 mM). In the eluate fraction with 500 mM imidazole, the final concentration of the recombinant N protein after the dialysis against PBS was brought up to 2 mg/cm^3.

The neutralization test (NT) was performed with the 45G37/35-K strain of the PPR virus according to the recommendation of the OIE (World Organization for Animal Health), 2016 [2].

The peroxidase conjugates of horseradish (Sigma, USA) with IgG or A protein (Sigma, USA) for the developed ELISA-based test systems were prepared according to the description [33].

For the competitive or indirect ELISA on strips (Eppendorf, Germany) the purified recombinant N protein at the concentration of 0.25 μg/m^3 was immobilized in the carbonate-bicarbonate buffer (pH 9.6) (50 μl per well during 16 hours at 4 °C; 96-well plates, Corning, USA). Then, the wells were washed three times for 1.0 minute with 300 μl of PBS with 0.1% Tween 20 (PBS-t), then the active sites of polystyrene were blocked with PBS-t with 1% casein (blocking solution, 100 μl per well) during 1 hour at 37 °C. Then the wells were washed once with PBS-t and moisture was removed. The sera were diluted in the blocking solution, poured into the wells (50 μl per well) and kept for 2 hours at 37 °C. Then, after three-time washing with PBS-t, the conjugate from the commercial kit or the investigated conjugates diluted in the blocking solution were poured into the wells and incubated for 1.5 hours at 22 °C, the wells were washed 3 times with PBS-t and then 100 μl of the chromogenic substrate ABTS, the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, (Thermo Scientific, USA) was added into the wells with H_2O_2. After 15-20 minutes of incubation in the dark at 22 °C, the results were recorded using a Sunrise spectrophotometer (Tecan, Austria) at λ = 405 nm (OD_{405}).

The commercial ID Screen® PPR Competition kit (IDvet, France) designed for detecting the antibodies to the PPR virus nucleoprotein in blood sheep and goats was the control test system. The conjugate in this kit is the peroxidase-labeled monoclonal murine antibodies to the recombinant N protein of the PPR virus.

The statistical processing of the obtained data was carried out using the Microsoft Excel 2010 software. We determined the mean values (*M*) and standard errors of the mean (±SEM). The average values of the indices were compared according to the Student's *t*-test. The differences were considered statistically significant at $p < 0.05$.

Results. Initially, in order to obtain the rabbit polyclonal antibodies to the nucleocapsid protein of the PPR virus, four immunization schemes differing in the dose of the N protein administered to the animal (40 and 200 μg) and the Freund's adjuvants type (complete or incomplete) were tested. The activity of

blood antibodies to the N protein in rabbits was investigated by the c-ELISA method using the commercial ID Screen® PPR Competition kit (Fig. 1). All the studied sera had the titer values 1:512-1:1024. When the dilution in these ratios the OD_{405} values were more than 2.5 times lower than when the last dilution at 1:8192 or with the control sera obtained before the immunization. The serum after immunization of rabbit No. 1 with N protein at the dose of 200 μg with the complete Freund's adjuvant turned to be the most preferred. When its dilution at 1:1024 the $OD_{405} = 0.2$, whereas the similar optical density according to the interpolation results was achieved at the dilution of 1:172 for No. 2, 1:768 for No. 3, and 1:256 for No. 4.

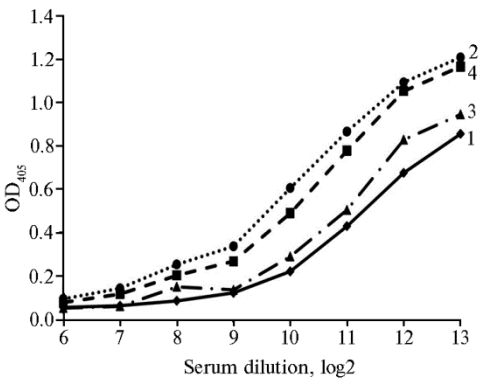

Fig. 1. Activity of blood antibody to the N protein in rabbits No. 1-4 (1-4) **immunized with the purified recombinant nucleocapsid protein of the peste des petits ruminants virus** (lab infection).

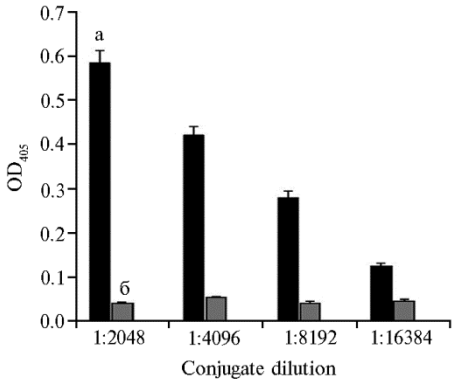

Fig. 2. Titration of protein A peroxidase conjugate using experimental strips with the negative (a) **and positive** (b) **blood sera of rabbit No. 1 diluted 1:1024** (indirect ELISA, $M \pm SEM$, $n = 4$, lab infection).

In the model experiment, the positive and negative blood sera of the rabbit No. 1 in different dilutions were added into the wells with the immobilized purified recombinant N protein. After the incubation and washing, protein A peroxidase conjugate in dilution of 1:500 was added. The results presented in Figure 2 confirm that the protein A peroxidase conjugate does not interact with the N protein directly and detects the rabbit antibodies positive to the N protein up to the serum dilution at 1:16384 that testifies about high analytical sensitivity of this indirect ELISA. The highest ratio of the OD_{405} for the positive and negative serum of the rabbit No. 1 was at their dilutions at 1:2048 (see Fig. 2).

In order to detect the antibodies to the PPR virus in goats, the strips were sensitized with the purified recombinant N protein. Then, the negative and positive goat sera taken from the commercial kit or from the rabbit No. 1 in the dilution of 1:2048 were separately added to the wells. After the incubation with protein A peroxidase conjugate, according to the reaction results, the ratio of the OD_{405} values with the positive and negative goat sera amounted to 5.54, and with the sera of the rabbit No. 1 to 7.99. It is important that in our experiments the protein A peroxidase conjugate reacted with the goat sera antibodies being a part of the immune complexes. Thus, the obtained results testify about the possibility to use the protein A peroxidase conjugate in the test systems for diagnosing PPR by the method of indirect ELISA (see Fig. 2).

When developing the c-ELISA, it was necessary to assess the correspondence of the antigenic determinants on the N protein, with which the antibodies from the goat sera and the antibodies from the serum of the rabbit No. 1 immunized with N protein interact. We found out, whether the antibodies of the positive goat serum from the commercial kit block the interaction of the anti-

bodies of the positive serum from the rabbit No. 1 with the antigenic determinants of the recombinant N protein. For this, the negative or positive goat sera were separately added into certain wells of strips with the immobilized N protein, and into other wells — first the negative or positive goat sera from the commercial kit were added, and then (after the incubation for 2 hours and three-time washing) the negative or positive serum of the rabbit No. 1 in dilution of 1:2048 was added. After washing, protein A peroxidase conjugate in dilution of 1:2000 was added into all wells. The results are presented in Figure 3.

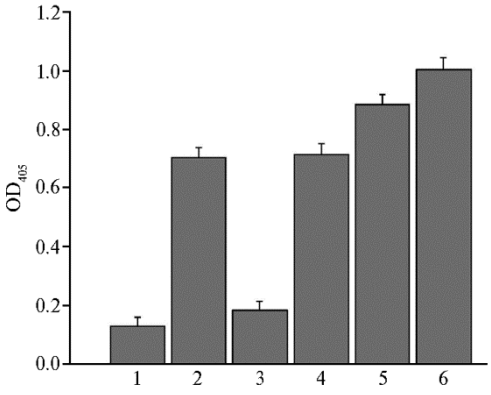

Fig. 3. Indirect ELISA with immobilized recombinant N protein, protein A peroxidase conjugate and different sera: 1 and 2 — the negative and positive goat sera, respectively, from the commercial ID Screen® PPR Competition kit (IDvet, France); 3 — adding the negative goat serum from the commercial kit followed by adding the negative serum of the rabbit No. 1; 4 — adding the positive goat serum from the commercial kit followed by the negative serum of the rabbit No. 1; 5 — adding the negative goat serum from the commercial kit followed by the positive serum of the rabbit No. 1; 6 — adding the positive goat serum from the commercial kit followed by the positive serum of the rabbit No. 1 (M±SEM, n = 4, lab infection).

The pre-incubation of the immobilized N protein with the negative or positive goat serum from the commercial kit followed by incubation with the negative blood serum of the rabbit No. 1 leads to an unreliable change ($p > 0.05$) of the OD_{405} indices. The successive incubation of the positive goat serum and the positive serum of the rabbit No. 1 led to the increase of OD_{405} from 0.70 to 1.00 ($p < 0.05$) (see Fig. 3). It follows from the aforesaid that in the rabbit immunized with the purified recombinant N protein the antibodies react mainly with the same antigenic determinants as the antibodies from the positive goat serum. At the same time, there is a probability that the antibodies from the serum of the rabbit № 1 react with the antigenic determinants which are inaccessible for the antibodies from the PPR-positive goat serum.

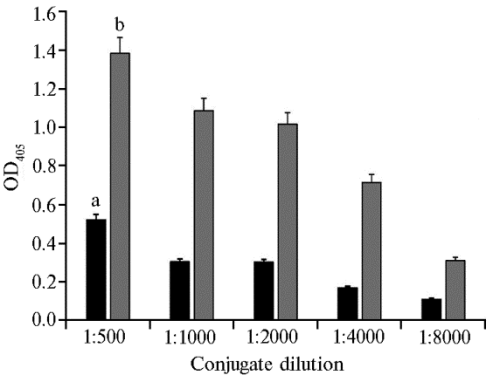

Fig. 4. Titration of the of rabbit IgG-based peroxidase conjugate on the experimental strips with the negative (a) **and positive** (b) **sera of the rabbit No. 1 in dilution of 1:1024** (competitive ELISA, M±SEM n = 4, lab infection).

For creating the test system for the PPR serodiagnosis in c-ELISA, the peroxidase conjugate based on the IgGs isolated from the positive serum of the rabbit No. 1 has been prepared. The prepared conjugate was titrated on the experimental strips with the purified recombinant N protein immobilized in wells in the dose of 0.0125 μg per well and with using the negative and positive sera of the rabbit No. 1 in dilution of 1:1024 (Fig. 4).

According to the results of c-ELISA, the ratio of OD_{405} with the negative and positive sera of the rabbit No. 1 with the conjugate amounted to 2.66 at 1:500 dilution, to 3.60in at dilution 1:1000 , 3.38 at 1:2000, 4.25 at 1:4000, and 1.59 at 1:8000. The 1:4000 dilution of the conjugate has been adopted as the operating dilution. The conjugate has not reacted with the control antigen pre-

pared from the untransformed 16-hour *E. coli* culture Rosetta 2(DE3)pLysS.

Then, in c-ELISA on the experimental strips with using the conjugate we prepared or the commercial conjugate, we compared the commercial goat sera with the sera of the rabbit No. 1 (Table 1). In case of both conjugates the results were positive, but the ratio of the OD_{405} values for the negative and positive goat sera with the commercial conjugate was higher than with the peroxidase conjugate of the IgGs of the rabbit No. 1.

1. Comparison of the negative and positive goat sera from the commercial ID Screen® PPR Competition kit (IDvet, France) and the sera of the rabbit No. 1 (competitive ELISA, $M \pm SEM$, $n = 3$, lab infection)

Serum	Experimental conjugate, 1:4000		Commercial conjugate	
	serum of the rabbit No. 1	commercial goat sera	serum of the rabbit No. 1	commercial goat sera
Negative (–)	0.63±0.04	0.46±0.03	0.56±0.05	0.59±0.03
Positive (+)	0.08±0.01	0.17±0.01	0.07±0.01	0.10±0.00
–/+	7.9	2.7	8.0	5.9

2. Comparison of neutralization test and competitive ELISA (experimental test system) for vaccinated goats and pigs inoculated with the peste des petits ruminants virus ($M \pm SEM$, $n = 3$, lab infection)

Serum	Animals						
	pigs				goats		
	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 3
	Neutralization test						
Titer	1:64	1:128	1:64	1:128	1:32	1:128	1:4
	Concurrent ELISA test						
Negative (–)	1.21±0.13	1.17±0.09	0.89±0.07	0.90±0.08	0.30±0.02	0.32±0.02	0.33±0.04
Positive (+)	0.33±0.05	0.32±0.02	0.30±0.02	0.33±0.01	0.18±0.01	0.10±0.01	0.17±0.01
–/+	3.7	3.6	3.0	2.7	1.7	3.2	1.9

Using the components of the experimental test system, i.e. the strips with the immobilized purified recombinant N protein and IgG peroxidase conjugate of the rabbit No. 1 immunized with N protein, the sera of four pigs inoculated with the purified vaccine PPR virus (Nos. 1-4), two vaccinated female goats (Nos. 1 and 2) and a 1-month-old baby goat (No. 3) born from the vaccinated goat (No. 1) were investigated (Table 2). All sera of the pigs inoculated with the purified PPR virus turned out to be positive in the c-ELISA because the ratio of the OD_{405} values for the negative and positive sera was more than 2.5. Among the two vaccinated goats, the antiserum of the goat No. 2 turned out to be positive. According to NT data, all investigated sera were positive, however, the titer of the virus-neutralizing antibodies in goat No. 2 was higher than in goat No. 1. The obtained data testify that in the competitive variant of ELISA, the peroxidase conjugates obtained on the basis of IgGs of the rabbits immunized with the purified recombinant N protein of the PPR virus can be used for diagnosing the PPR virus.

The advantage of serological analysis of PPR is that antibodies to the PPR virus can be detected in convalescence animals [34]. We compared the results of indirect ELISA obtained with the system developed by us and with the test system of Indian specialists, in which the purified virions of the attenuated PPR virus grown in the Vero cell culture were used as the antigen, and the peroxidase conjugate of the rabbit IgG was used against the goat IgG [17]. The test system included the standard controls (conjugate, negative goat and sheep serum with high and low antibodies' titer values). The average values of OD_{405} in goats in the case of negative sera and sera with high and low antibody titer values were respectively 0.19±0.07, 1.13±0.09 and 0.75±0.08. However, OD_{405} values with the samples of control panel of the sheep serum were 0.15±0.05, 0.95±0.08 and 0.49±0.07. In the opinion of the authors of that paper, this is due to the incom-

plete cross-reactivity of the anti-goat conjugate with the sheep antibodies. The average values of OD_{405} for the "zero" control reaction (the reaction between the convalescent goat antibodies to the PPR virus and the conjugate in the absence of the antigen) and for the control with the conjugate without antibodies were respectively 0.14±0.05 and 0.12±0.02 [17]. In our researches with the experimental test system for indirect ELISA with the negative and positive goat sera from the commercial kit, the OD_{405} value amounted to 0.13 and 0.70, and with the negative and positive rabbit sera No. 1 when the dilution in 1:1024 ratio, it was 0.09 and 0.74. It is known that goat immunoglobulins weakly bind to A protein [35]. It was also reported that binding of the antigen by the Fab fragments increases the affinity between the Fc fragment of IgG and A protein [36]. This expands the possibility of using A protein in ELISA because it makes it possible to use animal IgGs weakly interacting with it. Thus, the goat and sheep IgGs bound to the antigen interact with A protein 100 times more intensive than without the antigen. It is important to note that in our experiments, the A protein's peroxidase conjugate have reacted with the goat serum antibodies. So, the obtained results testify about the possibility of using of the A protein's peroxidase conjugate in the test systems for diagnosing PPR by the method of indirect ELISA.

In order to replace the neutralization test when wide-scale investigation of animal blood, it was previously proposed to use the c-ELISA based test system in which the lysate of the cell culture infected with the attenuated strain of the PPR virus and the monoclonal antibodies obtained for the neutralizing epitope of H protein were used as the antigen [37]. In the opinion of the cited publication's authors, the advantages of the said test system are the simplicity, quick obtaining of the result, less dependence on the serum samples' quality and convenience when investigating a large number of samples. Its sensitivity relative to the NT (neutralization test) was 92.2%, specificity – 98.4%. The authors note that usually in blood, the antibodies' titers in c-ELISA were by 1-3 $\log_2$ lower than in NT. Typically, the antibody titers in NT after the vaccination, especially in sheep, ranged from 1:8 to 1:16. The sera with low antibody titer values in NT were negative in c-ELISA. Therefore, for identifying positive samples it was proposed to choose the low cut-off (average negative value +2 standard deviations) [38].

So, it was found that in rabbits immunized with the purified recombinant N protein of the peste des petits ruminants (PPR) virus, the antibodies which react mainly with the same antigenic determinants of this protein as the antibodies of the PPR-positive goat serum are produced. We have created and tested the experimental test system for the PPR serodiagnostics by competitive ELISA (c-ELISA) based on the recombinant N protein and peroxidase conjugate based on IgGs from the N-protein-specific rabbit serum. When using the proposed test system, it was shown that the animal sera with titers of the antibodies to the PPR virus in the neutralization test of 1:64 and higher in c-ELISA were positive. In terms of the ratio of the OD_{405} values for negative and positive goat sera, the results obtained using the commercial ID Screen® PPR Competition test system (IDvet, France) were 2 times higher than with the experimental one. The obtained results allow us to positively evaluate the perspective of indirect and competitive ELISA-based test systems we created for the PPR serodiagnosis.

REFERENCES

1. Banyard A.C., Parida S., Batten C., Oura C., Kwiatek O., Libeau G. Global distribution of peste des petits ruminants virus and prospects for improved diagnosis and control. *Journal of General Virology*, 2010, 91(12): 2885-2897 (doi: 10.1099/vir.0.025841-0).

2. World Organization for Animal Health (OIE). Peste des petits ruminants (infection with Peste des petits ruminants virus). Chapter 2.7.10. In: *Manual of diagnostic tests and vaccines for terrestrial animals*. Cambridge, 2016.
3. Baron M.D., Diop B., Njeumi F., Willett B.J., Bailey D. Future research to underpin successful peste des petits ruminants virus (PPRV) eradication. *Journal of General Virology*, 2017, 98(11): 2635-2644 (doi: 10.1099/jgv.0.000944).
4. Schulz C., Fast C., Schlottau K., Hoffmann B., Beer M. Neglected hosts of small ruminant morbillivirus. *Emerging Infectious Diseases*, 2018, 24(12): 2334-2337 (doi: 10.3201/eid2412.180507).
5. Mariner J.C., Jones B.A., Rich K.M., Thevasagayam S., Anderson J., Jeggo M., Cai Y., Andrew R., Peters A.R., Roeder P.L. The opportunity to eradicate Peste des petits ruminants. *The Journal of Immunology*, 2016, 196(9): 3498-3506 (doi: 10.4049/jimmunol.1502625).
6. Amarasinghe G.K., Aréchiga N.G., Ashley C., Christopher C.B. Taxonomy of the order *Mononegavirales*: update 2018. *Archives of Virology*, 2018, 163(8): 2283-2294 (doi: 10.1007/s00705-018-3814-x).
7. *Virus taxonomy: classification and nomenclature of viruses. Ninth report of the International Committee on Taxonomy of Viruses*. A.M.Q. King, M.J. Adams, E.B. Carstens, E.J. Lefkowitz (eds.). Elsevier, 2012.
8. Libeau G., Diallo A., Parida S. Evolutionary genetics underlying the spread of Peste des petits ruminants virus. *Animal Frontiers*, 2014, 4(1): 14-20 (doi: 10.2527/af.2014-0003).
9. Dhar P., Sreenivasa B.P., Barrett T., Corteyn M., Singh R.P., Bandyopadhyay S.K. Recent epidemiology of Peste-des-petits-ruminants virus (PPRV). *Veterinary Microbiology*, 2002, 88(2): 153-159 (doi: 10.1016/S0378-1135(02)00102-5).
10. Liu F., Wu X., Zou Y., Li L., Wang Z. Peste des petits ruminants virus-like particles induce both complete virus-specific antibodies and virus neutralizing antibodies in mice. *Journal of Virological Methods*, 2015, 213: 45-49 (doi: 10.1016/j.jviromet.2014.11.018).
11. Durojaiye O.A. Precipitating antibody in sera of goats naturally affected with Peste des petits ruminants. *Tropical Animal Health and Production*, 1982, 14(2): 98-100 (doi: 10.1007/BF02282591).
12. Kozat S., Sepehrizadeh E. Peste des petit ruminants. *Journal of Istanbul Veterinary Sciences*, 2017, 1(2): 47-56 (doi: 10.30704/http-www-jivs-net.319937).
13. Durojaiye O.A., Taylor W.P. Application of countercurrent immuno-electro-osmophoresis to the serology of Peste des petits ruminants. *Revue d'élevage et de médecine vétérinaire des pays tropicaux*, 1984, 37(3): 272-276 (doi: 10.19182/remvt.8396).
14. Obi T.U., McCullough K.C., Taylor W.P. The production of Peste des petits ruminants hyperimmune sera in rabbits and their application in virus diagnosis. *Journal of Veterinary Medicine, Series B*, 1990, 37(1-10): 345-352 (doi: 10.1111/j.1439-0450.1990.tb01068.x).
15. Anderson J., Mckay J.A., Butcher R.N. The use of monoclonal antibodies in competition ELISA for detection of antibodies to rinderpest and peste des petits ruminants viruses. In: *Final research co-ordination meeting on the sero-monitoring of rinderpest throughout Africa, Bingerville (Cote d'Ivoire), 19-23 Nov 1990*. International Atomic Energy Agency (IAEA), Vienna, 1991: 43-53.
16. Abubakar M., Mahapatra M., Muniraju M., Arshed M.J., Khan E.A., Banyard A.C., Ali Q., Parida S. Serological detection of antibodies to Peste des petits ruminants virus in large ruminants. *Transboundary and Emerging Diseases*, 2017, 64(2): 513-519 (doi: 10.1111/tbed.12392).
17. Balamurugan V., Singh R.P., Saravanan P., Sen A., Sarkar J., Sahay B., Rasool T.J., Singh R.K. Development of an indirect ELISA for the detection of antibodies against Peste-des-petits-ruminants virus in small ruminants. *Veterinary Research Communications*, 2007, 31(3): 355-364 (doi: 10.1007/s11259-006-3442-x).
18. Singh R.P., Sreenivasa B.P., Dhar P., Shah L.C., Bandyopadhyay S.K. Development of a monoclonal antibody based competitive ELISA for detection and titration of antibodies to peste des petits ruminants (PPR) virus. *Veterinary Microbiology*, 2004, 98(1): 3-15 (doi: 10.1016/j.vetmic.2003.07.007).
19. Singh R.P., Screenivasa B.P., Dhar P., Roy R.N., Brandyopadhyay S.K. Development and evaluation of a monoclonal antibody based competitive enzyme-linked immunosorbent assay for the detection of rinderpest virus antibodies. *Revue Scientifique et Technique*, 2000, 19(3): 754-763 (doi: 10.20506/rst.19.3.1243).
20. Choi K.S., Nah J.J., Choi C.U., Ko Y.J., Sohn H.J., Libeau G., Kang S.Y., Joo Y.S. Monoclonal antibody-based competitive ELISA for simultaneous detection of rinderpest virus and peste des petits ruminants virus antibodies. *Veterinary Microbiology*, 2003, 96(1): 1-16 (doi: 10.1016/S0378-1135(03)00201-3).
21. Basagoudanavar S.H., Hosamani M., Muthuchelvan D., Singh R.P., Santhamani R., Sreenivasa B.P., Saravanan P., Pandey A.B., Singh R.K., Venkataramanan R. Baculovirus expression and purification of peste-des-petits-ruminants virus nucleocapsid protein and its application in diagnostic assay. *Biologicals*, 2018, 55: 38-42 (doi: 10.1016/j.biologicals.2018.07.003).
22. Liu F., Wu X., Li L., Liu Z., Wang Z. Use of baculovirus expression system for generation of virus-like particles: successes and challenges. *Protein Expression and Purification*, 2013, 90(2): 104-116 (doi: 10.1016/j.pep.2013.05.009).
23. Liu F., Wu X., Li L., Liu Z., Wang Z. Formation of peste des petits ruminants spikeless virus-like particles by co-expression of M and N proteins in insect cells. *Research in Veterinary Science*, 2014, 96(1): 213-216 (doi: 10.1016/j.rvsc.2013.10.012).

24. Berguido F.J., Bodjo S.C., Loitsch A., Diallo A. Specific detection of peste des petits ruminants virus antibodies in sheep and goat sera by the luciferase immunoprecipitation system. *Journal of Virological Methods*, 2016, 227: 40-46 (doi: 10.1016/j.jviromet.2015.10.008).
25. Bodjo S.C., Baziki J.D., Nwankpa N., Chitsungo E., Koffi Y.M., Couacy-Hymann E., Diop M., Gizaw D., Tajelser I.B.A., Lelenta M., Diallo A., Tounkara K. Development and validation of an epitope-blocking ELISA using an anti-haemagglutinin monoclonal antibody for specific detection of antibodies in sheep and goat sera directed against peste des petits ruminants virus. *Archives of Virology*, 2018, 163(7): 1745-1756 (doi: 10.1007/s00705-018-3782-1).
26. Lefevre P.C., Diallo A., Schenkel F., Hussein S., Staak G. Serological evidence of Peste des petits ruminants in Jordan. *Veterinary Record*, 1991, 128(5): 110 (doi: 10.1136/vr.128.5.110).
27. Cosby S.L., Weir L. Measles vaccination: threat from related veterinary viruses and need for continued vaccination post measles eradication. *Human Vaccines & Immunotherapeutics*, 2018, 14(1): 229-233 (doi: 10.1080/21645515.2017.1403677).
28. Munir M., Zohari S., Berg M. Current advances in molecular diagnosis and vaccines for peste des petits ruminants. In: *Molecular Biology and Pathogenesis of Peste des Petits Ruminants Virus. SpringerBriefs in Animal Sciences*. Springer, Berlin, Heidelberg, 2013: 105-133 (doi: 10.1007/978-3-642-31451-3_6).
29. Getachew B., Upmanyu V., Haq A.A., Santhamani R., Rajak K.K., Muthuchelvan D., Gupta S.K., Yousuf R.W., Mahapatra M., Parida S., Sharma B., Singh R.P. Monoclonal antibody resistant mutant of Peste des petits ruminants vaccine virus. *VirusDisease*, 2018, 29(4): 520-530 (doi: 10.1007/s13337-018-0483-z).
30. Clark J.D., Gebhart G.F., Gonder J.C., Keeling M.E., Kohn D.F. The 1996 guide for the care and use of laboratory animals. *ILAR Journal*, 1997, 38(1): 41-48 (doi: 10.1093/ilar.38.1.41).
31. Ashmarin I.P., Vasil'ev N.N., Ambrosov V.A. *Bystrye metody statisticheskoi obrabotki i planirovanie eksperimentov*. L., 1974.
32. Berzina A.G., Gamaleya N.B., Kapanadze G.D. *Biomeditsina*, 2013, 2: 95-102 (in Russ.).
33. Berg E.A., Fishman J.B. Conjugation of antibodies to horseradish peroxidase. *Cold Spring Harbor Protocols*, 2019, 9: pdb.prot099309 (doi: 10.1101/pdb.prot099309).
34. Wohlsein P., Singh R.P. Peste des petits ruminants in unusual hosts: epidemiology, disease, and impact on eradication. In: *Peste des Petits Ruminants Virus*. M. Munir (ed.). Springer, Berlin, Heidelberg, 2015: 95-118 (doi: 10.1007/978-3-662-45165-6_6).
35. *Antibodies: a laboratory manual*. E.A. Greenfield (ed.). Cold Spring Harbor Laboratory Press, 2013.
36. Verbov V.N. *Trudy instituta imeni Pastera*, 1998, 64: 3-27 (in Russ.).
37. Mapaco L., Monjane I., Fafetine J., Arone D., Caron A., Chilundo A., Quembo C., Carrilho M.D.C., Nhabomba V., Zohari S., Achá S. Peste des petits ruminants virus surveillance in domestic small ruminants, Mozambique (2015 and 2017). *Frontiers in Veterenary Science*, 2019, 6: 370 (doi: 10.3389/fvets.2019.00370).
38. Sreenivasa B. P., Dhar P., Singh R.P., Bandyopadhyay S.K. Development of peste des petits ruminants (PPR) challenge virus from a field isolate. In: *XIV annual conference and national seminar on management of viral diseases with emphasis on global trade and WTO regime of Indian Virological Society*. Pantnagar, India, 2002.

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1236-1246
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.3:619:578:577.2

doi: 10.15389/agrobiology.2019.6.1236eng
doi: 10.15389/agrobiology.2019.6.1236rus

EXPERIMENTAL SUBUNIT VACCINE AGAINST CLASSICAL SWINE FEVER DEVELOPMENT AND TRIAL

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The authors declare no conflict of interests
Received July 16, 2019

Abstract

Classical swine fever (CSF), the highly contagious viral disease, remains the major threat to pork industry in top ten pork producing countries save the United States. Disease outbreaks and following restrictions in international trade are causing major economic losses worldwide. Wild boars are the natural reservoir of the virus. They represent high danger to pork industry in regions with high density of wild boar population. Live attenuated vaccine has been used in Russia for decades for the total swine population vaccination. Today Russia belongs to world leaders in the pork production, but it still has to be recognized as CSF virus (CSFV) free country (or region) to be incorporated in a global market. The first step in this direction would be the implementation of non-replicating marker vaccines, allowing the differentiation between infected and vaccinated animals (DIVA). Here we first report results of recombinant E2 protein-based vaccine formulations trial in Russian Federation, where optimal administration protocol, adjuvant, dosage of specific component were selected. From the formulations tested, safe and effective vaccine formulation was selected, that needs to be tested for antigen stability and immunity duration in vaccinated animals, and then undergo clinical trial on farm. The aim of our study is the development of the vaccine based on CSFV recombinant surface glycoprotein E2 according to requirements for the country/region free of CSFV. We performed the series of animal trials on *Sus scrofa* Landrace-Duroc breed (total number of pigs 84, divided into 9 experimental and 2 control groups) to assess the vaccination schedule, antigen dosage, and choice of adjuvant. Highly pathogenic CSFV Shi-Men strain was used for challenge in 5×10^5 LD_{50} dose (ARRIAH collection). Double parenteral administration of 10 μg or 30 μg of antigen as well as single administration of 30 or 60 μg of E2 had not provided sufficient level of protection. Oil adjuvant was reactogenic at the inoculation spot even when used once, while polymeric adjuvant has not produced local or systemic reactions after the administration single time or twice. Double administration with the vaccine containing 60 rg of the antigen and polymeric adjuvant has completely protected pigs from the death after the challenge, while in non-vaccinated/challenged control group 5 out of 11 animals died within 14 days post-challenge. Vaccinated animals had less pronounced fever that lasted shorter (rise of the rectal temperature was delayed for 2 days, release of fever 2 days before the control challenge group), frequency and longevity of viremia and virus shedding in nasal swabs were significantly ($p \le 0.05$) reduced as compared to inoculated control piglets. Animals in this vaccinated group gained weight every day after the challenge, being slightly behind non-vaccinated/non-challenged controls. The high levels of antibodies against E2 protein were detected in sera of vaccinated animals before the challenge and they all were negative for antibodies to E^{rns} pro-

tein. After the challenge antibodies to E^{rns} proteins started to raise in sera of all animals save non-vaccinated/non-challenged controls, thus we developed the product that may be implemented as a marker vaccine against CSFV.

Keywords: Classical swine fever, subunit vaccine, CSF E2, adjuvant, vaccine development

Classical swine fever (CSF) is a highly contagious viral disease of domestic and wild pigs, which proceeds in acute, chronic and subclinical forms and causes significant economic damage to many countries with developed swine industry [1-3]. Thus, as the result of the CFS outbreaks in 1993-1998, more than 13 million pigs were eliminated out of necessity in a number of EU countries (Netherlands, Germany, Spain, Belgium, Italy), and the total damage exceeded 5 billion euros [4].

The causative agent of the disease is the RNA-containing virus belonging to the *Pestivirus* genus of the *Flaviviridae* family. The same genus includes the bovine diarrhea virus of I and II types and the border disease virus [5]. The genome of the CSF virus (CSFV) is represented by the single-stranded RNA molecule of positive polarity with the length of 12.3 thousand nucleotides, which encodes 4 structural and 8 non-structural proteins [6, 7]. The CSFV virions are in the form of spherical particles with the diameter of 40-60 nm. They consist of a nucleocapsid and a lipoprotein envelope. The nucleocapsid consists of RNA and C protein, the envelope is formed by three glycoproteins: E^{rns} (gp44/48), E1 (gp33) and E2 (gp55), which owing to disulfide links form the complexes (E^{rns} is homodimer, E1-E2 is heterodimer and E2 is homodimer) [8]. The protein of E^{rns} has a ribonuclease activity [9]. The heterodimeric complex E1-E2 ensures the virus's penetration into a cell [10]. CSFV E2 protein possesses antigenic epitopes on its surface that are involved in cellular and humoral immune responses to the virus: the monoclonal antibodies to this protein have the virus-neutralizing activity [11, 12]. Besides the antibodies to the E2 protein, the antibodies to E^{rns} and non-structural NS3 protein are detected in the infected animals' organisms [13].

The specific CSF prevention measures in Russia are based on using safe and highly immunogenic live vaccines. The most effective is the CS vaccine, which differs from other similar drugs with the high virus content in one vaccination dose (no less than 10^5 ImD_{50}) [14]. However, classical live vaccines do not allow differentiation of the vaccinated and infected animals [5, 15, 16]. This problem can be solved with using marked vaccines: subunit, chimeric, vector and DNA vaccines, among which only the subunit vaccine is available as commercial drug and the chimeric vaccine is licensed for use [15]. In terms of effectiveness, chimeric vaccines are comparable to traditional live vaccines [15, 16]. Implementation of live vaccines is related to the potential risks of recombination of the vaccine virus with the field one and sometimes is forbidden under the rules of importing animal products, therefore, the subunit recombinant vaccines based on the surface glycoprotein E2 of the CSF virus have been developed as a safe alternative to live vaccines. Subunit vaccines cause shorter immunity, require more time for its formation, are administered 2 times and do not ensure the sterile immunity [16]. Nevertheless, they are safe, ensure the animals protection from the control infection with the virulent virus and make it possible to apply the DIVA (differentiating infected from vaccinated animals) strategy [15, 17, 18]. In vaccinated pigs, by using the method of enzyme-linked immunosorbent assay (ELISA) the antibodies only to E2 protein can be detected, and in animals that were infected with field strains of the CSF virus the antibodies to both E^{rns} and E2 proteins can be detected [19, 20].

Previously, we have obtained and characterized the recombinant E2 en-

velope protein of the Shi-Men strain of the CSF virus [21], and the laboratory sample of the recombinant subunit vaccine against CSF, which protected pigs from infection with the virulent Shi-Men strain of the CSF virus has been developed basing on this protein [22]. In current study, we have improved the technology of obtaining of the CSF virus's recombinant E2 protein, created the construction for the coexpression of the genes of the surface E2 glycoprotein of two genotypes of the CSF virus, circulating in the territory of Russia, and have determined the composition and dosage of the preparation, which is safe and able to protect the experimental animals from infection with the highly pathogenic Shi-Men strain of the CSF virus.

Our objective was the evaluation of optimal composition of preparations, recombinant protein dosage, and administration schedule for the laboratory samples of the recombinant subunit vaccine against the classical swine fever.

Techniques. The laboratory samples of the recombinant subunit vaccine were prepared from the E2 protein of the 8Z, Shi-Men (genotype 1.1) and Alfort-Tübingen strains (genotype 2.3) previously obtained in the baculovirus gene expression system in our laboratory. The sequence encoding the ectodomain of the CSF virus's surface glycoprotein E2 was amplified from the field material (spleen) received by the laboratory in 1997. After determining the primary nucleotide sequence of the amplified fragment, the CSF virus's field isolate (working title 8Z) was classified to the genotype 1.1 along with the highly pathogenic Shi-Men strain (8Z differs from the latter by 8 amino acid substitutions). Then the commercial vector pFastBacHTc (Life Technologies, USA) was modified. On the CpoI and NcoI restriction sites we cut off a fragment between the promoter and the polylinker of the plasmid with the encoded histidine tag and the TEV-protease recognition site, and inserted the sequence encoding the signal peptide of secretion of the melittin bee venom toxin, which has been assembled from three synthetic oligonucleotides. Using the primers containing the NheI and EcoRI restriction sites, we amplified the part of the sequence of the E2 gene of the 8Z isolate (the fragment encoding the 31st amino acid at the C-terminus of the protein forming the transmembrane domain was excluded). As the result of expression of the obtained construct, we observed the production of the E2 surface glycoprotein ectodomain of isolate 8Z secreted into the culture medium. The construct, which simultaneously carries two genes encoding the E2 surface glycoproteins of the Shi-Men (genotype 1.1) and Alfort-Tübingen (genotype 2.3) CSF viruses, has been was obtained synthetically (Eurogen, Russia), both genes are preceded by the sequence of the signal peptide of the melittin secretion and also have been devoid of the region encoding the transmembrane domain. The transfection of insect cells by the recombinant baculovirus genome, infection of the cells, and accumulation of the recombinant protein in the Sf-9 cell culture has been performed according to the technique we described previously [22]. Besides, we used the commercial preparation of recombinant E2 (Prionics AG, Switzerland). The synthetic polyacrylate (Vet-Biohim LLC, Russia), and the incomplete Freund's adjuvant (Sigma Aldrich, USA) have been used as adjuvants. The obtained vaccines have been stored at 4 °C.

The effectiveness of different variants of recombinant subunit vaccine against CSF has been studied at the research facilities of the Lisiy Ostrov (branch of FSC VIEV RAS, Vyshny Volochyok). Piglets (n = 84) of 50-days age (taken from the CSF-free farm in which the prophylactic immunization with the live vaccine against CSF is carried out) have been used for the tests. According to the results of the enzyme immunoassay (ELISA), before the immunization, most piglets did not contain the antibodies to the CSF virus. The animals were distributed into groups depending on the vaccine composition, antigen content

and vaccination schedule.

The vaccine preparations (2 ml) were injected intramuscularly once or 2 times. In case of 2-times administration, the first vaccination of piglets was carried out at 50-days age, the second one in 21 days. In case of one-time administration, the animals were vaccinated 21 days before the challenge. 14 days after the second vaccination (or 21 days after the one-time vaccination), the animals of all groups, except for the X group, were intranasally and intramuscularly injected with 2 ml of the Shi-Men strain (1.25×10^5 LD_{50}/ml) (All-Russian Research Institute for Animal Health, Vladimir, Russia). Before the challenge, the animals of the X group (negative control) were transferred to other building in order to exclude the possibility of accidental infection with the CSF virus. After the control challenge, every second day during 2 weeks, the piglets' body temperature was measured, nasal swabs and blood samples were taken and weighing was performed. On day 14 after the challenge, all piglets were euthanized; the samples of spleen, tonsils and mesenteric lymph nodes were taken from each animal.

The authors confirm that the permission to conduct the experiments from the Committee on Ethics and Humane Treatment of Animals of the FSC VIEV RAS has been received, and all the requirements for working with animals have been met.

The blood sera have been investigated for the presence of the antibodies to the E2 protein of the CSF virus by the ELISA method in the commercial test systems (CSF-SEROTEST, Vetbiochem, Russia) and the antibodies to E^{rns} protein (Priochek CSFV E^{rns}, Thermo Fisher Scientific, USA) according to the attached instructions. For registering the reaction results, the iMark plan-table photometer with the 450 nm filter (Bio-Rad, USA) has been used. The results have been expressed as the coupling ratio ($C_{rat.}$) which has been calculated according to the instruction of the test system's manufacturer. The data have been interpreted (positive or negative serum) according to the criteria of the test system's manufacturer. The CSF virus RNA in the nasal swabs and sera were detected by the method of polymerase chain reaction (PCR) using the commercial test system for the detection of classical swine fever virus by the PCR method (Vetbiochem, Russia); the results were designated as "+" or "–".

The obtained data have been processed by the analysis of variance (ANOVA). The mean values (*M*) and standard errors of the mean (±SEM) have been calculated. In the Results section, the differences between the indices for the group I (the vaccine preparation recommended by us for further tests) and group IX (challenge control) were statistically significant at $p \le 0.05$.

Results. Animal assignment to groups, vaccine composition, antigen content and vaccination schedules are described in Table 1.

1. Design of the experiment on comparison of different vaccination schedules and subunit vaccines against the classical swine fever (CSF) (research facilities of Lisiy Ostrov, FSC VIEV RAS, Vyshny Volochyok)

Group	Vaccines	Piglets number per group	
		total	E2 seropositive on day of the 1st or single immunization
	Double vaccination		
I	E2 protein of8Z, 60 μg, synthetic polyacrylate	15	6
II	E2 protein of 8Z, 30μg, synthetic polyacrylate	10	4
III	E2 protein of 8Z, 10 μg, synthetic polyacrylate	5	2
IV	E2 protein of Shi-Men + Alfort-Tübingen, 30 μg, Freund's incomplete adjuvant	5	2
V	E2 protein of 8Z, 30 μg, Freund's incomplete adjuvant	15	5
VI	Commercial E2 preparation, 60 μg, synthetic polyacrylate	5	0

Continued Table 1

	Single vaccination		
VII	E2 protein of 8Z, 60 μg, synthetic polyacrylate	4	2
VIII	E2 protein of 8Z, 30 μg, Freund's incomplete adjuvant	5	2
	Controls		
IX	Challenge control (unvaccinated animals infected at the 84-days age)	11	4
X	Negative control (unvaccinated and uninfected animals)	8	5

Note. Under double vaccination of piglets, the 1st one was performed at the age of 50 days, the 2nd one — 21 days after the 1st vaccination; the control challenge was made 14 days after the second vaccination. Single vaccination was performed at the age of 64 days, the control challenge — 21 days after the vaccination.

2. Effectiveness of different vaccination schedules and CSF subunit vaccines (research facilities of Lisiy Ostrov, FSC VIEV RAS, Vyshny Volochyok)

Group	Vaccines	Piglets			
		infected	with fever above 41 °C	sick	dead
I (n = 15)	E2 protein of 8Z, 60 μg, synthetic polyacrylate	15	6	0	0
II (n = 10)	E2 protein of 8Z, 30μg, synthetic polyacrylate	10	3	1	0
III (n = 5)	E2 protein of 8Z, 10 μg, synthetic polyacrylate	5	5	5	1
IV (n = 5)	E2 protein of Shi-Men + Alfort-Tübingen, 30 μg, Freund's incomplete adjuvant	5	0	0	0
V (n = 15)	E2 protein of 8Z, 30 μg, Freund's incomplete adjuvant	15	4	2	2
VI (n = 5)	Commercial E2 preparation, 60 μg, synthetic polyacrylate	5	3	0	0
VII (n = 4)	E2 protein of 8Z, 60 μg, synthetic polyacrylate, single vaccination	4	4	4	3
VIII (n = 5)	E2 protein of 8Z, 30 μg, Freund's incomplete adjuvant, single vaccination	5	5	1	0
IX (n = 11)	Challenge control (unvaccinated animals infected at the 84-days age)	11	11	11	5
X (n = 8)	Negative control (unvaccinated and uninfected animals)	0	0	0	0

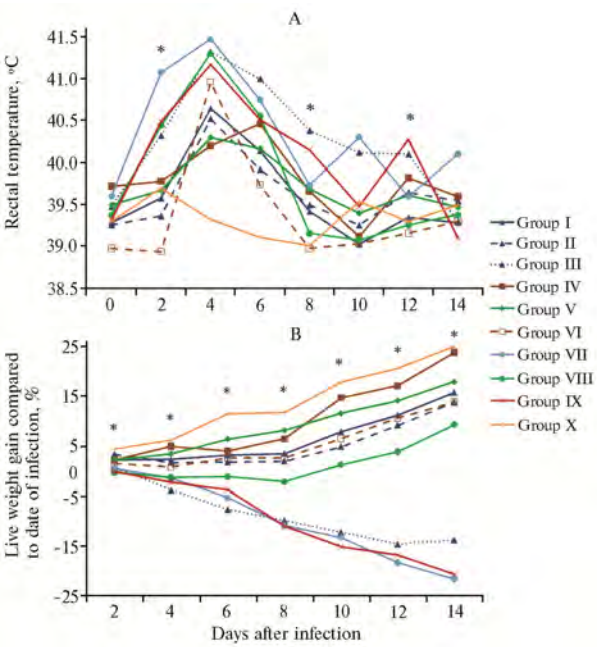

Fig. 1. Rectal temperature (A) **and increase in body weight** (B) **in piglets upon different vaccination schedules and CSF subunit vaccines** (research facilities of Lisiy Ostrov, FSC VIEV RAS, Vyshny Volochyok). See groups' descriptions in Table 1. The asterisks indicate the points for which the differences between groups I and IX are statistically significant at $p < 0.05$.

The experiment results showed (Table 2) that the injection of all samples of the vaccine against the CSF has not followed by general suppression of the condition and the increase in body temperature of the piglets. The control challenge of the unvaccinated animals (group IX) led to the development of the typical clinical manifestations. On day 14 after the challenge, 5 of 11 piglets in this group died (mortality 45.5%), the rest ones showed a significant decrease in body weight. In groups III and VII, the mortality rate was 20.0 and 70.0% respectively. Up to 8th day after the challenge, a fever was found in the animals

of group VIII, after that the temperature returned to normal value. In groups I, II, IV, V and VI, a moderate and short-term increase in temperature occurred, and only in some animals the temperature of 41°C was registered (Fig. 1, A, see Table 2).

The maximum decrease in body weight was observed in piglets of groups III and VII: the registered values were identical to those in group IX (challenge control) – the uniform decrease of these indices was observed from the 1st to the 14th day after the challenge (see Fig. 1, B). In groups I, II, IV, V, VI, and VIII, either there was no any decrease in the animals' body weight, or it manifested in a significantly lesser extent ($p \leq 0.05$). Moreover, from days 4-8 after the challenge, in groups I, IV, V and VI the complete restoration of the dynamics of increase in body weight occurred that is characteristic for the control (uninfected) piglets.

3. Blood level of antibodies to CSFV proteins E^{rns} and E2 ($C_{bind.}$ as per instructions of test kit manufacturers) **in piglets upon different vaccination schedules and CSF subunit vaccines** (research facilities of Lisiy Ostrov, FSC VIEV RAS, Vyshny Volochyok)

Group	Vaccines	Vaccination		Challenge with		Day 14 after the challenge		
		1st/single	2nd	E2	E^{rns}	E2	E^{rns}	
							$C_{bind.}$	"+", %
I	E2 protein of 8Z, 60 μg, synthetic polyacrylate	60.72 (+)	67.10 (+)	104.89 (+)	18.59 (–)	107.35 (+)	47.42 (+)	73.3
II	E2 protein of 8Z, 30μg, synthetic polyacrylate	53.17 (+)	57.05 (+)	105.29 (+)	17.94 (–)	108.37 (+)	45.95 (+)	70.0
III	E2 protein of 8Z, 10 μg, synthetic polyacrylate	70.00 (+)	57.90 (+)	81.44 (+)	23.14 (–)	108.40 (+)	52.98 (+)	80.0
IV	E2 protein of Shi-Men + Alfort-Tübingen, 30 μg, Freund's incomplete adjuvant	69.66 (+)	67.50 (+)	71.96 (+)	27.88 (–)	105.28 (+)	50.84 (+)	60.0
V	E2 protein of 8Z, 30 μg, Freund's incomplete adjuvant	62.95 (+)	71.11 (+)	86.51 (+)	22.48 (–)	103.65 (+)	31.88 (–)	53.8
VI	Commercial E2 preparation, 60 μg, synthetic polyacrylate	43.94 (–)	40.36 (–)	101.78 (+)	–0.12 (–)	112.26 (+)	35.10 (–)	60.0
VII	E2 protein of 8Z, 60 μg, synthetic polyacrylate, single vaccination	62.83 (+)	nd	47.93 (–)	24.78 (–)	107.40 (+)	55.70 (+)	100
VIII	E2 protein of 8Z, 30 μg, Freund's incomplete adjuvant, single vaccination	60.26 (+)	nd	81.64 (+)	25.10 (–)	108.84 (+)	44.50 (+)	60.0
IX	Challenge control (unvaccinated animals infected at the 84-days age)	51.00 (+)	41.15 (–)	39.89 (–)	14.25 (–)	51.07 (+)	30.30 (–)	33.3
X	Negative control (unvaccinated and uninfected animals)	63.64 (+)	50.17 (+)	47.58 (–)	5.51 (–)	41.38 (–)	9.70 (–)	0

N o t e. nd — no data; "+"/"–"— presence/absence of the relevant antibodies in ELISA test.

The average values of the relative content of antibodies ($C_{bind.}$, %) to E2 and E^{rns} proteins in the piglets' blood serum are given in Table 3. 2 weeks after the second immunization, the seroconversion to E2 protein was found in all vaccinated animals, while in unvaccinated animals by this time the status was seronegative. After the control challenge, in vaccinated animals, the content of antibodies to E2 continued to increase. As of the moment of the challenge, the antibodies to E^{rns} protein have not been detected in all piglets, however, on the 14th day after the challenge such antibodies were found in most infected animals. The minimum share of the animals which manifested the positive response to E^{rns} has been found in groups IV, V, VI, VIII and in the challenge control group. It is necessary to remember that seronegative animals should be vaccinated (in our case, most animals reached this status by the age of 50 days), because

a high level of maternal antibodies may adversely affect the development of the immune response after the vaccination [23, 24].

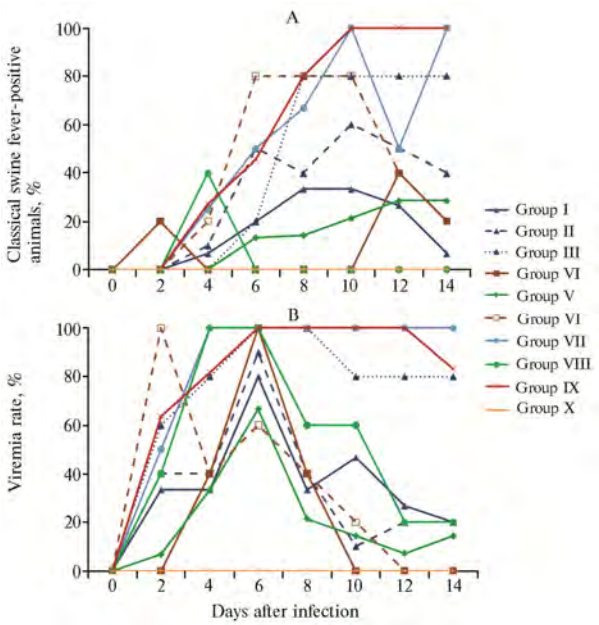

Fig. 2. Detection of CSF virus RNA in nasal swabs (A) **and viremia** (B) **in piglets upon different vaccination schedules and CSF subunit vaccines** (research facilities of Lisiy Ostrov, FSC VIEV RAS, Vyshny Volochyok). For PCR analysis; see the description in Table 1; the differences of the patterns when detecting the virus in the nasal swabs and blood of animal groups I and IX are statistically significant at $p < 0.05$.

The data on viremia and detection of CSF virus in nasal swabs after the control challenge of the piglets are given in Figure 2. The vaccine preparations have not ensured the sterile immunity: viremia and presence of CSF virus in nasal swabs were detected in all experimental groups. The highest values were in groups II, III, and VII, and the lowest in groups I, IV and V (see Fig. 2, A). It is noteworthy that the data on virus isolation are positively correlated with the clinical manifestations of CSF. In the blood serum of the animals of groups III, VII and IX, the CSF virus was detected on the 6th and 8th day, then the viremia was found in at least 80% of the animals of these groups. At the same time, in the groups I, II, IV, V and VI, the peak of viremia occurred on the 6th day, and already from the 8th day less than half of the animals contained the virus in the blood, after that the share of positively reacting animals continued to decrease (see Fig. 2, B). It is known that the absence of sterile immunity is characteristic of both live and subunit vaccines against CSF, however, a significant decrease in viral load allows us to conclude that this vaccine is highly effective [25].

The analysis of the obtained data shows that the vaccine preparations used in groups I, IV, and VI have ensured a sufficient degree of protection after the control challenge. However, the emulsified preparation with the antigen dose of 30 μg, having demonstrated good results in average, has not prevented the lethal development of the disease in two piglets. Moreover, even a single application of this preparation was followed by the development of reactogenicity (Fig. 3, A, B) that excludes the possibility of using such preparations in production conditions. The comparison of the efficiency of the application schedules with 1-time and 2-time vaccination testifies about the advantage of the latter one that is consistent with the data previously obtained from other researchers [1, 26].

Some authors report that single application of the E2-based recombinant subunit vaccine showed itself to be effective [27, 28] that may be due to the successful choice of the adjuvant, the introduction in the vaccine composition of additional immunostimulating molecules and using the piglets being completely naive to the CSF virus.

It is worth noting individually that it is necessary to include in the vaccine composition of the E2 proteins of both genotypes (group IV). Only one of

the four antigenic epitopes of E2 in the main genotypes of the CSFV is conservative [1, 29, 30] that may lead to lowered efficiency of the recombinant vaccine when infection with heterologous strains of the CSFV [31]. Considering the fact that in the territory of Russia the circulation of: 1 (historical) and 2 [32, 33] CSFV genotypes are shown, the necessity to include in the vaccine composition of the E2 proteins of the both CSFV genotypes is not in doubt. The researches we performed have shown that such vaccine by its efficiency is not inferior to the preparation based on the E2 protein of the genotype 1 of the CSF virus. However, the oil adjuvant used in the manufacture of this preparation caused a reactogenicity, therefore, it is necessary to compare the efficiency of the preparations based on synthetic polyacrylate including those when inoculation of the animals with highly virulent strains of different CSFV genotypes.

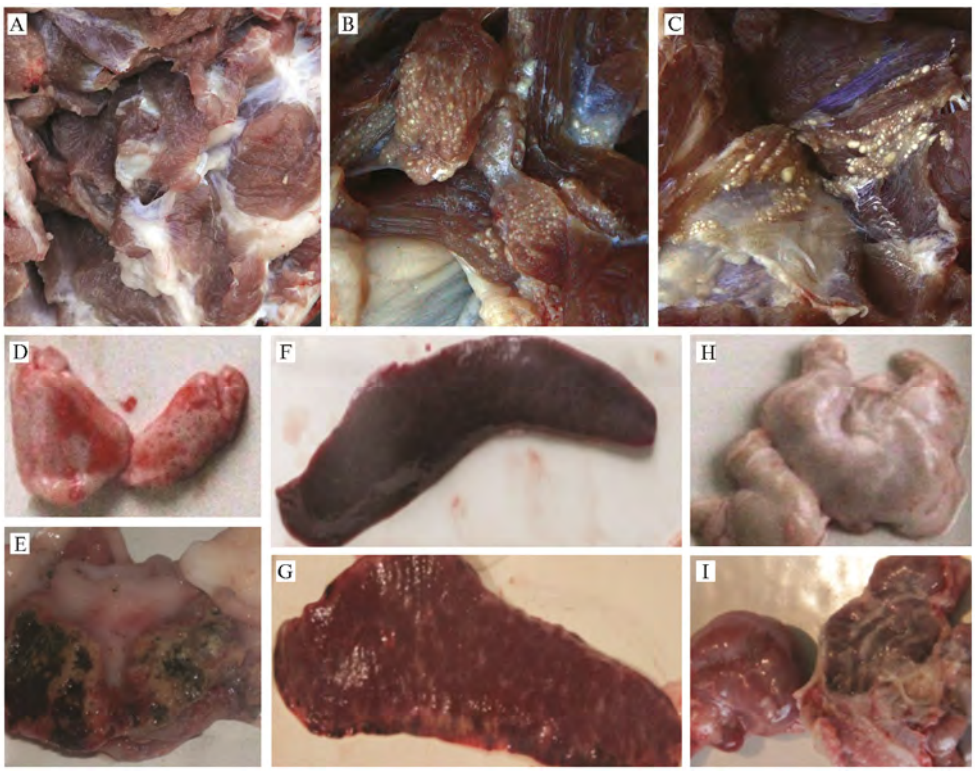

Fig. 3. Reactogenicity upon immunization and pathological changes in piglets challenged with CSF virus upon different vaccination schedules and CSF subunit oil adjuvant vaccines (research facilities of Lisiy Ostrov, FSC VIEV RAS, Vyshny Volochyok; see the description in Table 1).

A: The muscle tissue is unchanged, the animal of group VIII.

B: Muscle tissue, the injection site, the animal of group VIII. Diffusely spaced white milliary nodules are visible.

C: Muscle tissue, the injection site, the animal of group V. Diffusely spaced white diffuse miliary nodules are visible.

D: Tonsil of the animal of group I. Without changes.

E: Tonsil of the animal of group IX. Erosive-ulcerative lesion of the mucous coat with the area of necrosis (black) and the strongly pronounced inflammatory reaction around the pathological region.

F: Spleen of the animal of group I. Elastic consistency. The edges are sharp.

G: Spleen of the animal of group IX. From the parietal and visceral surface of the necrosis area of gray-white color, local regions of hemorrhages located mainly along the organ's edge.

H: Mesenteric lymph node of the animal of group I. Without changes.

I: Mesenteric lymph node of the animal of group IX. The capsule is sharply tense, the parenchyma at the section has extensive hemorrhages.

So, as the result of the researches, the preparation based on the recom-

binant vaccine against the classical swine fever (CSF), which combines safety and high efficiency, has been found. The two-time immunization with the vaccine containing E2 (60 μg/dose) of the 8Z strain of genotype 1 of CSFV and based on synthetic polyacrylate as an adjuvant ensured the apparent humoral immune response with the high degree of defense against the clinical manifestations of CSF and has not followed by the manifestation of reactogenicity. The application of the recombinant vaccine against the CSF has made it possible to differentiate the vaccinated and infected animals.

REFERENCES

1. Chander V., Nandi S., Ravishankar C., Upmanyu V., Verma R. Classical swine fever in pigs: recent developments and future perspectives. *Animal Health Research Reviews*, 2014, 15(1): 87-101 (doi: 10.1017/S1466252314000024).
2. Beltran-Alcrudo D., Falco J.R., Raizman E., Dietze K. Transboundary spread of pig diseases: the role of international trade and travel. *BMC Veterinary Research*, 2019, 15(1): 64 (doi: 10.1186/s12917-019-1800-5).
3. Zhou B. Classical swine fever in China — an update minireview. *Frontiers in Veterinary Science*, 2019, 6: 187 (doi: 10.3389/fvets.2019.00187).
4. Sergeev V.A., Orlyankin B.G., Alekseev K.P., Zaberezhnyi A.D., Aliper T.I., Nepoklonov E.A. *Veterinariya*, 2018, 4: 3-11.
5. Kirkland P.D., Le Potier M.-F., Vannier P., Finlaison D. *Pestiviruses*. In: *Diseases of swine*. J.J. Zimmerman, L.A. Karriker, A. Ramirez, K.J. Schwartz, G.W. Stevenson (eds.). Wiley-Blackwell, 2012: 538-553.
6. Rümenapf T., Meyers G., Stark R., Thiel H.J. Molecular characterization of hog cholera virus. In: *Ruminant Pestivirus infections. Archives of Virology (Supplementum 3), vol. 3*. B. Liess, V. Moennig, J. Pohlenz, G. Trautwein (eds.). Springer, Vienna, 1991: 7-18 (doi: 10.1007/978-3-7091-9153-8_2).
7. Lindenbach B.D., Muttay C.L., Thiel H.J. *Flaviviridae*. In: *Fields virology*. D.M. Knipe, P.M. Howley (eds.). Lippincott Williams and Wilkins, Philadelphia, 2013: 712-746.
8. Thiel H.J., Stark R., Weiland E., Rümenapf T., Meyers G. Hog cholera virus: molecular composition of virions from a pestivirus. *Journal of Virology*, 1991, 65: 4705-4712.
9. Wang F.I., Deng M.C., Huang Y.L., Chang C.Y. Structures and Functions of Pestivirus Glycoproteins: Not Simply Surface Matters. Viruses, 2015, 7(7): 3506-3529 (doi: 10.3390/v7072783)
10. Weiland E., Stark R., Haas B., Rьmenapf T., Meyers G., Thiel H.J. Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. *Journal of Virology*, 1990, 64(8): 3563-3569.
11. Nepoklonov E.A., Aliper T.I., Leneva I.A. *Voprosy virusologii*, 1999, 2: 54-60 (in Russ.).
12. Chang C.Y., Huang C.C., Lin Y.J., Deng M.C., Chen H.C., Tsai C.H., Chang W.M., Wang F.I. Antigenic domains analysis of classical swine fever virus E2 glycoprotein by mutagenesis and conformation-dependent monoclonal antibodies. *Virus Research*, 2010, 149(2): 183-189 (doi: 10.1016/j.virusres.2010.01.016).
13. Schroeder S., von Rosen T., Blome S., Loeffen W., Haegeman A., Koenen F., Uttenthal A. Evaluation of classical swine fever virus antibody detection assays with an emphasis on the differentiation of infected from vaccinated animals. *Revue Scientifique et Technique (International Office of Epizootics)*, 2012, 31(3): 997-1010 (doi: 10.20506/rst.31.3.2173).
14. Nepoklonov E.A. *Klassicheskaya chuma svinei: razrabotka metodov laboratornoi diagnostiki i sredstv spetsificheskoi profilaktiki. Doktorskaya dissertatsiya* [Classical swine fever: the development of laboratory diagnostic methods and means of specific prevention. DSc Thesis]. Moscow, 2000 (in Russ.).
15. Blome S., Mos C., Reimann I., König P., Beer M. Classical swine fever vaccines — State-of-the-art. *Veterinary Microbiology*, 2017, 206: 10-20 (doi: 10.1016/j.vetmic.2017.01.001).
16. Larson K.L., Zaabel P., Spickler A.R., Roth J.A. *NAHEMS Guidelines: Vaccination for Contagious Diseases, Appendix B: Vaccination for Classical Swine Fever*. USDA, 2017.
17. Hulst M.M., Westra D.F., Wensvoort G., Moormann R.J. Glycoprotein E1 of hog cholera virus expressed in insect cells protects swine from hog cholera. *Journal of Virology*, 1993, 67: 5435-5442.
18. König M., Lengsfeld T., Pauly T., Stark R., Thiel H.-J. Classical swine fevervirus: independent induction of protective immunity by two structural glycoproteins. *Journal of Virology*, 1995, 69: 6479-6486.
19. Huang Y.-L., Denga M.-C., Fun-In Wang F.-I., Huang C.-C., Chang C.-Y. The challenges of classical swine fever control: Modified live and E2 subunit vaccines. *Virus Research*, 2014, 179: 1-11 (doi: 10.1016/j.virusres.2013.10.025).
20. Dong X.N., Chen Y.H. Marker vaccine strategies and candidate CSFV marker vaccines. *Vaccine*, 2007, 25(2): 205-230 (doi: 10.1016/j.vaccine.2006.07.033).
21. Krivonos A.V., Zaberezhnyi A.D., Grebennikova T.V., Aliper T.I., Musienko M.I., Gibadulin

R.A., Tsibezov V.V., Bogdanova V.S., Kal'nov S.L., Aliper T.I., Nepoklonov E.A. *Voprosy virusologii*, 2000, 2: 29-36 (in Russ.).
22. Tsibezov V.V., Bogdanova V.S., Zaberezhnyi A.D., Grebennikova T.V., Krivonos A.V., Dudnikov L.A., Kal'nov S.L., Aliper T.I., Nepoklonov E.A. *Voprosy virusologii*, 2000, 2: 36-41 (in Russ.).
23. Klinkenberg D., Moormann R.J.M., de Smit A.J., Bouma A., de Jong M.C.M. Influence of maternal antibodies on efficacy of a subunit vaccine: transmission of classical swine fever virus between pigs vaccinated at 2 weeks of age. *Vaccine*, 2002, 20: 3005-3013 (doi: 10.1016/S0042-207X(02)00283-X).
24. Lipowski A., Drexler C., Pejsak Z. Safety and efficacy of a classical swine fever subunit vaccine in pregnant sows and their offspring. *Veterinary Microbiology*, 2000, 77(1-2): 99-108 (doi: 10.1016/S0378-1135(00)00266-2).
25. van Oirschot J.T. Vaccinology of classical swine fever: from lab to field. *Veterinary Microbiology*, 2003, 96(4): 367-384 (doi: 10.1016/j.vetmic.2003.09.008).
26. Bouma A., de Smit A.J., de Kluijver E.P., Terpstra C., Moormann R.J. Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever virus. *Veterinary Microbiology*, 1999, 66(2): 101-114 (doi: 10.1016/S0378-1135(99)00003-6).
27. Madera R., Gong W., Wang L., Burakova Y., Lleellish K., Galliher-Beckley A., Nietfeld J., Henningson J., Jia K., Li P., Bai J., Schlup J., McVey S., Tu C., Shi J. Pigs immunized with a novel E2 subunit vaccine are protected from subgenotype heterologous classical swine fever virus challenge. *BMC Veterinary Research*, 2016, 12(1): 197 (doi: 10.1186/s12917-016-0823-4).
28. Suárez M., Sordo Y., Prieto Y., Rodríguez M.P., Méndez L., Rodríguez E.M., Rodríguez-Mallon A., Lorenzo E., Santana E., González N., Naranjo P., Frías M.T., Carpio Y., Estrada M.P. A single dose of the novel chimeric subunit vaccine E2-CD154 confers early full protection against classical swine fever virus. *Vaccine*, 2017, 35(34): 4437-4443 (doi: 10.1016/j.vaccine.2017.05.028).
29. van Rijn P.A., Miedema G.K., Wensvoort G., van Gennip H.G., Moormann R.J. Antigenic structure of envelope glycoprotein E1 of hog cholera virus. *Journal of Virology*, 1994, 68(6): 3934-3942.
30. Yu M., Wang L.F., Shiell B.J., Morrissy C.J., Westbury H.A. Fine mapping of a C-terminal linear epitope highly conserved among the major envelope glycoprotein E2 (gp51 to gp54) of different pestiviruses. *Virology*, 1996, 222(1): 289-292 (doi: 10.1006/viro.1996.0423).
31. Huang Y.L., Deng M.C., Wang F.I., Huang C.C., Chang C.Y. The challenges of classical swine fever control: modified live and E2 subunit vaccines. *Virus Research*, 2014, 179: 1-11 (doi: 10.1016/j.virusres.2013.10.025).
32. Vlasova A., Grebennikova T., Zaberezhny A., Greiser-Wilke I., Floegel-Niesmann G., Kurinnov V., Aliper T., Nepoklonov E. Molecular epidemiology of classical swine fever in the Russian Federation. *Journal of Veterinary Medicine and Infectious Disease Veterinary Public Health*, 2003, 50(8): 363-367 (doi: 10.1046/j.1439-0450.2003.00695.x).
33. Titov I., Tsybanov S., Malogolovkin A. Genotyping of classical swine fever virus using high-resolution melt analysis. *Journal of Virological Methods*, 2015, 224: 53-57 (doi: 10.1016/j.jviromet.2015.08.012).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1247-1256
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.2/.3:619:578

doi: 10.15389/agrobiology.2019.6.1247eng
doi: 10.15389/agrobiology.2019.6.1247rus

DETECTION OF SCHMALLENBERG VIRUS IN CATTLE IMPORTED INTO THE RUSSIAN FEDERATION

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The authors declare no conflict of interests
Received December 27, 2018

Abstract

Schmallenberg disease, a transmissive vector-borne arbovirus infection affecting cattle, sheep and goats of all age groups, which may lead to significant economic losses. In the Russian Federation, specific antibodies against the causative agent of Schmallenberg disease were first detected in April 2013 and 2014 in the Andreapolsky and Konakovsky districts of the Tver region. In October 2014, specific antibodies were detected in cattle blood in one of the farms of the Novosokolnichesky district of the Pskov region. In this study we provide an overview of the serological and molecular surveillance for Schmallenberg virus (SBV) in the Russian Federation in 2014-2016. Testing of serum samples from cattle born and raised in regions sharing border with previously affected EU countries demonstrated seroconvertion in sentinel animals. The current findings raise concerns with regard to a possible SBV distribution and circulation in Russian cattle. We for the first time report the detection and isolation of SBV in cattle imported into Russia from EU. The virus was isolated on Vero cells and sequenced. The S gene sequence analysis showed 100 % identity of the recovered SBV strain to those isolated in Northern Europe in 2012.

Keywords: Schmallenberg virus (SBV), continuous Vero cell culture, polymerase chain reaction (PCR), phylogenetic analysis, sequencing

For the first time, the disease caused by the Schmallenberg virus was discovered in North Rhine-Westphalia (Germany) and in the northwest of the Netherlands. It is a transmissive arbovirus infection affecting cattle, sheep and goats of all age groups, which is characterized by fever, exhaustion and decrease of milk yield. The distinctive feature of the disease is that the contamination of animals at the stage of pregnancy leads to abortions and stillbirths that entails significant economic losses in case of mass spread of the infection [1].

The causative agent of the ShV belongs to the *Bunyaviridae* family of the *Orthobunyavirus* genus of the *Simbu* serogroup. ShV has the genome consisting of S (short), M (medium) and L (long) segments and is represented by single-stranded RNA [1, 2]. The disease's incubation period lasts from 1 to 4 days, and the viremia – from 1 to 5 days. Upon the experimental challenge of sheep and goats, the clinical signs appear on the 3rd and 5th days [3]. In infected fetuses, the virus is primarily found in the brain. In sick animals, the virus can be isolated from the blood. In molecular studies, the genome of the Schmallenberg virus can be detected in the blood, organs of infected fetuses, in placenta, amniotic fluid, and meconium [4].

The ShV is transmitted in a vector-borne way through blood-sucking insects which are spread throughout the territory of the Russian Federation [5].

The significance of the ShV transmitting agents under agroclimatic conditions of Russia (taking into account the found out biodiversity of biting midges) is covered in the review of A.V. Sprygin et al. [6]. The important role in the animals' contamination is played by the season of a year, depending on which the population of blood-sucking insects varies. The main biological transmitters of the Schmallenberg virus are the biting midges belonging to the *Ceratopogonidae* family of the *Culicoides* genus, which are ones of the smallest blood-sucking dipteran insects of gnats [5]. It was found out that this virus replicates in the salivary glands of female biting midges for 4-16 days depending on the ambient temperature and humidity [3, 7]. Previously, entomological studies showed that ShV has been found out in the pools of biting midges (*Culicoides obsoletus, C. dewulfi, C. pulicaris, C. punc-tatus*), selected in the territories of a number of European countries (Denmark, Norway, Netherlands, Germany, Belgium, Italy and Poland) [8-11]. It is known that ShV is transmitted also by bites of other bloodsucking insects including mosquitoes of the *Culicidae* family [12, 13]. To date, the vertical transmission of this virus through a placenta has been proven, wherein the direct transmission is unlikely. Also, ShV is found in the semen and embryos taken from infected farm animals [14]. The properties of ShV are described in more detail in the review of A.V. Sprygin et al. [7].

The distinctive epidemiological feature of ShV is a high interherd and low intraherd prevalence. The researches conducted in the period from June to September 2016 on sheep in Belgium showed the significant increase in the total (from 25 to 62%) and interherd (from 60 to 96%) seroprevalence in respect of the Schmallenberg virus that indicates the most widespread recirculation of this pathogen since its first appearance in 2011. The ShV circulation has been confirmed by the detection of the virus's RNAs in the pools of *Culicoides obsoletus* biting midges collected around the Antwerp city (Belgium) in August 2016, wherein the minimum morbidity rate of the animals reached 3% [15].

The ShV outbreaks were detected in 2012-2014 in the EU countries (Netherlands, Germany, Belgium, England, France, Italy, Spain, Denmark, Luxembourg, Switzerland, Sweden, Austria, Poland, Finland, Ireland, Norway, Greece, Slovenia, Latvia and other ones) [15, 16]. In the period of 2011-2012 in the territory of Russia, the monitoring of the imported and local animals had been carried out, which has not found the antibodies to ShV in the local farm animals [17]. For the first time in the territory of the Russian Federation, the specific antibodies to ShV were detected in April 2013 and 2014 in the Andreapolsky and Konakovsky districts of the Tver region when investigating the blood serum from cattle in the course of the epizootic monitoring [18, 19]. In October 2014, the specific antibodies to ShV were also found in the blood of cattle in one of the farms in the Novosokolniki district of the Pskov region [20].

The Schmallenberg virus is diagnosed basing on the clinical signs and detection of the viral genome by the PCR method in real-time [21], as well as basing on the virus isolation in insect cell cultures (KC), Syrian hamster kidney (ANC), African green monkey kidney (Vero) or its detection (as the virus kindred to the Akabane disease virus) when intracerebral infection of sucking mice. ShV can be isolated from the blood of infected adult animals and from different tissues of an infected fetus, in particular, from brain biomaterial [22]. For serological diagnostics, the enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence and the virus neutralization test are used [23].

The insufficient knowledge of the causative agent of ShV and unavailability of the precise data about its transmitters in the territory of Russia stimulate the monitoring studies of both the farm animals bred in the territory of the Russian Federation and the animals imported from the EU.

In this publication, for the first time we have summarized the results of the monitoring studies on ShV conducted in Russia from 2014 to 2016. Also, the detection of the PCR-positive animals imported from the EU in 2016 and the discovery in 2015 and 2016 of the seroconversion to Schmallenberg virus in cattle animals born and raised in the regions of Russia, which border on the EU countries where the situation with the Schmallenberg virus is adverse, are described for the first time.

The objective of our work is to accomplish the monitoring molecular-biological investigations of the samples of blood serum and blood from the imported and native cattle animals for the presence of the Schmallenberg virus.

Techniques. We used the samples of stabilized blood and serum from the quarantine animals (heifers) imported from EU countries to Russia, as well as from the local cattle animals. A total of 33,542 stabilized blood samples and 16,749 serum samples of the animals from Voronezh, Vladimir, Kaluga, Bryansk, Ryazan, Rostov, Kirov, Kaliningrad, Leningrad, Ivanovo, Kirov, Tyumen, Rostov, Yekaterinburg, Vologda, Moscow, Pskov, Smolensk, Volgograd, Tver, Lipetsk, Sverdlovsk, Novosibirsk, Tula, Nizhny Novgorod and Kursk regions, Altai Krai, Krasnoyarsk Krai, Karachay-Cherkess Republic, Republic of Bashkortostan, Republic of Crimea, Chuvash Republic, Republic of Dagestan, Republic of Tatarstan, from the Republic of Tajikistan, as well as from the EU countries: France, Germany, Hungary, Austria, Poland, Denmark, Netherlands, Czech Republic, Slovakia and Finland have been investigated. All the samples were taken within the period from 2014 to 2017.

The antibodies to ShV had been detected in these bovine sera using the ID Screen® Capripox Double Antigen Multi-species commercial kit according to the instructions of the manufacturer ("IDvet", France).

To assess the serological status of the cattle animals born and raised in the territory of the Russian Federation (aboriginal cattle) in some regions, the blood serum samples taken from the animals in the spring and autumn periods have been used. The cases when the antibodies were absent in such samples taken in the March-May period, but were detected in the autumn period in the animals which before were seronegative, have been considered as seroconversion.

The total RNA had being isolated from 100 μl of stabilized blood using the QIAamp Viral RNA kit ("Qiagen", Germany).

The PCR in real-time (RT PCR) has been performed according to the protocol described hereinbefore [21].

The isolate of Schmallenberg virus isolated from the cattle animals imported to the Kaliningrad region from Germany had being identified in regards to the S segment using the described primers [24]. The primary nucleotide sequence had being determined with the forward and reverse primers (Applied Biosystems® 3130l Genetic Analyzer, "Applied Biosystems, Inc.", USA). The nucleotide sequences had being equalized using the BioEdit program (https://softfamous.com/bioedit/), the dendrograms have been plotted using the ME-GA 4 software (http://www.megasoftware.net/).

The ShV was isolated from stabilized blood. The blood cells were precipitated by centrifugation for 10-15 minutes at 2,000 rpm, then resuspended with overbuffer saline (OBS, pH 7.2-7.4) and reprecipitated; this procedure was repeated three times. The washed cell fraction was resuspended in sterile OBS having adjusted the volume to the original value. In the obtained samples, the cells were destroyed by ultrasonic vibration (Sonopuls HD 3100, "Bandelin electronic GmbH & Co. KG", Germany) under the amplitude of 16-18 μm (2 times for 30 seconds each with the time interval of 60 seconds). The resulting material was used for the virus isolation in the passaged cell culture of the cells of African

green monkey (Vero). The stages of isolation had being monitored by the real time PCR method.

The cells were grown in 50 cm^3 plastic culturing bottles ("SPL Life Sciences Co., Ltd", Korea) until the formation of a monolayer. After that, the growth medium (manufactured by the FSBI ARRIAH (Federal State Budgetary Institution "All-Russian Research Institute for Animal Health") was removed, the virus-containing material was added and the resulting mixture was incubated for 1 hour at 37°C to adsorb the virus on the monolayer, then the supporting nutrient medium (SNM, manufactured by the FSBI ARRIAH) was added. The infected cell culture had been being examined daily using the microscope for the presence of the characteristic morphological changes. Upon the manifestation of the cytopathic effect (CPE) in 70-80% of the monolayer's area, the virus-containing material was frozen at the temperature of −80°C. The infectious activity of the viral material was determined by microtitring in the Vero cell culture using the conventional method. The virus's titer was calculated by the Reed and Mench method and expressed as lg TCD_{50}/cm^3. The presence of the virus's genome had been confirmed by the real time PCR [21].

Results. In order to monitor the spreading of the Schmallenberg virus, in the period from 2014 to 2016, the monitoring investigations of the bovine blood sera samples taken from the aboriginal animals from the regions of the Russian Federation, as well as of the blood serum samples taken from the animals imported to Russia from the EU countries (a total of 16,749 samples) had been being performed (Table 1, 2). The most part of seropositive animals have been identified in the Kaliningrad, Voronezh and Pskov regions (see Table 2).

1. Prevalence of blood antibodies to Schmallenberg virus in cattle imported into Russia (ELISA test, 2014)

Exporting country	Importing region	Test samples	
		total	seropositive ones, %
France	Voronezh region	522	73.9
Germany	Voronezh region, Kaliningrad region	137	5.8
Czechia	Kursk region	10	0
Sweden	Voronezh region, Tyumen region	160	47.5
Hungary	Kaliningrad region	140	2.8
Austria	Voronezh region	21	0
Poland	Republic of Tatarstan	74	40.5
Denmark	Republic of Tatarstan	408	12.5
Netherlands	Vladimir region	53	0
Slovakia	Kaliningrad region	32	0

2. Prevalence of blood antibodies to Schmallenberg virus in domestic cattle (ELISA test)

Region	Total/positive samples		
	2014	2015	2016
Voronezh region	1345/744	ni	ni
Vladimir region	340/0	ni	ni
Tyumen region	642/263	ni	ni
Tver region	ni	71/0	ni
Kaliningrad region	2273/1488	500/297	431/163
Pskov region	1756/368	645/196	504/50
Nizhny Novgorod Region	1476/107	ni	443/0
Kursk region	296/0	ni	ni
Republic of Tatarstan	1933/63	500/63	ni
The Republic of Dagestan	457/16	ni	ni
Republic of Crimea	ni	621/0	ni
Karachay-Cherkess Republic	959/106	ni	ni

N o t e. ni — not investigated.

Due to the fact of detection of the antibodies to ShV in the local cattle animals, which have been raised in the territory of Russia, in 2015-2016, within a framework of the state monitoring, the paired samples of blood sera taken

from the aboriginal cattle animals of the Kaliningrad and Pskov regions have been investigated (see Table 3).

3. Prevalence of antibodies to Schmallenberg virus in paired blood samples of domestic cattle from two Russian regions (ИФА-тест)

Province	District	Total/positive samples			
		2015		2016	
		spring	autumn	spring	autumn
Kaliningrad	Slavsk	36/0	36/2	36/0	36/7
	Nemansk	36/0	36/1	ni	ni
	Bagrationovsk	36/0	36/7	36/0	36/10
	Nesterov	36/0	36/0	36/0	36/0
	Pravdinsk	36/0	36/0	72/0	72/17
Pskov	Krasnoznamensk	ni	ni	36/0	36/9
	Ozyorsk	ni	ni	36/0	36/0
	Krasnogorodsk	32/0	32/2	36/0	36/0
	Sebezh	55/0	55/2	36/0	36/12
	Palkino	55/0	55/1	ni	ni
	Usvyaty	ni	ni	36/0	36/2
	Kunya	ni	ni	36/0	36/0
	Dedovichi	ni	ni	36/0	36/0

N o t e. ni — not investigated.

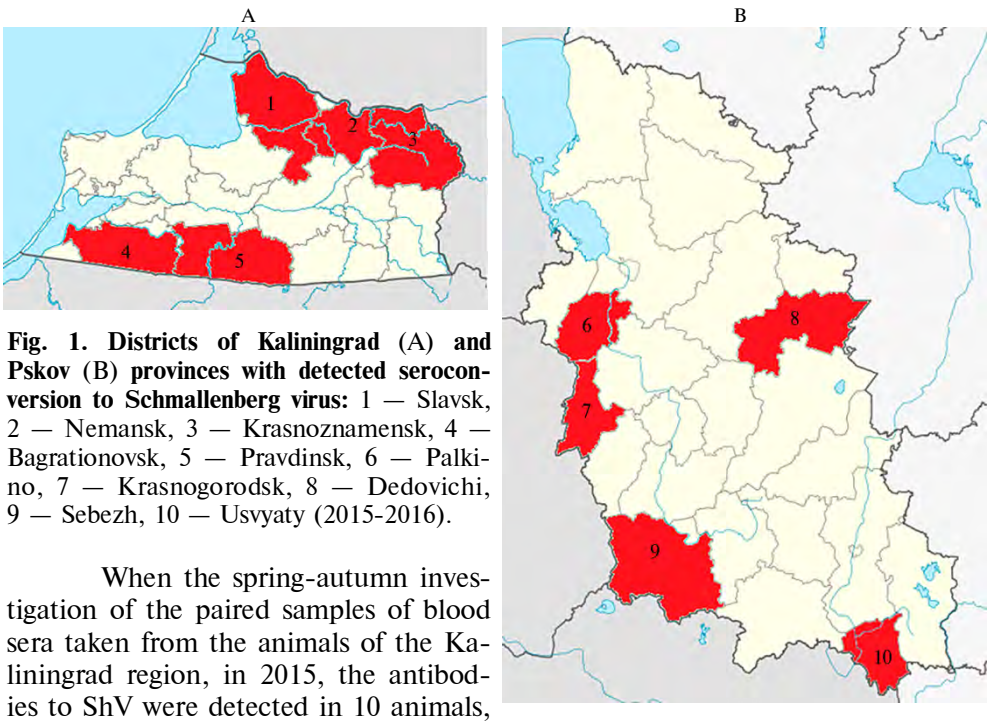

Fig. 1. Districts of Kaliningrad (A) **and Pskov** (B) **provinces with detected seroconversion to Schmallenberg virus:** 1 — Slavsk, 2 — Nemansk, 3 — Krasnoznamensk, 4 — Bagrationovsk, 5 — Pravdinsk, 6 — Palkino, 7 — Krasnogorodsk, 8 — Dedovichi, 9 — Sebezh, 10 — Usvyaty (2015-2016).

When the spring-autumn investigation of the paired samples of blood sera taken from the animals of the Kaliningrad region, in 2015, the antibodies to ShV were detected in 10 animals, in 2016 in 34 ones, and in the Pskov region in 5 and 14 animals, respectively. In each of the regions, the seroconversion was observed in 5 districts (Fig. 1).

When isolating ShV in the Vero cell culture, cytopathic changes were detected at 4th passage 96 hours after the inoculation. The virus's cytopathic effect in the Vero cell culture was initially manifested as the formation of pseudo-syncytium with the fusion of the outer membranes of the cells, later they rounded and merged into conglomerates. Upon the increase of the number of passages, the manifestation of cytopathic effect was found out after 48 hours of culturing (Fig. 2).

The matrasses with the infected cell culture of each passage were frozen, thawed, and the virus's infectious activity was determined in the Vero cell cul-

ture. The titer of infectious activity amounted to 2.83±0.14 lg TCD_{50}/cm^3 at the 5th passage, 3.33±0.00 lg TCD_{50}/cm^3 at the 6th passage, and 3.31±0.07 lg TCD_{50}/cm^3 at the 7th passage.

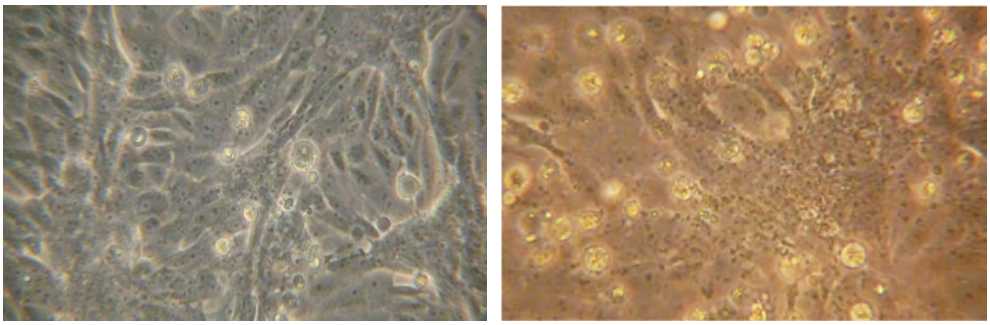

Fig. 2. Monolayers of the 2-day non-infected culture of Vero cells (leftward) **and the cells 48 hours after the infection with the Schmallenberg disease virus** (rightward). Light microscopy (Olympus microscope, Japan).

The 256 bp length fragment of the S segment encoding the nucleocapsid protein was amplified to confirm the isolates' belonging to certain species [24] (Fig. 3). The performed phylogenetic analysis showed that the Kaliningrad/2016 (diagnostic) isolate of ShV has 100% homology with the isolates of ShV found in the Northern Europe countries (see Fig. 3) (the isolate are deposited to the AR-RIAH strains collections).

Fig. 3. Phylogenetic position of the Schmallenberg virus Kaliningrad/2016 (diagnostic) isolate. The dendrogram shows the comparison of the nucleotide sequences of the 256 bp fragment of the genome S segment

Also, in the period from 2015 to 2017, the samples of bovine blood were examined for the presence of the Sh. virus's genome. The genome of this pathogen was found in three of 33,542 analyzed samples taken from the animals imported from Germany and Hungary in 2016-2017. Mainly the cattle animals imported from the Netherlands, Germany, Czech Republic, Hungary, Slovakia, France and the animals of local origin were investigated.

Since the detection of the Sh. virus in the territory of a number of EU countries in 2012 [1, 7], in the Russian Federation there has been a problem of the possible bringing in of this pathogen together with the breeding cattle. The course of infection is peculiar with the short duration of the viremia (3-5 days) [7] that significantly reduces the chances of detection of the virus's genome in sick animals during the intravital diagnostics [4]. Because of the absence of mandatory ShV notification in the EU, it is almost impossible to assess the risks of bringing in of this infection into our country due to the wide spread of the virus in the EU countries.

The situation with the spread of ShV is primarily due to the absence of dew attention to the problem from the veterinary and phytosanitary services of European countries despite the scale of the disease spread in these countries and the opinion of the experts of the European Food Safety Authority (EFSA) and the fact that without monitoring and mandatory notification of new cases of ShV it is impossible to assess the situation in a country in regard to this disease. The EU representatives continue to argue that the disease does not cause significant economic damage and does not pose a risk for the livestock sector. That is why the EU countries do not conduct transparent monitoring studies, and in a case of detection of the infection, a country just confines itself to recognizing its territory as endemic [15].

The results of researches of European scientists testify that since the cessation of the mass spread of Sh. virus, it has firmly entrenched itself in the territory of EU [15, 25-27]. In the period from March 3 to 10, 2017, in dairy herds of Ireland, the antibodies to this virus were detected in a total of 256 animals. Also, in the Belgian sheep population an increase in seroprevalence was also noted between June and September 2016 [15].

The Russian Federation is the main trading partner of a number of EU countries in the import of breeding cattle. Moreover, the transit of farm animals to Kazakhstan and other countries gets through the Russian Federation. Since the main transmitters of the Sh. virus are biting midges of the *Culicoides* genus, which are widespread throughout Russia, according to the reviews of the Russian entomofauna and analysis of the situation regarding the possible spread of ShV by its transmitters [6], this may potentially be the factor of the virus spreading in throughout the territory of the Russian Federation.

The serological researches performed in a number of regions of the Russian Federation (see Table 1) have showed that the aboriginal cattle animals are seropositive to the Schmallenber virus, however, according to the official statistics, any signs of congenital malformations in calves have not been found. In order to exclude the error, the analysis of paired samples of blood sera taken from the animals selected in the spring and autumn periods has been performed among the local (aboriginal) cattle animals of the Kaliningrad and Pskov regions. The results have demonstrated the circulation of the virus among the livestock without the manifestation of the clinical signs of the infection (see Table. 3) that testifies about necessity to perform in the listed regions of the entomological investigations of the biomaterial samples in the cases of clinical manifestation of teratogenic effect in pregnant animals. The largest number of seropositive aboriginal animals has been found in the Kaliningrad, Pskov and Voronezh regions in the areas bordering the EU countries. It is possible that the virus circulating in the border countries with the adverse situation is actively brought in to the territory of the Russian Federation by the transmitters or have already entrenched in the said regions. It is important to note that the results of the work of the scientists from the EU countries [15, 26-28] testify that the Schmallenber virus possibly became endemic in Europe.

The antibodies to Sh. virus have also been detected in cattle animals in the Republic of Dagestan (see Table 2). It is likely that the virus entered the territory of the republic from neighboring states (for example, Turkey), which reported the spread of ShV in their territory [27]. Currently numerous data about the spreading of the Sh. virus outside Europe and the Caucasus appear. Thus, in Ethiopia and Mozambique, a mass spread of this pathogen among cattle has been detected [29, 30]. Moreover, the antibodies to ShV have also been detected in cattle animals in China [31].

When investigating the blood sera for the presence of the Sh. virus's ge-

nome, it has been detected in the cattle animals imported from Hungary and Germany to the territory of the Kaliningrad and Tyumen regions. The detection of the antibodies to and the genome of the Schmallenber virus in cattle shows that this pathogen is already circulating in the territory of the Russian Federation and poses a serious threat in case of further spreading.

The S segment of the Kaliningrad/2016 isolate turned out to be 100% homologous to the S segment of the Schmallenber virus isolates found out in Europe (see Fig. 5). The Sh. virus is characterized by high genetic stability despite the fact that its genome is represented by single-stranded RNA. Previously, it was found out [32] that the Sh. virus isolates found in Hungary had 99.3% nucleotide homology. A group of scientists from Turkey also have found out that the Sh. virus was identical by its S segment to the isolates from Germany and Belgium (one nucleotide substitution) [24].

So, as the result of the researches we performed, for the first time since the detection of the Schmallenber virus (ShV) in the EU, it has been reported about detection of the seroconversion of this pathogen in the aboriginal animals in the regions of Russia which border on the EU countries where the situation with ShV is adverse, that testifies about possible bringing in and uncontrolled spreading of the virus in the territory of the Russian Federation. Moreover, the conclusion that the virus could have been brought in earlier both with the imported animals and by the virus transmitters has been confirmed by the fact of detection of the PCR-positive animals imported from the EU. The sequencing of S segment in the viral isolate we isolated showed that it is identical to the ShV isolates circulating in the EU countries. The firm conclusion can be made upon the performance of the relevant entomological studies and the detection of the Schmallenber virus's genome in the virus-transmitting blood-sucking insects. Therefore, we plan the further studying of the potential virus transmitters which support the virus's persistence in the areas where the cases of seroconversion in the animals born and raised in Russia including those obtained from the imported animals have been detected.

REFERENCES

1. Hoffmann B., Scheuch M., Höper D., Jungblut R., Holsteg M., Schirrmeier H., Eschbaumer M., Goller K.V., Wernike K., Fischer M., Breithaupt A., Mettenleiter T.C., Beer M. Novel orthobunyavirus in cattle, Europe, 2011. *Emerg. Infect. Dis.*, 2012, 18: 469-472 (doi: 10.3201/eid1803.111905).
2. Yanase T., Kato T., Aizawa M., Shuto Y., Shirafuji H., Yamakawa M., Tsuda T. Genetic reassortment between Sathuperi and Shamonda viruses of the genus *Orthobunyavirus* in nature: implications for their genetic relationship to Schmallenberg virus. *Arch. Virol.*, 2012, 157: 1611-1616 (doi: 10.1007/s00705-012-1341-8).
3. Muskens J., Smolenaars A.J., Van der Poel W.H., Mars M.H, van Wuijckhuizen L., Holzhauer M., van Weering H., Kock P. Diarree en productiedaling op Nederlandse melkbedrijven door het Schmallenbergvirus. *Tijdschr. Diergeneeskd.*, 2012, 137: 112-115.
4. Kolbasov D., Sal'nikov N., Nikitina E., Lunitsin A. *Zhivotnovodstvo Rossii*, 2012, 11: 35-36 (in Russ.).
5. Glukhova B.M. *Fauna SSSR. Nasekomye dvukrylye* [Fauna of the USSR. Insects Diptera]. Leningrad, 1989 (in Russ.).
6. Sprygin A.V., Fedorova O.A., Babin Yu.Yu., Kononov A.V., Karaulov A.K. Blood-sucking midges from the genus *Culicoides* (*Diptera: Ceratopogonidae*) act as filed vectors of human and animal diseases (review). *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2015, 50(2): 183-197 (doi: 10.15389/agrobiology.2015.2.183eng).
7. Sprygin A.V., Kononov A.V., Babin Yu.Yu., Mishchenko V.A. Schmallenberg virus disease: molecular biology and clinical presentation (review). *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2012, 6: 24-34 (doi: 10.15389/agrobiology.2012.6.24rus) (in Russ.).
8. Barber J., Harrup L.E., Silk R., Veronesi E., Gubbins S., Bachanek-Bankowska K., Carpenter S. Blood-feeding, susceptibility to infection with Schmallenberg virus and phylogenetics of *Culicoides* (*Diptera*:*Ceratopogonidae*) from the United Kingdom. *Parasites & Vectors*, 2018, 11(1):

116 (doi: 10.1186/s13071-018-2650-x).
9. De Regge N. Akabane, Aino and Schmallenberg virus — where do we stand and what do we know about the role of domestic ruminant hosts and *Culicoides* vectors in virus transmission and overwintering? *Curr. Opin. Virol.*, 2017, 27: 15-30 (doi: 10.1016/j.coviro.2017.10.004).
10. Pagиs N., Talavera S., Verdún M., Pujol N., Valle M., Bensaid A., Pujols J. Schmallenberg virus detection in *Culicoides* biting midges in Spain: first laboratory evidence for highly efficient infection of *Culicoides* of the *Obsoletus complex* and *Culicoides imicola*. *Transbound. Emerg. Dis.*, 2018, 65(1): 1-6 (doi: 10.1111/tbed.12653).
11. Rasmussen L.D., Kristensen B., Kirkeby C. *Culicoides* as vectors of Schmallenberg virus. *Emerg. Infect. Dis.*, 2012, 18: 1204-1206 (doi: 10.14202/vetworld.2018.30-33).
12. Elliott R.M., Blakqori G. Molecular biology of orthobunyaviruses. In: *Bunyaviridae: molecular and cellular biology*. A. Plyusnin, R.M. Elliott (eds.). Norfolk, UK, 2011: 1-39.
13. Saeed M.F., Li L., Wang H., Weaver S.C., Barrett A.D. Phylogeny of the Simbu serogroup of the genus *Bunyavirus*. *J. Gen. Virol.*, 2001, 82(9): 2173-2181 (doi: 10.1099/0022-1317-82-9-2173).
14. Kęsik-Maliszewska J., Larska M. Detection of Schmallenberg virus RNA in bull semen in Poland. *Pol. J. Vet. Sci.*, 2016, 19(3): 655-657 (doi: 10.1515/pjvs-2016-0083).
15. Sohier C., Deblauwe I., VanLoo T., Hanon J.B., Cay A.B., DeRegge N. Evidence of extensive renewed Schmallenberg virus circulation in Belgium during summer of 2016 — increase in arthrogryposis-hydranencephaly cases expected. *Transbound. Emerg. Dis.*, 2017, 64(4): 1015-1019 (doi: 10.1111/tbed.12655).
16. Beer M., Conraths F.J., van der Poel W.H. 'Schmallenberg virus' — a novel orthobunyavirus emerging in Europe. *Epidemiology and Infection*, 2013, 141(1): 1-8 (doi: 10.1017/S0950268812002245).
17. Byadovskaya O.P., Zimina E.E., Piskunov A.V., Babin Y.Y., Sprygin A.V., Kononov A.V. Serological surveillance of Schmallenberg virus infection in local and import cattle in the Russian Federation. *Proc. Int. Conf. «Primed for tomorrow», Denmark*. Copenhagen, 2014: 6.
18. *Epizooticheskaya situatsiya: informatsionnoe soobshchenie № 84 ot 29.04.2014 g. Informatsionno-analiticheskii tsentr Rossel'khoznadzora (elektronnyi resurs)* [Epizootic situation: information message No. 84 dated April 29, 2014. Information and Analytical Center of the Rosselkhoznadzor (electronic resource)]. Available: http://www.fsvps.ru/fsvps/iac/messages/1554.html. No date (in Russ.).
19. *Epizooticheskaya situatsiya: informatsionnoe soobshchenie № 58 ot 9.04.2013 g. Informatsionno-analiticheskii tsentr Rossel'khoznadzora (elektronnyi resurs)* [Epizootic situation: information message No. 58 dated April 9, 2013. Information and Analytical Center of the Rosselkhoznadzor (electronic resource)]. Available: http://www.fsvps.ru/fsvps/iac/messages/1271.html. No date (in Russ.).
20. *Epizooticheskaya situatsiya: informatsionnoe soobshchenie № 224 ot 23.10.2014 g. Informatsionno-analiticheskii tsentr Rossel'khoznadzora (elektronnyi resurs)* [Epizootic situation: information message No. 224 dated October 23, 2014. Information and Analytical Center of the Rosselkhoznadzor (electronic resource)]. Available: http://www.fsvps.ru/fsvps/iac/messages/1222.html. No date (in Russ.).
21. Bilk S., Schulze C., Fischer M., Beer M., Hlinak A., Hoffmann B. Organ distribution of Schmallenberg virus RNA in malformed newborns. *Vet. Microbiol.*, 2012, 159(1-2): 236-238 (doi: 10.1016/j.vetmic.2012.03.035).
22. *OIE. Manual of diagnostic tests and vaccines for Terrestrial animals 2018 (elektronnyi resurs)*. Available: http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/. No date.
23. Zentis H.J., Zentis S., Stram Y. Schmallenberg virus: lessons from related viruses. *Vet. Rec.*, 2012, 171(8): 201-202 (doi: 10.1136/vr.e5653).
24. Yilmaz H., Hoffmann B., Turan N., Cizmecigil U.Y., Richt J.A., Van der Poel W.H. Detection and partial sequencing of Schmallenberg virus in cattle and sheep in Turkey. *Vector-Borne and Zoonotic Diseases*, 2014, 14(3): 223-225 (doi: 10.1089/vbz.2013.1451).
25. Collins Á.B., Barrett D.J., Doherty M.L., McDonnell M., Mee J.F. Significant re-emergence and recirculation of Schmallenberg virus in previously exposed dairy herds in Ireland in 2016. *Transbound. Emerg. Dis.*, 2017, 64(5): 1359-1363 (doi: 10.1111/tbed.12685).
26. Gache K., Zientara S., Collin E., Authié E., Dion F., Garin E., Zanella G., Calavas D. Spatial and temporal patterns of Schmallenberg virus in France in 2016. *Vet Rec.*, 2018, 182(20): 575 (doi: 10.1136/vr.104769).
27. Azkur A.K., Albayrak H., Risvanli A., Pestil Z., Ozan E., Yılmaz O., Tonbak S., Cavunt A., Kadı H., Macun H.C., Acar D., Özenç E., Alparslan S., Bulut H. Antibodies to Schmallenberg virus in domestic livestock in Turkey. *Trop. Anim. Health. Prod.*, 2013, 45(8): 1825-1828 (doi: 10.1007/s11250-013-0415-2).
28. Hoffmann B., Schulz C., Beer M. First detection of Schmallenberg virus RNA in bovine semen, Germany. *Vet. Microbiol.*, 2013, 167(3-4): 289-295 (doi: 10.1016/j.vetmic.2013.09.002).
29. Blomstrцm A.L., Stenberg H., Scharin I., Figueiredo J., Nhambirre O., Abilio A.P., Fafetine J., Berg M. Serological screening suggests presence of Schmallenberg virus in cattle, sheep and goat in the Zambezia Province, Mozambique. *Transbound. Emerg. Dis.*, 2014, 61(4): 289-292 (doi: 10.1111/tbed.12234).
30. Sibhat B., Ayelet G., Gebremedhin E.Z., Skjerve E., Asmare K. Seroprevalence of Schmal-

lenberg virus in dairy cattle in Ethiopia. *Acta Tropica*, 2018, 178: 61-67 (doi: 10.1016/j.actatropica.2017.10.024).
31. Zhai S.L., Lv D.H., Wen X.H., Zhu X.L., Yang Y.Q., Chen Q.L., Wei W.K. Preliminary serological evidence for Schmallenberg virus infection in China. *Trop. Anim. Health Prod.*, 2018, 50(2): 449-453 (doi: 10.1007/s11250-017-1433-2).
32. Fehér E., Marton S., Tyth Á.G., Ursu K., Wernike K., Beer M., Dán Б., Bányai K. Sequence analysis of Schmallenberg virus genomes detected in Hungary. *Acta Microbiologica et Immunologica Hungarica*, 2017, 64(4): 373-384 (doi: 10.1556/030.64.2017.038).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1257-1266
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.2:619:579

doi: 10.15389/agrobiology.2019.6.1257eng
doi: 10.15389/agrobiology.2019.6.1257rus

PHENOTYPIC RESISTANCE TO ANTIBIOTICS OF *Staphylococcus aureus* STRAINS ISOLATED FROM COW MILK

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The authors declare no conflict of interests
Supported financially by the Russian Science Foundation, project No. 15-16-00020-P
Received September 27, 2019

Abstract

The emergence of multiresistant strains has sharply escalated in recent decades due to the wide use of antimicrobials. The infections caused by *Staphylococcus aureus* are most common in highly productive dairy animals. The aim of our work was to assess the phenotypic resistance of *S. aureus* strains isolated from cow milk to the main antibacterial drugs used to treat various forms of mastitis in the Russian Federation, as well as to determine their minimum inhibitory concentrations (MIC, MIC_{50} and MIC_{90}). From January to December 2018 milk samples were taken from cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation. A total of 314 milk samples were examined and 447 potential staphylococci were isolated. All isolates were evaluated by conventional phenotypic methods. Only 103 isolates met all the identification criteria and were used for further analysis. The disk diffusion method (DDM) was used to test the susceptibility of the isolated strains to the following antibiotics: penicillin (PEN, 10 U), oxacillin (OX, 10 μg), gentamicin (GN, 10 μg), erythromycin (ER, 15 μg), and lincomycin (LN, 15 mcg), rifampicin (RF, 5 mcg), ciprofloxacin (CP, 5 mcg), vancomycin (VA, 30 μg), fusidine (FZ, 10 μg) (Pharmacotherapy Research Center, Russia; Mueller Hinton Agar, HiMedia Laboratories Pvt. Ltd., India). A method of double (Log_2) serial dilutions of antibiotics (penicillin, erythromycin, gentamicin, and ciprofloxacin) in TSB medium (from 64 to 0.125 mg/l) with 5×10^5 CFU/ml inoculum was used to determine the minimum inhibitory concentrations (MIC, MIC_{50}, MIC_{90}) and LD_{50}. The incubation was carried out at 37 °C for 20 hours (220 rpm). The bacterial growth in TSB medium with antibiotics was evaluated by plating on Baird Parker Agar medium (HiMedia Laboratories Pvt. Ltd., India). The interpretation of the results was carried out according to the recommendation of The European Committee on Antimicrobial Susceptibility Testing (EUCAST). The highest degree of resistance was observed to erythromycin (82.5 %) and fusidine (75.7 %). Seven of 103 strains tested were susceptible to all studied antibiotics, 96 isolates were resistant to at least one of them. When assessing multiple resistance (MAR), 65 (63.1 %) strains were resistant to four or more antibiotics. The predominant phenotypes for the isolates were ER + LN + FZ and ER + CP (58.3 % and 47.6 %, respectively). A total of 31 strains of the 47 isolates DDM-resistant to penicillin grew at ≥ 64 mg/l penicillin, while in the concentration range of ≤ 1.0 mg/l, the growth was observed in 33.0 % of the strains. Fifteen of the 85 erythromycin-resistant isolates showed growth at MIC ≥ 64 mg/l; only 7 strains out of 18 DDM-susceptible ones were in the MIC ≤ 0.5 mg/l range. A total of 29 (28.2 %) of the 103 studied strains were DDM-resistant to gentamicin, 72 (60.2 %) showed growth at MIC ≥ 0.5 mg/l, and 5 strains at MIC ≥ 64.0 mg/l. Only 2 of the 49 DDM-resistant strains grew at MIC ≥ 64 mg/l. It is important to point out that the MIC_{50} was not in the susceptible range for any of the studied antibiotics, and the MIC_{90} showed the susceptible range for penicillins, aminoglycosides, and fluoroquinolones (> 0.5, > 0.25, and > 0.25 mg/l, respectively). In our study the high phenotypic resistance indices of *S. aureus* isolates from cow milk emphasizes the importance of routine screening of *S. aureus* isolates for inducible phenotype resistance.

Keywords: mastitis, *Staphylococcus aureus*, antibiotics, antimicrobial susceptibility, MIC,

phenotypic resistance

Although emergence and spread of resistant bacteria is a natural and an inevitable process, its rate is directly related to selective pressure of antibiotics, which expression, in turn, is associated with rate of their application both in medicine and in agriculture (animal production and health) [1]. The infections caused by *Staphylococcus aureus* are most common in highly productive dairy animals. The emergence of multiresistant strains has sharply escalated in recent decades due to the constant use of antimicrobials, increasing the relevance of antibiotic treatment of mastitis.

Macrolide, aminoglycoside, and penicillin groups, including such antibiotics as benzyl-penicillin, erythromycin, amoxicillin, and gentamicin are often used to treat mastitis in cows in the Russian Federation [2-4]. Findings of recovery studies in vitro are an important tool at selection of the most effective antimicrobial agent for therapeutic and preventative interference. Subinhibitory concentrations (sub-MIC) of antibiotics result in broad transcriptional changes in a bacterial cell, affecting the adhesive ability and development of antibiotic resistance [5, 6]. In such a case, antibiotics are acting as signal molecules and are able to modulate bacterial phenotypes. It is known that sub-MIC of antibiotics, underlying mechanism of which lies in DNA damage, reinforce mutations in bacteria. Gene expression controlling formation of bio-film (BF) is induced in *S. aureus* in presence of macrolides (erythromycin, clarithromycin, and azithromycin) and β-lactam antibiotics (penicillin, oxacillin, cephalexin, cephalothin, and vancomycin) [7-9]. Cephalosporins initiate protein synthesis but do not influence on gene expression of Quorum Sensing (QS) system modulating BF formation [10, 11]. Formed bio-films are resistant to wide spread antimicrobials, which calls for more effective approach and development of BF control strategies in medicine and animal health fields.

Availability of representative sample during the microbiological analysis of milk is of paramount importance for making a correct diagnosis [12]. Mechanisms for developing antibiotic resistance vary from spontaneous producing of enzyme and antimicrobial selectivity of a cell wall to efflux-systems [13, 14]. Traits of resistance to antimicrobial agents are encoded by genes, which may be transferred on bacterial chromosome, plasmids, transposone or on gene cassettes included in integrons [15-17]. Resistance genes *rpoB*, *blaZ*, *mecA* [18], *aacA-D*, *tetK*, *tetM*, *ermA* [19, 20], *msrA* (21), *linA*, *vatA*, *vatB*, *dfrA*, *gyrA*, *grlA*, *cat1* to antibiotics of ansamycin, penicillin, aminoglycoside, tetracycline, macrolide-lincosamide-streptogramin B, lincosamide, streptogramin, and fluoroquinolone groups were found in *S. aureus* strains [22, 23]. Penicillin resistant isolates bear plasmid-located gene *blaZ*, which encodes β-lactam enzyme called penicillinase [24, 25].

Emergence and spreading of penicillinase resistance in *S. aureus* is called the first wave of resistance, whilst development of methicillin resistance is called the second wave. The latter is mediated by presence of *mecA* gene encoding low affine protein binding penicillin (PBP2a) [26]. Quinolone resistance in *S. aureus* emerges stepwise due to point mutation (mainly, in topoisomerase IV subunit GrlA gene and helicase subunit GyrA gene), as well as expression of *norA* gene encoding NorA protein with main pumping function (NorA efflux pump). *S. aureus* isolates resistant to aminoglycosides produce three types of aminoglycoside-modifying enzymes (AME) [27] — aminoglycoside-3″-O-phosphoryltransferase III [aph(3′)-III] [28], aminoglycoside-4′-O-phosphoryltransferase I [ant(4′)-I] [29], and aminoglycoside-6′-N-acetyltrans-ferase/2″O-phosphoryltransferase

[aac(6')/aph(2″)] [30], which accordingly modify aminoglycosides kanamycin, tobramycin, and gentamicin [31, 32]. Study of macrolide resistance in *S. aureus* shows that *erm* encoding ribosomal methylase are the most common genes determining the erythromycin resistance [33, 34].

Current work for the first time describes daily activity dynamics of *S. aureus* strains isolated from milk of highly productive cows in the environment with high concentrations of antimicrobials. Direct correlation between the strain resistances was found at evaluation by disk diffusion method (DDM) and by minimum inhibitory concentrations. This work introduces information on combined antibiotic resistance in *S. aureus* strains found in cow milk in the Central region of Russia.

The aim of our work was to assess the phenotypic resistance of *Staphylococcus aureus* strains from cow's milk to the main antibacterial drugs used to treat various forms of mastitis in the Russian Federation, as well as to determine their minimum inhibitory concentrations (MIC, MIC_{50} and MIC_{90}).

Techniques. From January to December 2018 milk samples were collrcted from cows of black-motley Holstein breed *(Bos taurus taurus)* at the farms of the Central region of the Russian Federation. Samples were taken aseptically in milking period (from each cow in 60-70 days after calving). Samples were delivered to the laboratory in 2 hours at temperature of +4 °C or in frozen state at temperature of –20 °C.

All isolates were assessed by commonly accepted phenotypic methods: microscopy evaluation of colonies by Gram coloration; growth in Baird Parker Agar medium (HiMedia Laboratories Pvt. Ltd., India); hemolysis in Azide Blood Agar Pronadisa medium (Conda, Spain); coagulase positive take (CJSC ECOlab, Russia); biochemical identification with the use of microbiological mediums and API 20 STAPH panel (BioMerieux, France). Strains for further studies were kept in vials with Trypticase Soy Broth (TSB, Merck, Germany) with 30 % sterile glycerin (Sigma, USA) at –18 °C.

Strain sensitivity to penicillin (PEN, 10 U), oxacillin (OX, 10 μg), gentamicin (GN, 10 μg), erythromycin (ER, 15 μg), and lincomycin (LN, 15 μg), rifampicin (RF, 5 μg), ciprofloxacin (CP, 5 μg), vancomycin (VA, 30 μg), fusidine (FZ, 10 μg) (Pharmacotherapy Research Center, Russia) was tested by disk diffusion method (DDM) on Mueller Hinton agar medium (HiMedia Laboratories Pvt. Ltd., India). Screening of methycillin-resistant isolates of *S. aureus* (MRSA) was performed using disks with cefoxitin (CF, 30 μg) [35].

Minimum inhibitory concentrations (MIC, MIC_{50}, MIC_{90}) and LD_{50} of antibiotics were determined by method of serial dilutions in TSB medium. To that effect, inoculum of tested *S. aureus* strains with density of 0.5 based on McFarland Standard was made from agar culture. Antibiotics were diluted in physiological solution. Double (Log_2) serial dilutions of antibiotics (penicillin, erythromycin, gentamicin, and ciprofloxacin) in concentrations from 64 to 0.125 mg/l were made according to methodological guidelines [36]. Approximately 5×10^5 CFU/ml of tested microorganism was tested in 1 ml of each dilution of antimicrobial drug (AMD). The incubation was carried out at nutator at 37 °C for 20 hours (220 rpm). The bacterial growth in TSB medium with antibiotics was evaluated by plating on Baird Parker Agar medium. The results were interpreted based on recommendation of The European Committee on Antimicrobial Susceptibility Testing (EUCAST) [37].

Biometrical processing was carried out with the use of Microsoft Excel 2010, SAS version 6.12 (SAS Institute, USA).

Results. A total of 314 milk samples from high productive cows were examined and 447 potential staphylococci were isolated, of which only 103 met all identification criteria and were used for further analysis. Such level of contamination is similar to that noted during previous studies in Russia [37, 38] and other countries [39-41].

The highest degree of *S. aureus* isolate resistance in vitro was observed in case of erythromycin (82.5 %) and fusidine (75.7 %) (Table 1). Sensitivity to all studied antibiotics was found only in 7 isolated strains (6.8 %), whilst 96 isolates were resistant to at least one of them. Other authors found evident development of *S. aureus* resistance to penicillin, ampicillin, and erythromycin [40, 42, 43].

1. Antibiotic sensitivity of *Staphylococcus aureus* strains isolated from cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation (2018)

Antibiotic	Concentration	MSSA (n = 47)				Total strain number (n = 103)			
		resistant		susceptible		resistant		susceptible	
		n	%	*n*	%	*n*	%	*n*	%
Pen	10 U	47	100	0	0	47	45.6	56	54.4
OX	10 μg	0	0	47	100	0	0	103	100
GN	10 μg	20	42.6	27	57.4	29	28.2	74	71.8
ER	15 μg	41	87.2	6	12.8	85	82.5	18	17.5
LN	15 μg	31	66.0	16	34.0	61	59.2	42	40.8
RF	5 μg	30	63.8	17	36.2	49	47.6	54	52.4
CP	5 μg	37	78.7	10	21.3	49	47.6	54	52.4
VA	30 μg	0	0	47	100	0	0	103	100
FZ	10 μg	42	89.4	5	10.6	78	75.7	25	24.3
CF	30 μg	0	0	47	100	0	0	103	100

Note. Pen — penicillin, OX — oxacillin, GN — gentamicin, ER - erythromycin, LN — lincomycin, RF — rifampicin, CP — ciprofloxacin, VA — vancomycin, FZ — fusidine, CF — cefoxitin; MSSA — methycillin-susceptible *S. aureus*.

Frequency of staphylococci resistance related to production of β-lactamase comprises from 55.7 to 92.6 % worldwide [44]. In our studies, phenotype traits of resistance to β-lactam antibiotics were found in 47 out of 103 isolates. All 47 (45.6 %) strains were resistant to penicillin (100 %), which is consistent with findings of Shi et al. [45] that had tested 206 strains of *S. aureus* in Mongolia, but which is inconsistent with findings of Aarestrup et al. [46] denoting low frequency (10 %) of benzyl-penicillin resistance in Denmark, Northway, and Sweden. Share of penicillin resistant isolates in other part of Europe varied from 23 to 69 % [47].

We had not found resistance to oxacillin and cefoxitin in all tested strains, making them methycillin-susceptible *S. aureus* MSSA. In case of MSSA, it is assumed that penicillin surpasses oxacillin by its effect, but only if strains do not produce penicillinase [48]. A total of 12 out of 47 strains (25.5 %) had simultaneous resistance to 7 antibiotics (gentamicin, erythromycin, lincomycin, rifampicin, ciprofloxacin, and fusidine).

It was found that 100 % strains isolated from cow's milk in the Central region of the Russian Federation were sensitive to vancomycin. Such drug is not used in veterinary medicine anymore in many countries, including Russia, which may explain the presented findings.

Multiple antibiotic resistance (MAR) phenotypes were identified for 103 strains of *S. aureus* (Table 2). Hence, 65 (63.1 %) strains were resistant to four and more antibiotics. Maximum frequency of combined resistance was observed in case of erythromycin + lincomycin + fusidine) (58.3 %) and erythromycin + ciprofloxacin (47.6 %). Penicillin resistant strains had higher phenotype resistance that resistance to other antibiotics. Multiple resistances were developed in 60 out of 103 strains.

2. Combined resistance to various antibiotics in *Staphylococcus aureus* strains isolated from cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation (n = 103, 2018)

Antibiotic	Susceptible strains per sample	
	n	%
Pen + GN	20	19.4
Pen + ER	41	39.8
Pen + LN	31	30.1
Pen + CP	37	35.9
Pen + FZ	42	40.8
ER + CP	49	47.6
Pen + GN + ER	20	19.4
Pen + GN + ER + LN	16	15.5
Pen + GN + ER + LN + RF + CP + FZ	12	11.7
ER + LN + FZ	60	58.3

N o t e . Pen — penicillin, 10 U; OX — oxacillin, 10 μg; GN — gentamicin, 10 μg; ER - erythromycin, 15 μg; LN — lincomycin, 15 μg; RF — rifampicin, 5 μg; CP — ciprofloxacin, 5 μg; VA — vancomycin, 30 μg; FZ — fusidine, 10 μg; CF — cefoxitin, 10 μg.

3. Minimum inhibitory concentrations (MIC, mg/ml) **for coagulose-positive *Staphylococcus aureus* strains isolated from cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation** (n = 103, 2018)

Antibiotic	MIC		Suppressed isolates for different MIC										MIC_{50}	MIC_{90}
	TV	PV	≥ 64	32	16	8	4	2	1	0,5	0,25	≤ 0,125		
ER	0.5	0.25-1.0	15	10	27	14	10	9	11	3	4	0	> 16.0	> 1.0
Pen	0.25-0.5	0.125-1.0	31	10	7	6	8	7	14	12	8	0	> 8.0	> 0.5
GN	0.25	0.125-0.5	5	13	14	13	11	6	10	13	10	8	> 4.0	> 0.25
CP	0.5-1.0	0.25-2.0	2	8	12	12	13	10	12	14	11	9	> 2.0	> 0.25

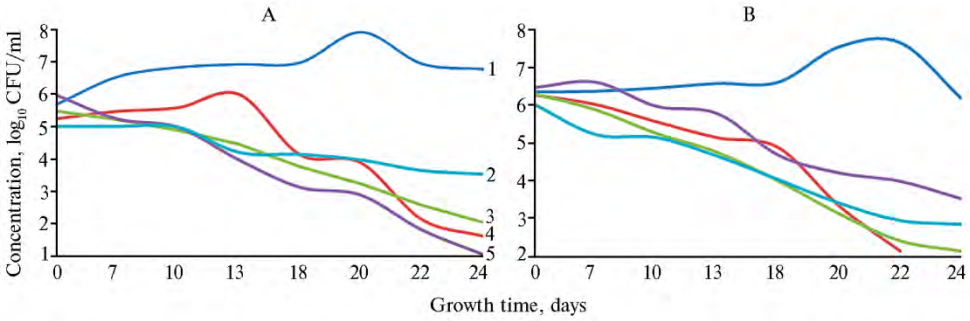
N o t e . TV — target values, AV — permitted values; ER erythromycin, Pen — penicillin, GN — gentamicin, CP — ciprofloxacin; $MIC_{50\ and}$ MIC_{90} — concentrations at which 50 and 90% strains were killed, accordingly. Target values were calculated by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) [49]. Permitted values are consistent to ISO 20776-1: 2006 (as updated based on the latest version of CLSI M100).

MIC values for coagulate positive *S. aureus* strains are provided in Table 3. Due to insignificant difference (2-3 %) in specificity and sensitivity of *S. aureus* strains to cefoxitin at assessment by MIC and DDM methods, which reliably predicts the resistance to methycillin, MIC studies for such antibiotic were not carried out [50]. A total of 31 strains of the 47 isolates DDM-resistant to penicillin grew at ≥ 64 mg/l penicillin, while in the concentration range of ≤ 1.0 mg/l, the growth was observed in 33.0 % of the strains. MIC_{50} and MIC_{90} for penicillin comprised > 8.0 and > 0.5 mg/l, much higher than the established standard for susceptible strains. High penicillin resistance among *S. aureus* strains could be due to the use of intramammary drugs containing combinations of various broad-spectrum antibiotics and antimicrobials [51].

A total of 15 out of the 85 erythromycin-resistant isolates showed growth at MIC ≥ 64 mg/l; only 7 strains out of 18 DDM-susceptible ones were in the MIC ≤ 0.5 mg/l range. MIC_{50} and MIC_{90} for erythromycin were maximal as compared to all studied antibiotics (see Table 3). A total of 29 (28.2 %) of the 103 studied *S. aureus* strains were DDM-resistant to gentamicin, 72 (60.2 %) showed growth at MIC ≥ 0.5 mg/l, and 5 strains at MIC ≥ 4.0 mg/l. Ciprofloxatin was the most effective regarding the isolated *S. aureus* strains. Thus, only 2 of the 49 DDM-resistant strains grew at MIC ≥ 64 mg/l; MIC_{50} and MIC_{90} for such antibiotic comprised > 2.0 and > 0.25 mg/l, respectively. Finally, it is important to point out that the MIC_{50} was not in the susceptible range for any of the studied antibiotics, and the MIC_{90} showed the susceptible range for penicillins, aminoglycosides, and fluoroquinolones.

A total of 12 multiresistant *S. aureus* strains, susceptibility of which was described above, were selected out of 103 strains as samples to study the antibiotic effect as anti-growth factor. Active growth at average for all strains began by the 7^{th} hour of culturing (Fig.). Antibiotics in the medium suppressed or inhibit-

ed growth. The observed effect depended upon the time of culture growth and type of antibiotics. Penicillin in concentration of 64 mg/l resulted in decrease of the number of viable cells in 83.3 % cases, whilst complete suppression of growth by 20 hour was observed only in two cultures. Gentamicin, ciprofloxatin, and erythromycin in concentration of 64 mg/l did not render full suppressive effect on any of 12 strains. After 24 hours, number of *S. aureus* cells in the medium with gentamicin was not lower than 2.84 $\log_{10}$ CFU/ml, and with erythromycin not lower than 2.97 $\log_{10}$ CFU/ml. Viability of *S. aureus* in ciprofloxatin-containing medium comprised from 18.5 to 84.0% for various strains, which implies a presence of cells with genes resistant to such group of antibiotics. Similar dependence in antibiotic effect we observed in all 12 studied strains.



Growth of *Staphylococcus aureus* strains 615 (A) **and 1839** (B) **isolated from cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation depending on time and type of antibiotics:** 1 — control, 2 — ciprofloxatin, 64 mg/l, 3 — gentamicin, 64 mg/l, 4 — penicillin, 64 mg/l, 5 — erythromycin, 64 mg/l.

In our work, high phenotype resistance of *S. aureus* isolates from cow's milk emphasizes the importance of routine screening of *S. aureus* isolates for identification of inducible resistance of phenotypes. Observations of changes in count of viable cells exposed to high antibiotic concentrations during 24 hours allow tracing development of the resistance.

Therefore, 23.0% of all isolates from milk of cows of black-motley Holstein breed at the farms of the Central region of the Russian Federation were classified as *Staphylococcus aureus*, of which 63.1 % strains were resistant to four and more antibiotics. MIC_{50} and MIC_{90} for penicillin comprised > 8.0 and > 0.5 mg/l, much higher than the established standard for susceptible strains. Out of DDM-resistant *Staphylococcus aureus* to penicillin, erythromycin, gentamicin, and ciprofloxatin at MIC ≥ 64.0 mg/l growth potential was confirmed in 65.96; 17.65; 17.24, and 4.08 % respectively. Antibiotics in maximum dosages (64 mg/l) did not render complete suppressive effect in viable strains: cell count for all 12 studied isolates was not lower than 2.84 $\log_{10}$ CFU/ml in gentamicin medium and 2.97 $\log_{10}$ CFU/ml in erythromycin medium. Count of viable cells comprised 16.7 % for penicillin and 84.0 % for ciprofloxatin. Practical use of phenotypic estimates makes it possible to identify antibiotic resistance in mastitis etiologic agent in high productive cows. This findings allowed us to select strains for further studies aimed at searching resistance genes.

REFERENCES

1. *Programma SKAT (Strategiya Kontrolya Antimikrobnoi Terapii) pri okazanii statsionarnoi meditsinskoi pomoshchi: Rossiiskie klinicheskie rekomendatsii* /Pod redaktsiei S.V. Yakovleva, N.I. Briko, S.V. Sidorenko, D.N. Protsenko [SCAT (Antimicrobial Therapy Control Strategy) for inpatient care: Russian clinical guidelines. S.V. Yakovlev, N.I. Briko, S.V. Sidorenko, D.N. Protsenko (eds.)]. Moscow, 2018 (in Russ.).
2. Koba I.S., Reshetka M.B. *Effektivnoe zhivotnovodstvo*, 2013, 3(89): 22-23 (in Russ.).

3. Gamayunov V.M., Amirov A.Kh. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii, posvyashchennoi 85-letiyu so dnya rozhdeniya G.A. Cheremisova i 50-letiyu sozdaniya Voronezhskoi shkoly veterinarnykh akusherov «Sovremennye problemy veterinarnogo akusherstva i biotekhnologii vosproizvedeniya zhivotnykh»* [Proc. Int. Conf. dedicated to the 85th birthday of G.A. Cheremisov and the 50th anniversary of the Voronezh school of veterinary obstetricians «Modern problems of veterinary obstetrics and animal reproduction biotechnology»]. Voronezh, 2012: 143-146 (in Russ.).
4. Klimov N.T. *Eksperimental'naya i klinicheskaya farmakologiya lekarstvennykh preparatov na osnove dioksidina i doksitsiklina i ikh effektivnost' pri mastite u korov. Avtoreferat doktorskoi dissertatsii* [Experimental and clinical pharmacology of drugs based on dioxidine and doxycycline and their effectiveness in mastitis in cows. DSc Thesis]. Voronezh, 2009 (in Russ.).
5. Bulgakova V.G., Vinogradova K.A., Orlova T.I., Kozhevin P.A., Polin A.N. *Antibiotiki i khimioterapiya*, 2014, 59(1-2): 36-43 (in Russ.).
6. Tsai S.H., Lai H.C., Hu S.T. Subinhibitory doses of aminoglycoside antibiotics induce changes in the phenotype of *Mycobacterium abscessus*. *Antimicrobial Agents and Chemotherapy*, 2015, 59(10): 6161-6169 (doi: 10.1128/AAC.01132-15).
7. Wang Q., Sun F.-J., Liu Y. Xiong L.-R., Xie L.-L., Xia P.-Y. Enhancement of biofilm formation by sub inhibitory concentrations of macrolides in icaADBC-positive and -negative clinical isolates *of Staphylococcus epidermidis*. *Antimicrobial Agents and Chemotherapy*, 2010, 54(6): 2707-2711 (doi: 10.1128/AAC.01565-09).
8. Kaplan J.B. Antibiotic-induced biofilm formation. *The International Journal of Artificial Organs*, 2011, 34(9): 737-751 (doi: 10.5301/ijao.5000027).
9. Sanchez C.J. Jr., Shiels S.M., Tennent D.J., Hardy S.K., Murray C.K., Wenke J.C. Rifamycin derivatives are effective against staphylococcal biofilms in vitro and elutable from PMMA. *Clinical Orthopaedics and Related Research*, 2015, 473(9): 2874-2884 (doi: 10.1007/s11999-015-4300-3).
10. Subrt N., Mesak L.R., Davies J. Modulation of virulence gene expression by cell wall active antibiotics in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, 2011, 66(5): 979-984 (doi: 10.1093/jac/dkr043).
11. Viana D., Comos M., McAdam P.R., Ward M.J., Selva L., Guinane C.M., González-Muñoz C.M., Tristan A., Foster S.J., Fitzgerald J.R., Penadés J.R. A single natural nucleotide mutation alters bacterial pathogen host tropism. *Nature Genetics*, 2015, 47(4): 361-367 (doi: 10.1038/ng.3219).
12. Rodriges Kh. *Meditsinskii sovet*, 2017, 1: 34-44 (doi: 10.21518/2079-701X-2017-1-34-44) (in Russ.).
13. Koymans K.J., Vrieling M., Gorham R.D. Jr., van Strijp A.G.J. Staphylococcal immune evasion proteins: structure, function, and host adaptation. In: *Staphylococcus aureus. Current Topics in Microbiology and Immunology, vol. 409*. F. Bagnoli, R. Rappuoli, G. Grandi (eds.). Springer, Cham, 2015: 441-489 (doi: 10.1007/82_2015_5017).
14. Koop G., Vrieling M., Storisteanu D.M.L., Lok L.S.C., Monie T., van Wigcheren G., Raisen C., Ba X., Gleadall N., Hadjirin N., Timmerman A.J., Wagenaar J.A., Klunder H.M., Fitzgerald J.R., Zadoks R., Paterson G.K., Torres C., Waller A.S., Loeffler A., Loncaric I., Hoet A.E., Bergström K., de Martino L., Pomba C., de Lencastre H., Slama K.B., Gharsa H., Richardson E.J., Chilvers E.R., Haas C., Kessel K., Strijp J.A., Harrison E.M., Holmes M.A. Identification of LukPQ, a novel, equid-adapted leukocidin of *Staphylococcus aureus*. *Scientific Reports*, 2017, 7: 40660 (doi: 10.1038/srep40660).
15. Löffler B., Hussain M., Grundmeier M., Brück M., Holzinger D., Varga G., Roth J., Kahl B.C., Proctor R.A., Peters G. *Staphylococcus aureus* panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathogens*, 2010, 6(1): e1000715 (doi: 10.1371/journal.ppat.1000715).
16. Vrieling M., Boerhout E. M., van Wigcheren G.F., Koymans K.J., Mols-Vorstermans T.J., de Haas C.J.C., Aerts P.C., Daemen I.J.J.M., van Kessel K.P.M., Koets A.P., Rutten V.P.M.G., Nuijten P.J.M., van Strijp J.A.G., Benedictus L. LukMF' is the major secreted leukocidin of bovine *Staphylococcus aureus* and is produced in vivo during bovine mastitis. *Scientific Reports*, 2016, 6(1): 37759 (doi: 10.1038/srep37759).
17. de Jong N.W.M., Vrieling M., Garcia B.L., Koop G., Brettmann M., Aerts P.C., Ruyken M., van Strijp J.A.G., Holmes M., Harrison E.M., Geisbrecht B.V., Rooijakkers S.H.M. Identification of a staphylococcal complement inhibitor with broad host specificity in equid *Staphylococcus aureus* strains. *Journal of Biological Chemistry*, 2018, 293(12): 4468-4477 (doi: 10.1074/jbc.RA117.000599).
18. Kozlova Yu.N., Fomenko N.V., Morozova V.V., Saranina I.V., Tikunov A.Yu., Ganichev D.A., Samokhin A.G., Pavlov V.V., Rozhnova O.M., Bondar' I.A., Zenkova E.V., Nimaev V.V., Klimontov V.V., Tikunova N.V. *Vavilovskii zhurnal genetiki i selektsii*, 2017, 21(8): 952-958 (doi: 10.18699/VJ17.318) (in Russ.).
19. Safarpoor Dehkordi F., Gandomi H., Akhondzadeh Basti A., Misaghi A., Rahimi E. Phenotypic and genotypic characterization of antibiotic resistance of methicillin-resistant Staphylococcus aureus isolated from hospital food. *Antimicrob. Resist. Infect. Control.*, 2017, 6(1): 104 (doi: 10.1186/s13756-017-0257-1).
20. Safarpoor Dehkordi F., Basti A.A., Gandomi H., Misaghi A., Rahimi E. Pathogenic Staphylococcus aureus in hospital food samples; prevalence and antimicrobial resistance properties. *Journal of Food Safety*, 2018, 38(6): e12501 (doi: 10.1111/jfs.12501) (Retracted).

21. Momtaz H., Safarpoor Dehkordi F., Rahimi E., Asgarifar A., Momeni M. Virulence genes and antimicrobial resistance profiles of *Staphylococcus aureus* isolated from chicken meat in Isfahan province, Iran. *Journal of Applied Poultry Research*, 2013, 22(4): 913-921 (doi: 10.3382/japr.2012-00673).
22. Hasanpour Dehkordi A., Khaji L., Sakhaei Shahreza M.N., Mashak Z., Safarpoor Dehkordi F., Safaee Y., Hosseinzadeh A., Alavi I., Ghasemi E., Rabiei-Faradonbeh M. One-year prevalence of antimicrobial susceptibility pattern of methicillin-resistant *Staphylococcus aureus* recovered from raw meat. *Tropical Biomedicine*, 2017, 34(2): 396-404.
23. Albano M., Karau M.J., Greenwood-Quaintance K.E., Osmon D.R., Oravec C.P., Berry D.J., Abdel M.P., Patel R. In vitro activity of rifampin, rifabutin, rifapentine, and rifaximin against planktonic and biofilm states of staphylococci isolated from periprosthetic joint infection. *Antimicrobial Agents and Chemotherapy*, 2019, 63(11): e00959-19 (doi: 10.1128/AAC.00959-19).
24. Chambers H.F. The changing epidemiology of *Staphylococcus aureus*? *Emerg. Infect. Dis.*, 2001, 7(2): 178-182 (doi: 10.3201/eid0702.010204).
25. Lowy F.D. Antimicrobial resistance: the example of *Staphylococcus aureus*. *The Journal of Clinical Investigation*, 2003, 111(9): 1265-1273 (doi: 10.1172/JCI18535).
26. Torimiro N., Moshood A.A., Eyiolawi S.A. Analysis of beta-lactamase production and antibiotics resistance in *Staphylococcus aureus* strains. *Journal of Infectious Diseases and Immunity*, 2013, 5(3): 24-28 (doi: 10.5897/JIDI2013.0118).
27. Chandrakanth R.K., Raju S., Patil S.A. Aminoglycoside-resistance mechanisms in multidrug-resistant *Staphylococcus aureus* clinical isolates. *Current Microbiology*, 2008, 56(6): 558-562 (doi: 10.1007/s00284-008-9123-y).
28. Ubukata K., Yamashita N., Gotoh A., Konno M. Purification and characterization of aminoglycoside-modifying enzymes from *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrobial Agents and Chemotherapy*, 1984, 25(6): 754-759 (doi: 10.1128/AAC.25.6.754).
29. Hosseini S.A.A.M., Khoramrooz S.S., Marashifard M., Parhizgari N., Mansouri F. Frequency of the genes encoding aminoglycoside modifying enzymes in *Staphylococcus aureus* isolated from hospitalized burn patients. *Journal of Mazandaran University of Medical Sciences*, 2016, 25(134): 147-157.
30. Rouch D.A., Byrne M.E., Kong Y.C., Skurray R.A. The aacA-aphD gentamycin and kanamycin resistance determinant of Tn4001 from *Staphylococcus aureus*: expression and nucleotide sequence analysis. *Journal of General Microbiology*, 1987, 133(11): 3039-3052 (doi: 10.1099/00221287-133-11-3039).
31. Hancock R.E.W. Aminoglycoside uptake and mode of action — with special reference to streptomycin and gentamicin. I. Antagonists and mutants. *Journal of Antimicrobial Chemotherapy*, 1981, 8(4): 249-276 (doi: 10.1093/jac/8.4.249).
32. Turutoglu H., Hasoksuz M., Ozturk D., Yildirim M., Sagnak S. Methicillin and aminoglycoside resistance in *Staphylococcus aureus* isolates from bovine mastitis and sequence analysis of their *mecA* genes. *Veterinary Research Communications*, 2009, 33(8): 945-956 (doi: 10.1007/s11259-009-9313-5).
33. Wang Z., Han R., Liu Y., Du Q., Liu J., Ma C., Li H., He Q., Yan Y. Direct detection of erythromycin-resistant *Bordetella pertussis* in clinical specimens by PCR. *Journal of Clinical Microbiology*, 2015, 53(11): 3418-3422 (doi: 10.1128/JCM.01499-15).
34. Li Y., Liu X., Zhang B., He Q., Wang Z. Where macrolide resistance is prevalent. *APMIS*, 2015, 123(4): 361-363 (doi: 10.1111/apm.12357).
35. *Performance Standards for Antimicrobial Susceptibility Tests; Approved Standard — Twelfth Edition. CLSI Supplement M02-A12.* Clinical and Laboratory Standards Institute, USA, 2015.
36. *Opredelenie chuvstvitel'nosti mikroorganizmov k antibakterial'nym preparatam. MUK 4.2.1890-04* [Determination of the sensitivity of microorganisms to antibacterial drugs. MUK 4.2.1890-04]. Moscow, 2004 (in Russ.).
37. Shurduba N.A., Sotnikova V.M., Osipova I.S. *RZh «Problemy veterinarnoi sanitarii, gigieny i ekologii»*, 2013, 2(10): 56-57 (in Russ.).
38. Nikulin D.M. *Nivy Zaural'ya*, 2015, 6: 74-77 (in Russ.).
39. Klibi A., Maaroufi A., Torres C., Jouini A. Detection and characterization of methicillin-resistant and susceptible coagulase-negative staphylococci in milk from cows with clinical mastitis in Tunisia. *International Journal of Antimicrobial Agents*, 2018, 52(6): 930-935 (doi: 10.1016/j.ijantimicag.2018.07.026).
40. Haran P., Godden S.M., Boxrud D., Jawahir S., Bender J.B., Sreevatsan S. Prevalence and characterization of *Staphylococcus aureus*, including methicillin-resistant *Staphylococcus aureus*, isolated from bulk tank milk from Minnesota dairy farms. *Journal of Clinical Microbiology*, 2012, 50(3): 688-695 (doi: 10.1128/JCM.05214-11).
41. Hamiroune M., Berber A., Boubekeur S. Contribution à l'étude de la contamination du lait bovin par les staphylocoques dans certaines fermes de la région d'Alger et son impact sur la santé humaine. *Revue scientifique et technique (International Office of Epizootics)*, 2014, 33(3): 1027-1034.
42. Peles F., Wagner M., Varga L., Hein I., Rieck P., Gutser K., Keresztúri P., Kardos G., Turcsányi I., Béri B., Szabó A. Characterization of *Staphylococcus aureus* strains isolated from bovine milk in Hungary. *International Journal of Food Microbiology*, 2007, 118(2): 186-193 (doi: 10.1016/j.ijfoodmicro.2007.07.010).
43. Srednik M.E., Usongo V., Lépine S., Janvier X., Archambault M., Gentilini E.R. Characterisa-

tion of *Staphylococcus aureus* strains isolated from mastitis bovine milk in Argentina. *Journal of Dairy Research*, 2018, 85(1): 57-63 (doi: 10.1017/s0022029917000851).
44. Bidya S., Suman R.S. Comparative study of three β lactamase test methods in Staphylococcus aureus isolated from two Nepalese hospitals. *Open Journal of Clinical Diagnostics*, 2014, 4: 47-52 (doi: 10.4236/ojcd.2014.41009).
45. Shi D., Hao Y., Zhang A., Wulan B., Fan X. Antimicrobial resistance of Staphylococcus aureus isolated from bovine mastitis in China. *Transboundary and Emerging Diseases*, 2010, 57(4): 221-224 (doi: 10.1111/j.1865-1682.2010.01139.x).
46. Aarestrup F.M., Jensen N.E. Development of penicillin resistance among *Staphylococcus aureus* isolated from bovine mastitis in Denmark and other countries. *Microbial Drug Resistance*, 1998, 4(3): 247-256 (doi: 10.1089/mdr.1998.4.247).
47. Nunes S.F., Bexiga R., Cavaco L.M., Vilela C.L. Technical note: antimicrobial susceptibility of Portuguese isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* in subclinical bovine mastitis. *Journal of Dairy Science*, 2007, 90(7): 3242-3246 (doi: 10.3168/jds.2006-739).
48. Kaase M., Lenga S., Friedrich S., Szabados F., Sakinç T., Kleine B., Gatermann S.G. Comparison of phenotypic methods for penicillinase detection in *Staphylococcus aureus*. *Clinical Microbiology and Infection*, 2008, 14(6): 614-616 (doi: 10.1111/j.1469-0691.2008.01997.x).
49. *The European Committee on Antimicrobial Susceptibility Testing. Routine and extended internal quality control as recommended by EUCAST. Version 8.0, 2018.* Rezhim dostupa http://www.eucast.org/ast_of_bacteria/previous_versions_of_documents/. Available 18.12.2019.
50. Farahani A., Mohajeri P., Gholamine B., Rezaei M., Abbasi H. Comparison of different phenotypic and genotypic methods for the detection of methicillin-resistant *Staphylococcus aureus*. *North American Journal of Medical Sciences*, 2013, 5(11): 637-640 (doi: 10.4103/1947-2714.122305).
51. Pitkälä A., Haveri M., Pyörälä S., Myllys V., Honkanen-Buzalski T. Bovine mastitis in Finland 2001 — prevalence, distribution of bacteria, and antimicrobial resistance. *Journal of Dairy Science*, 2004, 87(8): 2433-2441 (doi: 10.3168/jds.S0022-0302(04)73366-4).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1267-1280
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Biologicals and producers

UDC 579.64:632.937.15

doi: 10.15389/agrobiology.2019.6.1267eng
doi: 10.15389/agrobiology.2019.6.1267rus

INSECTICIDAL PROPERTIES of *Bacillus thuringiensis* var. *israelensis*. I. THE ACTIVITY SPECTRUM OF A LARVICIDAL PREPARATION BASED ON INDUSTRIAL STRAIN 7-1/23A

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The authors declare no conflict of interests
Acknowledgements:
The work was carried out on the equipment of the ARRIAM Center for Genomic Technologies, Proteomics and Cell Biology.
Supported financially by the project of applied research and experimental development (PNER) batch 2017-14-579-0030 on the topic "Creation of microbiological preparations for expanding the adaptive capacity of agricultural crops for nutrition, resistance to stress and pathogens" (code of the application 2017-14-579-0030-013), Agreement No. 14.607.21.0178, a unique identifier (project) RFMEFI60717X0178
Received August 2, 2019

Abstract

Blood-sucking mosquitoes and blackflies belonging to the order *Diptera* cause sanitary-epidemiological and veterinary-epizootological damages in humans and animals and serve as the carriers of various dangerous transmissible diseases. The productivity and milk yield of livestock as well as egg production in poultry birds are significantly affected by harmful *Diptera* species. The problem of bioprotection from harmful insects is extremely relevant in crop production, including the cultivation of seeded fodder crops. Currently, the spore-forming bacterium *Bacillus thuringiensis* Berliner (Bt) represents the most common agent for biological control of the number of insect species and is considered as the basis for the production of insecticides. Biological preparations are of particular importance due to their significant advantages over chemical pesticides and are considered in modern agricultural systems as environmentally and socially beneficial alternatives to agrochemicals. A liquid form of larvicidal preparation against blood-sucking and herbivorous mosquitoes based on the original strain *Bacillus thuringiensis* var. *israelensis* 7-1/23A was developed at the ARRIAM. Under registration number RCAM 00626 (RF patent 2539732 dated 12/09/2014), the strain was deposited in the ARRIAM Collection of beneficial agricultural microorganisms (RCAM). The composition of the liquid form of the biopreparation includes a spore-crystalline complex, 5 % sodium chloride, 2 % coniferous extract (as a preservative and perfume, respectively), and the remains of a nutrient medium. The titer was 4.25×10^9 CFU/ml. Larvicidal activity expressed in LC_{50} for *Aedes aegypti* IV instar larvae was 0.11×10^{-3} % (biotest proposed by the World Health Organization). The strain was previously isolated from an anophelogenic reservoir in the Leningrad region, studied for physiological and cultural features, and characterized according to H. De Barjac's and A. Bonnefoi's classification. In this work, for the first time, a comprehensive analysis of a larvicidal preparation based on BtH_{14} 7-1/23A was performed, including the molecular characterization of the strain, determination of its larvicidal activity against a number of harmful dipterans, and an assessment of its effects on the growth and development of non-target objects (oyster mushroom mycelium and champignon). Sequencing of the gene encoding B subunit of the DNA gyrase (GyrB) confirmed that the isolated strain belongs to *B. thuringiensis* var. *israelensis*. The presence of the *cry4* and *cry11* genes encoding protein insecticidal toxins was detected by PCR analysis. We also studied the spectrum of action of

the larvicidal biopreparation against the blood-sucking mosquitoes of the genera *Aedes*, *Anopheles*, *Culex*, and harmful herbivorous dipterans: rice (*Cricotopus sylvestris* Fabr.) and mushroom (*Lycoriella fucorum* Frey) flies. The analysis was carried out in laboratory and field conditions (2014-2016, Leningrad and Moscow regions, Krasnodar Territory, aedogenic, anofelogenic ponds, raw basement rooms, rice checks) and also included an assessment of the effect of the preparation on the growth of mycelium of oyster mushrooms (*Pleurotus ostreatus*) and champignon (*Agaricus campestris*) as bio-eco indicators. LC_{50} values in laboratory tests were 0.11×10^{-3} and 0.12×10^{-3} % for *Culex* and *Aedes*, respectively, and 0.29×10^{-3} % for *Anopheles maculipennis* Meigen. Field tests in water reservoirs against malarial (*Anopheles maculipennis*) and non-malarial mosquitoes of the genus *Aedes* — *communis*, *dorsalis*, *punctor*, *caspius*, and *flavescens*; in moist basement rooms (*Culex pipiens* Linnaeus f. *molestus* Forskel), rice fields (*Cricotopus sylvestris* Fabr.) showed 90.2-100 % mortality of larvae. The effect of the biological preparation on the growth of mycelium of oyster mushrooms and champignon was studied in three concentrations (5, 10, and 20 %). The most efficient was 5 % concentration which stimulated the growth of fungal mycelium by 28.2-32.5 %. The analysis of the separate and combined applications of the 7-1/23A-based biopreparation and the chemical insecticide, chitin synthesis inhibitor Dimilin («Arysta LifeScience S.A.S.», France) against the larvae of the mushroom fly of the family *Lycoriidae* (*L. fucorum* Frey) demonstrated that combined use of these insecticides in 4-8 times reduced dozes caused death of 97.2 % larvae and led to an increase in the yield by 38.6 %. Thus, a preparation based on the BtH_{14} 7-1/23A is promising for sanitary ecology and veterinary. In addition, it is advisable to study the possibilities of its use against insect pests of seeded forage crops.

Keywords: *Bacillus thuringiensis* var. *israelensis* (BtH_{14}), titer, larvicidal activity, growth-promoting activity, biological preparation

Blood-sucking dipteran species cause significant economic damage to livestock. In the regions of its outbreaks dairy yield is decreased by 20-30 % and over, body weight gain in growing stock is decreased by 20-40 %, and morbidity and loss of stock from exhaustion, especially in reindeers and fur animals, is increased. Besides, such insects cause sanitary-epidemiological damage as infective and invasive sources and agents in humans and animals (malaria, yellow fever, dengue fever, filariasis, cattle anaplasmosis, anthrax, tularaemia, brucellosis, plague, onchocerciasis, and etc.) [1]. For a long time, blood-sucking mosquitoes were combated by variety of ways, up to petrolization and treatment by non-specific high-toxic chemicals. It resulted in adverse consequences: massive loss of fish, birds, amphibian, and many other organisms, in making ponds unsuitable for water supply, watering, and bathing purposes, as well as in development of chemical insecticide resistance in mosquito populations [2, 3].

A highly promising alternative to conventional chemicals for control of the population of harmful diptera are biological preparations based on *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) [4]. Following the discovery of insecticide effect of Bti on blood-sucking mosquitoes and flies in 1976, many studies confirming safety of such bacteria for vertebrates and non-target invertebrates sharing single habitat with mosquito larvae have been carried out [4, 5]. Selective effect of BtH_{14} is insured by availability of certain intestinal pH, specialized enzymes, and specific BtH_{14} toxin attachment receptors in susceptible mosquito larvae. Thus, toxins responsible for pathogenic effect in mosquito larvae do not affect non-target organisms and, accordingly, are suitable for common use in the recommended dosages [4, 6].

B. thuringiensis forms spores, produces crystalline endotoxins [7], thermoresistant exotoxins [8], and other proteins and combinations with insecticide and antifungal properties [9-11], whilst some of them have growth stimulating effect [12, 13]. *B. thuringiensis* bacteria were found in Europe, North America, Middle East, India, and Japan [14-16]. Bt-based preparations take the leading place by production volumes [17-19]; its share at the biopesticide market exceeds 60 %. Among its advantages are technological effectiveness, broad-spectrum [20-22], and safety for human beings and environment [23, 24], and

non-target insects [25-27]. Nowadays, this line of research in the scientific world is one of the most relevant and prioritized [28, 29]. Recently, scientists from different countries had isolated and identified over 70 variants of Bt effective against *Lepidoptera*, *Coleoptera*, *Diptera,* and *Hymenoptera* orders [30, 31], with high technologic effectiveness, virulence, and broad-spectrum effect.

The novelty of our work is due to molecule-genetic identification of phylogenic position of *B. thuringiensis* var. *israelensis* 7-1/23A (BtH_{14} 7-1/23A) strain by sequencing of *gyrB* gene, identification of *cry4* and *cry11* genes encoding protein insecticide toxins by polymerase chain reaction, as well as by data on assessment of BtH_{14} 7-1/23A effect on growth and development of non-target objects (oyster mushroom mycelium and champignon), carried out for enhancement of preparation based on such strain.

Our purpose was to study the spectrum of effect of insecticide preparation based on BtH_{14} 7-1/23A strain.

Techniques. Bacillus thuringiensis var. *israelensis* 7-1/23A (BtH_{14} 7-1/23A) strain was isolated from sample taken in anophelogenous pond in the Leningrad Region and was characterized according to H. De Barjak and A. Bonnefoi classification [32] (patent of the Russian Federation No. 2539732 dated 09.12.2014).

For isolation of genomic DNA, BtH_{14} 7-1/23A strain was grown on agar Luria-Bertani plates (LB, 2 % bacteriological agar) at 30 °C during 16-18 hours. Afterwards, bacterial cells were suspended in Tris-EDTA buffer (1 M Tris-HCl, pH 7.5 + 0.5 M EDTA, pH 8.0) and heated during 10 minutes at 102 °C. Cell debris was removed by centrifugation (15000 g during 3 minutes). Supernatant fluid was transferred to clean Eppendorf tubes and used in further studies (33). Concentration of obtained DNA was measured by Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Inc., USA).

Protocol for polymerase chain reaction (PCR) was selected based on primer annealing temperature and size of amplified fragment. Initial denaturation lasted 5 min at 95 °C, followed by 30 cycles including 30 sec denaturation at 95 °C, 30 sec annealing at selected temperature, elongation at 72 °C during the time meeting the time set for primers (target fragments); 10 min final elongation at 72 °C ended by storage at 12 °C (amplifier T100, Bio-Rad, USA). Reaction mixture (20 μl) contained genome DNA (100 ng), 10 μl FermentasDreamTaq green PCR master mix (Thermo Fisher Scientific, Inc., USA), and primers in final concentration of 1 pmol/μl each. PRC products were analyzed electrophoretially in 1 % agarose (staining with 0.002 % ethydium bromide, molecular weight marker λ DNA/HindIII, Thermo Fisher Scientific, Inc., USA). *B. thuringiensis* var. *darmstadiensis* 56 (BtH_{10} 56) strain was a negative control.

Amplified fragment of g*yrB* gene was sequenced by Sanger method [34] (CEQ 8000, Beckman Coulter, USA). The Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to analyze nucleotide sequences.

For examination by light microscopy method (Zeiss Axio Imager A2, Carl Zeiss, Germany), BtH_{14} 7-1/23A culture was plated on fish agar (FA, State Research Center of Applied Microbiology and Biotechnology, Russia), grown at 28-30 °C until formation of spores and crystalline endotoxin, and stained by aniline black (Lucar, Russia) [35]. Strain productive capacity was determined in a modified yeast-polysaccharide medium (ratio of components and additives is a commercial secret) by a submerged culture in Erlenmeyer flasks with aeration

(220 rpm) after 68-hour growth at 30 °C. Cell titer was counted by commonly accepted serial dilution method with further counting on FA. Liquid BtH_{14} 7-1/23A-based biopreparation containing spore-crystal complex, 5% NaCl (preservative), 2% spruce extract (flavor), and the medium residues was produced (Ecos Branch of the All-Russian Research Institute for Agricultural Microbiology — ARRIAM, St. Petersburg-Kolpino).

Larvicidal activity of the preparation was tested on larvae of *Aedes aegypti* IV mosquitoes at the age of insect population, as well as on larvae of mosquitoes genus *Aedes*, *Culex pipiens pipiens* forma *molestus*, *Anopheles maculipennis* II and IV from the natural populations. Larvae of *Aedes* genus was caught during May in aedogenic ponds, *Anopheles maculipennis* larvae — during July in anophelogenous ponds, *Culex pipiens pipiens* f. *molestus* — in raw basement rooms during a year. On collection date, larvae were separated by ages and were tested according to recommendations of the World Health Organization (as revised in 2002). Preparation based on *B. thuringiensis* var. *israelensis* 33 strain with titer $3{,}12\times10^9$ CFU/ml and LC_{50} for *A. aegypti* 0.17×10^{-3} % was used as control [36].

At obtainment of larvae of insect population *Aedes aegypti* (population is maintained in ARRIAM for nearly 40 years), imago was kept at 28 °C and moisture of 70-75 % accounting for day and night intervals. Eggs laid by imago on wet blotting paper disks were put in glazed crystallizers and poured by settling running water of room temperature. Larvae was fed by 0.5-1.0 ml of 5 % sterile protein-vitamin concentrate (PVC, Kirishskiy Biochemical Plant, Russia) and left in thermostat at 28 °C. Larvae (IV instar) were selected and placed in test bacterial suspension. The suspension was made based on tap water (dilutions 1:200000, 1:400000, 1:800000, and 1:1600000, which corresponded to conventional bacterial count 0.5×10^{-3}; 0.25×10^{-3}; 0.125×10^{-3} and 0.0625×10^{-3} %, or 5.0; 2.5; 1.25; 0.625 μl/l). A total of 50 ml of relevant diluted fluid was poured and 25 mosquito larvae species were placed in Petri dish (4-fold replication). Dishes were left in thermostat at 28-30 °C with count of larvae loss in 24 hours. Death rate for each concentration adjusted by death of larvae in control was calculated by formulae:

$$X = (M_o - M_c) : (100 - M_c) \times 100\ \%,$$

where Mt и Mc are arithmetic means of dead insects in test and control, respectfully. LC_{50} expressed in percentage of larva death was calculated by formulae:

$$\lg LC_{50} = \lg C_m - \sigma(\sum X_2 - 0.5),$$

where C_m is maximum concentration amongst tested; σ is dilution factor logarithm (ratio of each previous dilution to the next one); $\sum X_2$ is the sum of ratios of the dead insects to total insects exposed to corresponding dilution of preparations [37].

Field studies of preparation effectiveness against blood-sucking mosquitoes of *Aedes* and *Anopheles* genes were carried out in ponds at the territory of Saint Petersburg conservation parks (Pushkin and Pavlov parks) in places of massive breeding, against *Culex pipiens* Linnaeus f. *molestus* Forskel in raw basement rooms in the Leningrad Region, against rice flies *Cricotopus sylvestris* Fabr. on rice fields (rice checks) in the Krasnodar Territory. Application rate of the preparation against *Culex pipiens molestus*, genus *Aedes*, *Anopheles maculipennis* Meigen and *Cricotopus sylvestris* was 0.25; 0.35; 0.50 and 0.30 ml/m^2, accordingly. Ponds settled by mosquito larvae were selected for field studies, their area was measured, larvae were counted (equivalent to 1 m^2 of water surface) prior to

processing, and treatment was carried out by manual sprayer accounting for the preparation application dosage. The untreated pond settled by larvae served as control. Preparation efficacy (E) was assessed on the 3rd day after treatment by formulae:

$$E = (C_1 - C_2) \cdot C_1^{-1} \times 100\ \%,$$

where C_1 and C_2 are larva counts prior and post treatment, respectfully.

To assess preparation effect on growth of oyster mushroom (*Pleurotus ostreatus*) and champignon (*Agaricus campestris*), fungal mycelium on wheat grain was soaked in bacterial suspension with concentration 5, 10, 20 % (in yeast-polysaccharide medium in control) for 2 hours. After soaking, one grain at a time was placed in the center of Petri dish with medium (2 % wort agar) and transferred to thermostat (25 °C, moisture 85 %; 4-fold biological replication). Diameter of fungal mycelium colonies was measured on day 7. Preparation and chemical insecticide, the inhibitor of chitin synthesis Dimilin® (250 g/kg diflubezuron, Arysta LifeScience S.A.S., Austria) were tested in mushroom house against mushroom flies (*Lycoriidae fucorum* Frey) in dosages of 200 ml and 3 g/m^2, respectively, at separate application [38], and 50 ml and 0.375 g/m^2 at joint application. Larvae were counted before treatment in a 5 m^2 area (mean count of larvae per one sample of 5 g substrate; calculated as 5 samples per 1 m^2). First treatment was carried out by spraying in 3 days after application of cover mix according to mushroom cultivation technology (0.5 l of working suspension per 1 m^2), second treatment was performed in 2 weeks after the first one. Preparation efficacy (E) was assessed by formulae:

$$E = [1 - (a \times b)/(c \times d)] \times 100\ \%,$$

where a is mean arithmetic count of live insects after treatment, b is mean arithmetic count of live control insects before treatment, c is mean arithmetic count of live test insects before treatment, d is mean arithmetic count of live control insects after treatment.

Findings were processed by standard dispersion analysis method at confidence interval of 95 % [39]. Mean (*M*) and standard error mean (±SEM) were calculated. Statistical significance of differences was assessed by *t*-Student test at confidence interval of 95 % ($p < 0.05$).

Results. Based on classic approach proposed by H. De Barjac and A. Bonnefoi, we had characterized BtH_{14}7-1/23A [32] strain as *B. thuringiensis* var. *israelensis*. To confirm systematic position of the studied strain, PCR-product of *gyrB* gene was obtained and sequenced by using relevant primers (Table 1).

1. Primers used in the study of *Bacillus thuringiensis* var. *israelensis* 7-1/23A genome characteristics

Primer	Gene	Oligos (5′→3′)	PCR product, bp	T_m, °C	Elongation, sec	Reference
GyrB	*gyrB*	CTTGAAGGACTAGARGCAGT (f) CCTTCACGAACATCYTCAC(r)	1500	55	90	[40]
Cry11	*cry11*	TTAGAAGATACGCCAGATCAAGC(f) CATTTGTACTTGAAGTTGTAATCCC (r)	305	45	50	[41]
Cry4	*cry4*	GCATATGATGTAGCGAAACAAGCC(f) ACCTGGAACATCTGACAACCAATC (r)	439	62	60	[42] [43]

As noted, analysis of nucleotide sequence of gene encoding 16S RNA used for separation of most bacteria does not allow differentiating *B. thuringiensis* subspecies due to conservative property of such gene [43]. At the same time, sequence analysis of *gyrB* gene by BLAST program had shown full sequence identity of *gyrB* gene in 7-1/23A strain and sequences of the same gene

in reference *B. thuringiensis* var. *israelensis* AM65-52 strain out of GenBank NCBI (https://www.ncbi.nlm.nih.gov/, accession number CP013275.1) and had revealed significant deviations from sequence of relevant fragment of gene *B. thuringiensis* var. *thuringiensis* (accession number CP004123.1) selected as negative control (Fig. 1). Thus, it was established that studied strain is in fact *B. thuringiensis* var. *israelensis*.

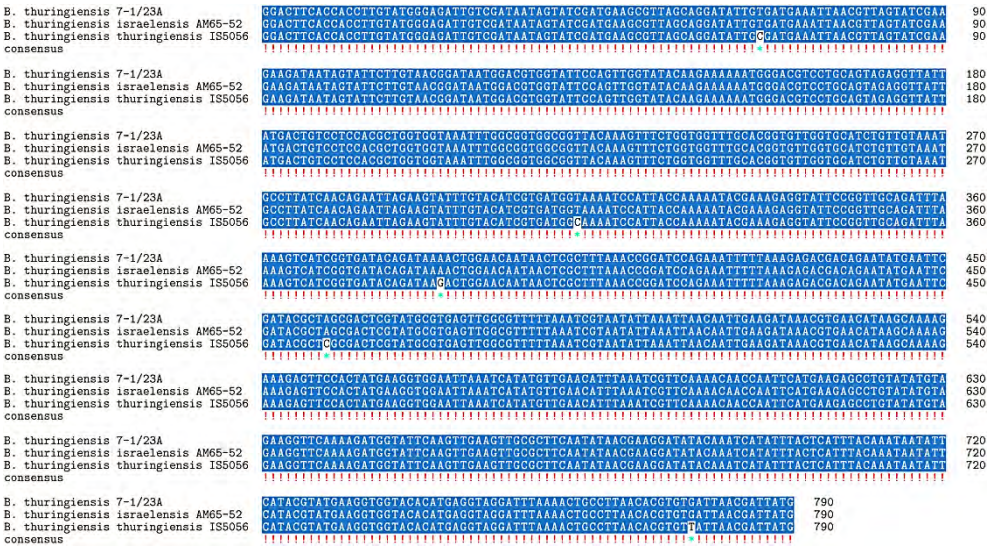

Fig. 1. Alignment of nucleotide sequences of *Bacillus thuringiensis* var. *israelensis* 7-1/23A gene *gyrB* and reference strains AM65-52 *B. thuringiensis* var. *israelensis* and IS5056 *B. thuringiensis* var. *thuringiensis*. Positions completely identical in all three strains are colored in blue, single nucleotide replacements in white.

Positive amplifications with Cry 4 и Cry 11 primers occurred for genomic DNA of *B. thuringiensis* var. *israelensis* 7-1/23A. As expected, DNA amplification of *B. thuringiensis* var. *darmstadiensis* strain 56 (negative control) did not render positive result with these primers (Fig. 2).

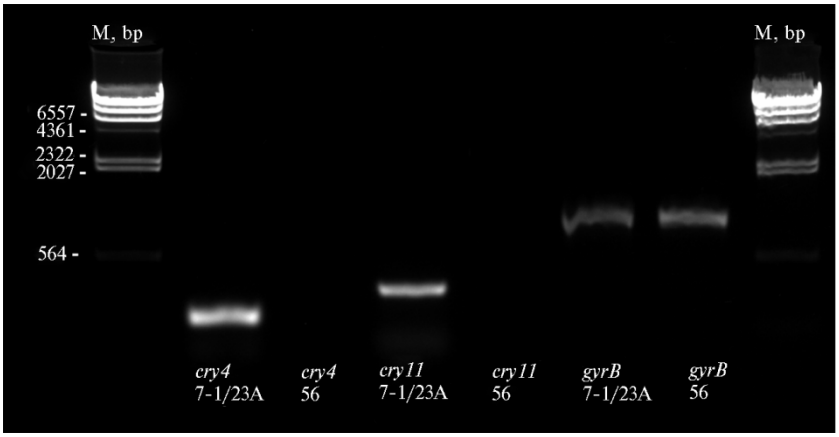

Fig. 2. Gel electrophoresis of PCR products with primers to *cry4*, *cry11* and *gyrB* genes for strains *Bacillus thuringiensis* 7-1/23A и 56. M — marker of molecular weight λ DNA/HindIII (Thermo Fisher Scientific, Inc., USA). Genes (subject to used primers), strain numbers, and weight of control DNA fragments are specified.

Such findings confirm presence of *cry4* and *cry11* genes encoding protein insecticide toxins in 7-1/23A strain. Based on references, both toxins relate to main Bt toxins specific for *Diptera*. Herewith, Cry4 toxin is highly toxic for lar-

vae of *Anopheles* and *Aedes* mosquito genus (to the lesser extent it had effect against *Culex* larvae), and Cry11 manifests high efficacy regarding *Aedes* and *Culex* and low against *Anopheles* [44]. Therefore, composition of toxins identified in our work in 7-1/23A strain ensures its effect against three key mosquito genera, the vectors of dangerous transmissive diseases (*Anopheles*, *Aedes* and *Culex*).

2. Morphocultural traits of *Bacillus thuringiensis* var. *israelensis* 7-1/23A strain

Trait	Description
Gram staining	Gram-positive
Form and size of vegetative cells	Even or slightly sulcated rods of 2.9-3.8×0.9-1.1 μm
Mobility	Peritrithous flagellation
Cell connection	Chains (short, long)
Spore formation	Sub-terminal
Spore form and size	Elliptic, 0.9-1.1 μm in diameter
Form and size of crystalline protein endotoxin	Odd, from 0.4-0.5 to 1.2-1.4 μm
Colony characteristic:	
diameter	0.8-1.2 cm
surface	Finely-rough
profile	Flat
optical properties	Matted
color	Grayish-white
substrate color	Persistent
edge	Rolling
structure	Fine-grained
consistence	Viscous

Analysis of morphocultural traits of 7-1/23A strain (Table 2) revealed that, as per complex of featurea, the strain is similar to reference strain *B. thuringiensis* var. *israelensis* AM65-52. In 48 hours of growth on FA at 30 °C, *B. thuringiensis* var. *israelensis* 7-1/23A formed large colonies of uneven form, while formation of spores and crystalline endotoxin occurred on day 5 (Fig. 3). Productive capacity of BtH_{14} 7-1/23A in yeast-polysaccharide medium comprised 4.25×10^9 CFU/ml.

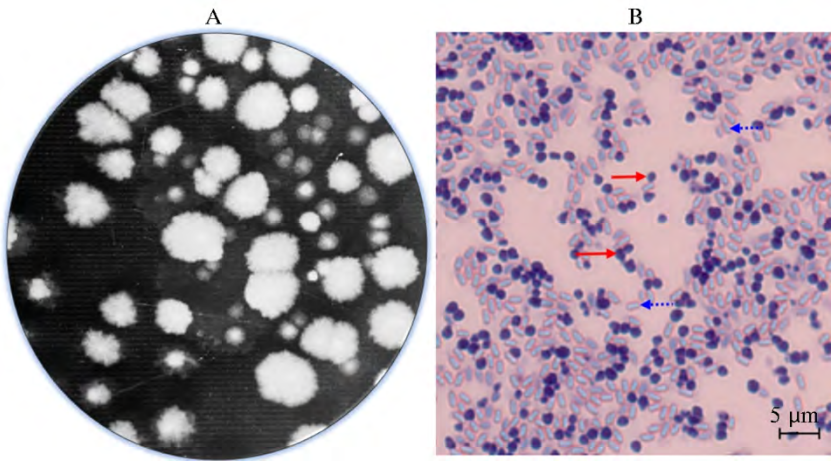

Fig. 3. Growth of colonies in 48 hours (A) **and formation of spores and endotoxin on day 5 of growth** (B) **of *Bacillus thuringiensis* var. *israelensis* 7-1/23A strain on fish agar.** Staining by aniline black (Lucar, Russia); Zeiss Axio Imager A2 microscope (Carl Zeiss, Germany, ×2000 zoom); spores are marked by blue arrows, crystals by red arrows.

In lab tests high activity of biopreparation based on BtH_{14} 7-1/23A strain against the natural populations of mosquito larvae of various genus *Aedes*, *Anopheles maculipennis* and *Culex pipiens molestus* was established (Table 3). Such data of laboratory biotests align with results of PCR identification of the toxic composition of the strain. Larva of genus *Aedes* and *Culex pipiens mo-*

lestus were the most susceptible. Activity of the preparation expressed in LC_{50}, for L_4 of mosquitoes genus comprised $(0.12\pm0.015)\times10^{-3}$ and $(0.11\pm0.015)\times10^{-3}$ % for *Aedes* and *Culex pipiens molestus*, respectively, and $(0.29\pm0.01)\times10^{-3}$ % for *Anopheles maculipennis*, thus yielding to the first two in 2.4-2.6 times (differences were statistically significant at $p < 0.05$). The same trend was demonstrated by preparation based on BtH_{14} 33 (taken as control), but its activity was 1.27-1.40 times lower than in BtH_{14} 7-1/23A-based preparation.

3. Larvicidal activity of liquid on *Bacillus thuringiensis* var. *israelensis*-based preparations against natural populations of blood-sucking mosquitoes ($M\pm$SEM, lab test, $n = 4$)

Mosquitoes	LC_{50} for mosquito larvae, $\times10^{-3}$ %			
	II instar		IV instar	
	BtH_{14} 7-1/23A	BtH_{14} 33 (etalon)	BtH_{14} 7-1/23A	BtH_{14} 33 (etalon)
Genus *Aedes*	0.078±0.010	0.096±0.015	0.120±0.015	0.180±0.015
Culex pipiens Linnaeus f. *molestus* Forskel	0.062±0.015	0.084±0.020	0.110±0.015	0.140±0.015
Anopheles maculipennis Meigen	0.180±0.020	0.250±0.020	0.290±0.010	0.400±0.020

N o t e. Natural population of *Aedes* genus included *A. communis*, *A. dorsalis*, *A. punctor*, *A. flavescens*, *A. caspius species*. Data was processed by dispersion method at 95 % confidence interval. LC_{50} for L_4 *Anopheles maculipennis* in 2.4-2.6 times yielded LC_{50} for L_4 of mosquitoes genus *Aedes* and *Culex pipiens molestus* (the differences were statistically significant at $p < 0.05$).

Mosquitoes susceptibility to BtH_{14} was high species-specific, which, evidently, relates to specific features of the composition of toxin complex in various strains of the bacteria. Thus, the BtH_{14} 7-1/23A-based preparation was highly effective against blood-sucking mosquito genus *Aedes*, *Anopheles,* and *Culex* (90.2-100 % death). Susceptibility of the above insect genus, according to the literature sources, is due to presence of main insecticide toxins Cry4, Cry11 and Cyt and Cry10 in strains [44]. PCR analysis (see Fig. 1) revealed presence of *cry4* и *cry11* genes in BtH_{14} 7-1/23A, which are in charge for production of the same toxins determining the spectrum of entomocide action and efficacy towards dipteral larva stages.

According to Kandybin et al. [2], bactoculicide (preparation based on *B. thuringiensis* var. *israelensis*) LC_{50} for L_4 towards *Aedes cantans* and *A. vexans* is 0.035 mg/l, *Anopheles messeae* 1.05 mg/l (over 3-fold excess). Bactocolucide in dosage of 0.02 g/m^2 water surface caused 59 and 80 % loss in larvae of *Anopheles culicifaciens* and *A. stephensi* in 48 hours and 99 % of loss of larvae *Culex quinquefasciatus* in 24 hours [2]. Liquid bactoculicide in dosages 0.25; 0.40 and 0.50-0.75 ml/m^2 caused 90-96 % of loss of larvae of *Culex pipiens molestus*, *Aedes communis*, and *Anopheles maculipennis*, respectively [45]. BtH_{14} preparation in concentration of 1 ml/l caused 72% death of *Anopheles maculipennis* III instar larvae of Crimean population. It should be noted that the activity of this preparation was 1.7 times inferior to BtH_{14} 7-1/23A-based preparation with LC_{50} against L_4 *Anopheles maculipennis* of 0.29×10^{-3} % [46].

4. Effectiveness of *Bacillus thuringiensis* var. *israelensis* 7-1/23A-based liquid preparation against blood-sucking mosquitoes and rice flies ($n = 3$, 2013-2015, field trials)

Genus, species	Test sites	Biological effectiveness, %
Aedes	Aedogenic ponds (Saint Petersburg)	92.8-100
Anopheles maculipennis Meigen	Anofelogenous ponds (Saint Petersburg)	94.8-98.4
Culex pipiens Linnaeus f. *molestus* Forskel	Moist basement premises (Leningrad Region)	90.2-100
Cricotopus sylvestris Fabr.	Rice checks (Krasnodar Territory)	94.9-97.8

N o t e. Untreated sites were used for each mosquito species at the test territory as a negative control.

Field trials of biopreparation showes (Table 4) that its effectiveness in

ponds against mosquito genera *Aedes* and *Anopheles*, *Culex pipiens molestus* and rice flies *Cricotopus sylvestris* comprises 90.2-100 %.

Polyfunctionality of insecticide biopreparations based on *B. thuringiensis* strains deserves special attention. It was reported that cultures of *B. thuringiensis* strains promote development of aerial parts of salad plant by 24.5 %, roots by 7.2 % and an 84.8 % increase of plant weight [47]. Similar effect is inherent in several other microorganisms: endophytic bacteria genus *Pseudomonas* renders positive effect on plant growth [48], growth acceleration and vegetative reproduction of wild strawberry occur under the effect of *B. subtilis* strains [49]. *B. thuringiensis* may also affect various fungi groups. Previously we have found fungistatical activity of BtH_{10}, BtH_1 and BtH_{14} on model fungi *Botrytis cinerea* [50] and shown that it varies for various serotypes (in BtH_{14} inhibition of *B. cinerea* is lower than in BtH_{10} and BtH_1).

In this work, to analyze the perspectives for extension of the scope of 7-1/23A-based biopreparation, we examined its effect on growth of mycelium in edible fungus, the oyster mushroom and champignon (Table 5), in lab tests. It was noted that BtH_{14} 7-1/23A-based preparation in concentration of 5 % did not suppress but, rather, accelerate fungal growth by 28.2 and 32.5 % ($p < 0.05$), respectively.

5. Effect of liquid *Bacillus thuringiensis* var. *israelensis* 7-1/23A-based preparation on growth of oyster mushroom mycelium and champignon ($M \pm$SEM, $n = 4$, lab test)

Concentration, %	Growth of fungus colonies on day 7			
	diameter, cm	of control, %	diameter, cm	of control, %
5.0	5.30±0.25	132.5	4.50±0.25	128.2
10.0	5.00±0.50	125.0	4.20±0.20	119.7
20.0	4.70±0.25	117.5	–	–
Control (yeast-polysaccharide medium)	4.00±0.35		3.51±0.35	

N o t e. Dashes mean absence of data. The data were processed by dispersion method at 95 % confidence interval. All differences from control are statistically significant at $p < 0.05$.

Of critical importance is that use of bioinsecticides in combination with chemical pesticides allows decreasing ecological load of each preparation. We have compared the effectiveness of BtH_{14} 7-1/23A-based preparation and chemical inhibitor of chitin synthesis (Dimilin® WP) against moss fly *Lycoriella fucorum* when used separately and jointly. Biological effectiveness at joint use of preparation in dosages decreased by 4-8 times was at least equal to chemical preparation (97.2 % death of larvae *Lycoriella fucorum*) while providing a 38.6 % yield gain. The observed high protective effect was due to synergic effect of preparations [51-53]. The fact that combination of biologicals and chemicals decreases their application rates and, consequently, reduces costs, making their co-use more favorable not only ecologically, but also economically. Ability of the proposed BtH_{14} 7-1/23A-based preparation not only to suppress larvae of dipteral agricultural crop pests, but also positively affect plant growth and development in combination with a reduced pesticide load will be in demand in integrated plant protection.

Our findings allow us in the aggregate to consider BtH_{14} 7-1/23A strain as a perspective producer of larvicidal biopreparation with multifunctional properties. It should be noted that, in global, the practice of use of *B. thuringiensis* var. *israelensis*-based preparations to control various blood-sucking dipteral species constantly grows due to high efficacy, specificity, and safety for many economically valuable organisms [54-56]. The same is confirmed by findings of this study.

Therefore, the suggested *Bacillus thuringiensis* var. *israelensis* 7-1/23A-

based preparation has high larvicidal activity (90.2-100 %) against blood-sucking mosquitoes, the vectors of dangerous infections in humans and animals, and is perspective for sanitary ecology and animal health control. It could also be used for rice crop protection from rice fly *Cricotopus sylvestris* Fabr. and against *Lycoriella fucorum* Frey on commercial mushroom cultures (over 90% effectiveness). Herewith, the strain renders positive effect on growth of oysters and champignons. Also, it is reasonable to learn possibilities of use of the biopreparation based on such strain against feed pest insects. Joint use of developed biopreparation with chemical pesticide is economically reasonable due to reduction of the application rates of preparations and yield increase.

REFERENCES

1. Akbaev M.Sh., Vodyanov A.N., Kosminkov N.E., Yatusevich A.I., Pashkin P.I., Vasilevich F.I. *Parazitologiya i invazionnye bolezni zhivotnykh* [Parasitology and invasive animal diseases]. Moscow, 2000 (in Russ.).
2. Kandybin N.V., Patyka T.I, Ermolova V.P., Patyka I.F. *Mikrobiokontrol' chislennosti nasekomykh i ego dominanta Bacillus thuringiensis* [Insect microbiocontrol and its dominant *Bacillus thuringiensis*]. St. Petersburg—Pushkin, 2009 (in Russ.).
3. Hemingway J., Hawkes N.J., McCarroll L., Ranson H. The molecular basis of insecticide resistance in mosquitoes. *Insect Biochemistry and Molecular Biology*, 2004, 34(7): 653-665 (doi: 10.1016/j.ibmb).
4. Lacey L.A. *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *Journal of the American Mosquito Control Association*, 2007, 23(sp2): 133-163 (doi: 10.2987/8756-971X(2007)23[133:BTSIAB]2.0.CO).
5. Boisvert M., Boisvert J. Effects of *Bacillus thuringiensis* var. *israelensis* on target and nontarget organisms: a review of laboratory and field experiments. *Biocontrol Science and Technology*, 2000, 10(5): 517-561 (doi: 10.1080/095831500750016361)
6. Bravo A., Gill S.S., Soberyn M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*, 2007, 49(4), 423-435 (doi: 10.1016/j.toxicon.2006.11.022).
7. Choi Y.S., Cho E.S., Je Y.H., Roh J.Y., Chang J.H., Li M.S., Seo S.J., Sohn H.D., Jin B.R. Isolation and characterization of a strain of *Bacillus thuringiensis* subsp. *morrisoni* PG-14 encoding delta-endotoxin Cry1Ac. *Current Microbiology*, 2004, 48(1): 47-50 (doi: 10.1007/s00284-003-4102-9).
8. Kim Y.T., Huang H.T. The β-exotoxins of *Bacillus thuringiensis.* I. Isolation and characterization. *Journal of Invertebrate Pathology*, 1970, 15(1): 100-108 (doi: 10.1016/0022-2011(70)90103-5).
9. Raddadi N., Cherif A., Ouzari H., Marzorati M., Brusetti L., Boudabous, A., Daffonchio D. *Bacillus thuringiensis* beyond insect biocontrol: plant growth promotion and biosafety of polyvalent strains. *Annals of Microbiology*, 2007, 57(4): 481-494 (doi: 10.1007/BF03175344).
10. Donmez V.F., Uysal B.D., Erkol E.F., Sezai C.R. Biological control of root rot disease caused by *Rhizoctonia solani* Kuhn on potato and bean using antagonist bacteria. *Acta scientiarum Polonorum. Hortorum cultus = Ogrodnictwo,* 2015, 14(5): 29-40.
11. Malovichko Y.V., Nizhnikov A.A., Antonets K.S. Repertoire of the *Bacillus thuringiensis* virulence factors unrelated to major classes of protein toxins and its role in specificity of host-pathogen interactions. *Toxins*, 2019, 11(6): e11060347 (doi: 10.3390/toxins11060347).
12. Choudhary D.K., Johri B.N. Interactions of *Bacillus* spp. and plants — with special reference to induced systemic resistance. *Microbiol. Res.*, 2009, 164(5): 493-513 (doi: 10.1016/j.micres.2008.08.007).
13. Kumar P., Dubey R.C., Mahshwari D.K. *Bacillus* strain isolated from rhizosphere showed plant growth promoting and antagonistic activity against phythopathogens. *Microbiol. Res.*, 2012, 167(8): 493-499 (doi: 10.1016/j.micres.2012.05.002).
14. Chatterjee S.N., Bhattacharya T., Dangar T.K., Chandra G. Ecology and diversity of *Bacillus thuringiensis* in soil environment. *African Journal of Biotechnology*, 2007, 6(13): 1587-1591.
15. Das J., Dangar T.K. Diversity of *Bacillus thuringiensis* in the rice field soils of different ecologies in India. *Indian J. Microbiol.*, 2007, 47(4): 364-368 (doi: 10.1007/s12088-007-0065-z).
16. Ramalakshmi A., Udayasuriyan V. Diversity of *Bacillus thuringiensis* isolated from Western Ghats of Tamil Nadu state, India. *Current Microbiology*, 2010, 61(1): 13-18 (doi: 10.1007/s00284-009-9569-6).
17. Pane C., Villecco D., Campanile F., Zaccardelli M. Novel strains of *Bacillus* isolated from compost and compost-amended soils as biological control agents against soil-borne phytopathogenic fungi. *Biol. Sci. Technol.,* 2012, 22(12): 1373-1388 (doi: 10.1080/09583157.2012.729143).
18. Akram W., Mahboob A., Javed A.A. *Bacillus thuringiensis* strain 199 can induce systemic resistance in tomato against Fusarium wilt. *Eur. J. Microbiol. Immunol.*, 2013, 3(4): 275-280 (doi:

10.1556/EuJMI.3.2013.4.7).
19. Tao A., Pang F., Huang S., Yu G., Li B., Wang T. Characterization of endophytic *Bacillus thuringiensis* strains isolated from wheat plants as biocontrol agents against wheat flagsmut. *Biocontrol. Sci. Technol.*, 2014, 24(8): 901-924 (doi: 10.1080/09583157.2014.904502).
20. Lacey L.A., Grywaczet D., Shapiro-Ilan D., Frutos R., Brownbridge M., Goettel M.S. Insect pathogens as biological control agents: back to the future. *Journal of Invertebrate Pathology*, 2015, 132: 1-41 (doi: 10.1016/j.jip.2015.07.009).
21. Eswarapriya B., Gopalsamy B., Kameswari B., Meera R., Devi P. Insecticidal activity of *Bacillus thuringiensis* IBt-15 strain against *Plutella xylostella*. *Int. J. Pharm. Tech. Res.*, 2010, 2(3): 2048-2053.
22. Patel K.D., Bhanshali F.C., Chaudhary A.V., Ingle S.S. A new enrichment method for isolation of *Bacillus thuringiensis* from diverse sample types. *Appl. Biochem. Biotechnol.*, 2013, 170: 58-66 (doi: 10.1007/s12010-013-0145-y).
23. Rakhmanin Yu.A., Novikov S.M., Rumyantsev G.I., Ivanov S.I. *Gigiena i sanitariya*, 2006, 5: 10-13 (in Russ.).
24. Raddadi N., Cherif A., Ouzari H., Marzorati M., Brusetti L., Boudabous A., Daffonchio D. *Bacillus thuringiensis* beyond insect biocontrol: plant growth promotion and biosafety of polyvalent strains. *Annals of Microbiology,* 2007, 57(4): 481-494 (doi: 10.1007/BF03175344).
25. Narayanasamy P. *Biological management of diseases of crops. Progress in biological control* (Book 15). Springer, Dordrecht, 2013: 295-429 (doi: 10.1007/978-94-007-6380-7).
26. Patel K.D., Bhanshali F.C., Ingle S.S. Diversity and characterization of *Bacillus thuringiensis* isolates from alluvial soils of Mahi river basin, India. *J. Adv. Dev. Res.*, 2011, 2(1): 14-20.
27. Ermolova V.P. *Bacillus thuringiensis* strains from natural sources in the Leningrad region: isolation and identification. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2016, 51(1): 128-131 (doi: 10.15389/agrobioligy.2016.1.128eng).
28. Bravo A., Likitvivatanavong S., Gill S.S., Soberyn M. *Bacillus thuringiensis*: a story of a successful bioinsecticide. *Insect Biochemistry and Molecular Biology*, 2011, 41(7): 423-431 (doi: 10.1016/j.ibmb.2011.02.006).
29. Jouzani G.S., Valijanian E., Sharafi R. *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. *Appl. Microbiol. Biotechnol.*, 2017, 101(7): 2691-2711 (doi: 10.1007/s00253-017-8175-y).
30. Al-KHamada A.D. Vydelenie entomopatogenov *Vacillus thuringiensis* (*Bt*) iz regiona Deir Ezzor Sirii i ikh biotestirovanie. *Vestnik zashchity rastenii,* 2009, 4: 54-62.
31. Grishechkina S.D., Ermolova V.P., Romanova T.A., Nizhnikov A.A. Search for natural isolates of *Bacillus thuringiensis* for development of ecologically friendly biologicals. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2018, 53(5): 1062-1069 (doi: 10.15389/agrobiology.2018.5.1062eng).
32. de Barjac H., Bonnefoi A. A classification of strains of *Bacillus thuringiensis* Berliner with a key to their differentiation. *Journal of Invertebrate Pathology*, 1968, 11: 335-347.
33. Hansen B.M., Hendriksen N.B. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Applied and Environmental Microbiology*, 2001, 67(1): 185-189 (doi: 10.1128/AEM.67.1.185-189.2001).
34. Sanger F., Nicklen S., Coulson A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 1977, 74(12): 5463-5467 (doi: 10.1073/pnas.74.12.5463).
35. Smirnoff U.A. The formation of crystals in *Bacillus thuringiensis* var *thuringiensis* Berliner before sporulation of temperature inculcation. *J. Insect Pathol.*, 1965, 2: 242-250.
36. *Kollektsiya shtammov bakterii-simbiontov vrednykh nasekomykh i gryzunov, prigodnykh dlya biokontrolya chislennosti vreditelei sel'skokhozyaistvennykh rastenii* [Collection of bacterial symbionts of harmful insects and rodents suitable for biocontrol of the number of pests of agricultural plants]. St. Petersburg, 2014 (in Russ.).
37. *Metodicheskie ukazaniya po primeneniyu i metodam kontrolya kachestva insektitsidnogo mikrobiologicheskogo sredstva «Baktitsid»* [Guidelines for the use and quality control methods of the insecticidal microbiological agent «Bacticid»]. Moscow, 2001 (in Russ.).
38. Mokrousova E.P., Glazunova I.N., Kandybin N.V., Ermolova V.P. V sbornike: *Problemy entomologii v Rossii* [In: Entomological problems in Russia]. St. Petersburg, 1998, tom 2: 40-41 (in Russ.).
39. Dospekhov B.A. *Metodika polevogo opyta* [Methods of field trials]. Moscow, 1973 (in Russ.).
40. Punina N.V., Zotov V.S., Parkhomenko A.L. Genetic diversity of *Bacillus thuringiensis* from different geo-ecological regions of Ukraine by analyzing the 16S rRNA and gyrB genes and by AP-PCR and saAFLP. *Acta Naturae*, 2013, 5(1): 90-100.
41. Bravo A., Sarabia S., Lopez L., Ontiveros H., Abarca C., Ortiz A., Quintero R. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Applied and Environmental Microbiology*, 1998. 64(12): 4965-4972.
42. Ben-Dov E., Zaritsky A., Dahan E., Barak Z., Sinai R., Manasherob R., Margalith Y. Extended screening by PCR for seven cry-group genes from field-collected strains of *Bacillus thurin-*

giensis. Applied and Environmental Microbiology, 1997, 63(12): 4883-4890.
43. Guidi V., Patocchi N., Lüthy P., Tonolla M. Distribution *of Bacillus thuringiensis* subsp. *israelensis* in soil of a Swiss Wetland reserve after 22 years of mosquito control. *Applied and Environmental Microbiology*, 2011, 77(11): 3663-3668 (doi: 10.1128/AEM.00132-11).
44. Ben-Dov E. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins*, 2014, 6(4): 1222-1243 (doi: 10.3390/toxins6041222).
45. Rakhman A.I., Ermolova V.P. V knige: *Infektsionnye bolezni* [In: Infectious diseases]. St. Petersburg, 2015: 203-207 (in Russ.).
46. Kandybin N.V., Stus' A.A., Kuznetsova L.N. *Byulleten' VNIISKHM*, 1981, 33: 54-57 (in Russ.).
47. Kryzhko A.V., Kuznetsova L.N. *Vestnik Voronezhskogo gosudarstvennogo universiteta*, 2017, 4: 51-53 (in Russ.).
48. Chebotar' V.K., Shcherbakov A.V., Shcherbakova E.N., Maslennikova S.N., ZaplatkinA.N., Mal'fanova N.V. Biodiversity of endophytic bacteria as a promising biotechnological resource. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2015, 50(5): 648-654 (doi: 10.15389/agrobiology.2015.5.648eng).
49. Shternshis M.V., Belyaev A.A., Tsvetkova V.P., Shpatova T.V., Lelyak A.A., Bakhvalov S.A. *Biopreparaty na osnove bakterii roda Bacillus dlya upravleniya zdorov'em rastenii* [Bacillus-based biological products for plant health management]. Novosibirsk, 2016 (in Russ.).
50. Smirnov O.V., Grishechkina S.D. Polyfunctional activity of *Bacillus thuringiensis* Berliner. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2011, 3: 123-126 (in Russ.).
51. Salama H.S., Foda M.S., Zaki F.N., Moawad S. Potency of combinations of *Bacillus thuringiensis* and chemical insecticides on *Spodoptera littoralis* (*Lepidoptera*: *Noctuidae*). *Journal of Economic Entomology*, 1984, 77(4): 885-890 (doi: 10.1093/jee/77.4.885).
52. Grishechkina S.D., Ermolova V.P., Kovalenko T.K., Antonets K.S., Belousova M.E., Yakhno V.V., Nizhnikov A.A. Polyfunctional properties of the *Bacillus thuringiensis* var. *thuringiensis* industrial strain 800/15. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2019, 54(3): 494-504 (doi: 10.15389/agrobiology.2019.3.494eng).
53. Zhang L., Qiu S., Guan X. Effect of chemical additives on *Bacillus thuringiensis* (*Bacillales: Bacillaceae*) against *Plutella xylostella* (*Lepidoptera*: *Pyralidae*). *Journal of Economic Entomology*, 2013, 106(3): 1075-1080 (doi: 10.1603/ec1228).
54. Tetreau G., Alessi M., Veyrenc S., Périgon S., David J.-Ph., Reynaud S., Després L. Fate of *Bacillus thuringiensis* subsp. *israelensis* in the field: evidence for spore recycling and differential persistence of toxins in leaf litter. *Applied and Environmental Microbiology*, 2012, 78(23): 8362-8367 (doi: 10.1128/AEM.02088-12).
55. Derua Y.A., Kweka E.J., Kisinza W.N. Bacterial larvicides used for malaria vector control in sub-Saharan Africa: review of their effectiveness and operational feasibility. *Parasites Vectors*, 2019, 12: 426 (doi: 10.1186/s13071-019-3683-5).
56. Lacey L.A. *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *Journal of the American Mosquito Control Association,* 2007, 23(2 Suppl): 133-163 (doi: 10.2987/8756-971X(2007)23[133:BTSIAB]2.0.CO;2).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1281-1289
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 579.64:632.937.15

doi: 10.15389/agrobiology.2019.6.1281eng
doi: 10.15389/agrobiology.2019.6.1281rus

INSECTICIDAL PROPERTIES OF *Bacillus thuringiensis* var. *israelensis*. II. COMPARATIVE MORPHOLOGICAL AND MOLECULAR GENETIC ANALYSIS OF THE CRYSTALLOGENIC AND ACRYSTALLOGENIC STRAINS

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by the project of applied research and experimental development (PNER) batch 2017-14-579-0030 on the topic: «Creation of microbiological preparations for expanding the adaptive capacity of agricultural crops for nutrition, resistance to stress and pathogens» (code of the application 2017-14-579-0030-013), Agreement No. 14.607.21.0178, a unique identifier (project) RFMEFI60717X0178
Received July 19, 2019

Abstract

Currently, the bacterium *Bacillus thuringiensis* var. *israelensis* represents a key agent for biological protection against dipteran species, which are harmful to livestock and crop production and transmit infectious diseases of economically important animals. The production strains can be obtained by isolation from natural resources, selection of previously used isolates, screening of genetic collections, and genetic or genomic engineering. The issue of preservation and control of practically valuable properties of strains is of high importance. Biologicals are of significant interest due to their substantial advantages over chemical pesticides and are considered in modern agricultural systems as environmentally and socially priority alternatives to agrochemicals. In the present work, we performed the first comprehensive comparative analysis of crystallogenic and acrystallogenic variants of *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) isolated after storage of the strain in different modes. For crystallogenic variants, genes encoding the target insecticidal toxins, Cry4 and Cry11, were detected by the polymerase chain reaction (PCR), and it was shown that the acrystallogenic variants are devoid of these genes. It was found that the culture fluid of crystallogenic variants is approximately 7000 times more active against the *Aedes aegypti* larvae than the same of acrystallogenic. The aim of this work was to compare the morphological, biochemical, technological, larvicidal properties of the crystal-forming and acrystallogenic variants of the strains of *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) and testing for the presence of genes encoding Cry insecticidal toxins, which are key determinants of virulence. We studied the strains 404 and 87 stored for 28 years by freeze-drying, then 2 years in fish agar (FA) slant tubes with re-inoculation every 6 months; the 7-1/23 strain stored for 28 years in crystals of NaCl, then 2 years in culture liquid (CL) at 3 °C. Bacterial strains were inoculated on Petri dishes to obtain separate colonies. On day 7 of growth, the 404/14, 87/21, 7-1/23-4 (crystal-forming) and 404/19, 87/33, 7-1/23-8 (acrystallogenic) variants were selected by microscopic analysis using aniline black dye. The differences in the colony morphology were not revealed: the colonies were flat, opaque, grayish-white, rough, rounded, the structure was fine-grained, and the consistency was viscous. The differences either in the morphology of the vegetative cultures, or in the main biochemical properties (the formation of acetylmethyl carbinol, lecithinase, the use of carbohydrates, the splitting of starch, etc.), or in the titer on the yeast-polysaccharide medium were not shown as well. The productivity of the 404/14, 87/21, 7-1/23-4 and 404/19, 87/33, 7-1/23-8 strains varied from 3.36×10^9 CFU/ml to 4.02×10^9 CFU/ml and from 3.74×10^9 CFU/ml to 4.13×10^9 CFU/ml, respectively. The larvicidal activity of the crystal-forming variants, expressed in LC_{50} for L4 *Aedes aegypti*, was $(0.12\text{-}0.16)\times10^{-3}$ %, while acrystallogenic variants were inactive within the standard dilutions ($\times10^{-3}$ %) 1.0; 0.5; 0.25; 0.125; 0.06. Only their 1 % suspension (7000-fold higher concentration) caused 22-39 % death of the *Aedes* larvae after 24 hours; the same concentration of

active variants resulted in 100 % death in 15 minutes. It was established that cultural liquid of the acrystallogenic variants formed a precipitate and a supernatant layer after 12 hours, while the crystal-forming variants remained suspended. The investigated variants of BtH_{14} were analyzed for the presence of genes encoding insecticidal toxins. The results of the PCR analysis with the Bti-specific primers confirmed the belonging of the both crystal-forming and acrystallogenic variants to BtH_{14}. It has been found that the 404/14, 87/21, 7-1/23-4 strains carry genes encoding the Cry4 and Cry11 insecticidal toxins, while 404/19, 87/33, 7-1/23-8 acrystallogenic variants are devoid of these genes agreeing with the absence of larvicidal activity against *A. aegypti*.

Keywords: *Bacillus thuringiensis*, culture liquid, larvicidal activity, insecticidal toxins, Cry4, Cry11

Blood-sucking mosquitoes and flies of the order *Diptera*, transmitters of dangerous infections in humans and animals, significantly damage animal husbandry. *Bacillus thuringiensis* subsp. *israelensis* (BtH_{14}) is an effective bacterial agent of dipteral larvae control used worldwide. Preparations based on such bacteria are used in wide range of habitat of target insects, not causing their resistance and not rendering negative effect on ecosystem [1]. BtH_{14} is considered as priority bioagent in fighting blood-sucking mosquitoes, the parasites of animals and humans and transmitters of diseases, including anaplasmose in cattle stock. Blood-sucking dipteral species can cause a 20-30 % decrease in milk yields and 20-40 % decrease in body weight gain [2].

In plants, including seeded forages, “green” biologicals against harmful organisms based on bacteria [3-5], actinomycetes [6], entomorphtorales and entomopathogenic nematodes [7, 8] as a factor of optimization of agricultural plant protection and nutrition is also an important trend. One of such bioagents is spore-forming bacteria *Bacillus thuringiensis* (Bt) [9, 10] with a complex of useful features. Bt can protect agricultural crops from phytophagans [11, 12] and plant pathogens [13-15], may stimulate plants growth [16, 17], whilst being safe for humans [18] and useful entomofauna [19, 20]. Bt-based biopreparations take the priority place at the market as the most effective and safe for environment [21-23]. Their active substance is spore-crystalline complex [24, 25], in some cases also the thermostable exotoxin [26, 27], as well as a series of other lesser studied metabolites and protein factors of virulence [28-30].

Optimal profitable preparations are obtained at use of producer strain of high virulence, technological effectiveness, and safety. However, any culture of producers is subjected to population diversity [31, 32]. Long storage, frequent reinoculations on solid agar mediums result in formation of colonies of various morphotypes and lead to slight decrease of virulence [33]. Thus, Bt strains stored for 28 years in crystals of sodium chloride or in lyophilized form, and 10 years (observation term) in cryopreserved state remained 100 % viable, but manifested larvicidal activity at a level of 78-90 % as compared to the initial values.

In presented work we for the first time performed comprehensive analysis of isolated crystallogenic and acrystallogenic variants of *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) by polymerase chain reaction (PCR) method at early development stage of culture (18 hours). Genes encoding insecticide toxins Cry4 and Cry11 were identified in studied crystallogenic variants, whereas such genes were not found in acrystallogenic ones. Herewith, variants did not differ by main biochemical properties, culture morphology, and colonies on fish agar (FA), as well as by productive capacity. It was established that cultural fluid (CF) in 12 hours after incubation does not form sediment in crystallogenic variants as opposed to acrystallogenic ones, and is approximately 7000 times more active against *Aedes aegypti* larvae.

The purpose of the study was to compare morphological, biochemical, technological, and larvicidal features and presence of entomocidal toxins Cry in vilurent and avirulent variants of BtH_{14} strains.

Techniques. Strains BtH_{14} 404 and 87 were stored for 28 years in lyophilized state, and afterwards 2 years on fish agar (FA) slant tubes with reinoculation every 6 months; 7-1/23 strain was stored for 28 years in NaCl crystalls, and afterwards for 2 years in its CF at 3 °C. Initial productive capacity was characterized by the following values ($\times 10^9$ CFU/ml): 3.96±0.28; 4.4±0.22; 4.25±0.30; LK_{50} for L_4 *Aedes aegypti* comprised $(0.135\pm0.01)\times10^{-3}$ %; $(0.115\pm0.015)\times10^{-3}$ %; $(0.128\pm0.01)\times10^{-3}$ %, respectively.

BtH_{14} strains were cultured on slant FA at 30 °C until full formation of spores and crystals.

Morphology of BtH_{14} colonies was studied on standard fish agar (FA) in Petri dishes using streak plate method on day 7 of growth at 30 °C. Preparations for light microscopy (microscope Zeiss Axio Imager A2, Carl Zeiss, Germany, immersion lens ×100) were stained with aniline black (Lucar, Russia). Crystallogenic and acrystallogenic variants [34] were selected to analyze for crystalline toxin genes [35].

Technological efficacy of BtH_{14} was assessed in yeast-saccharine medium, after 68-hour submerged growing at 30 °C and 220 rpm aeration. The number of cells plated on FA was determined by standard serial dilutions. LK_{50} (larvicidal activity) for *Aedes aegypti* mosquitoes was assessed as described [33].

Biochemical properties (utilization of carbohydrates, formation of acetyl methyl carbinol, indol, etc.) were studied with indicator disks (Paper-Based Systems for Identification of Microorganisms, Microgen, Russia) according to the attached instruction. Cultures grown on FA for 1 day at 30 °C were collected by microbiological loop and placed into sterile sodium chloride (0.85 %). Biochemical analysis of strains was performed in 5-18 hours.

For genomic DNA extraction of for PCR analysis, bacteria were cultured on standard Luria-Bertani (LB) medium during 16-18 hours at 30 °C. Cells were suspended in Tris-EDTA buffer (1 M Tris-HCl, pH 7.5 + 0.5 M EDTA, pH 8.0) and heated during 10 minutes at 102 °C. Cell debris was removed by sedimentation during 3 minutes at 15000 g. Supernatant fluid containing genomic DNA was transferred to eppendorfs and used for PCR analysis (a DNA amplifier T100, Bio-Rad, USA) [35, 36]. Program for PCR analysis was selected based on the annealing temperature of known primers and size of the amplified DNA fragment. Reaction mix (20 μl) contained 1 μl water solution of bacterial DNA (80 ng DNA), 10 μl of Fermentas DreamTaq green PCR master mix (Thermo Fisher Scientific, USA), and 0.3 μl of each primer, final concentration of 1 pmol/μl). Virulent strain BtH_{10} 56 of *B. thuringiensis* var. *darmstadiensis* group was a negative control. PCR products were analyzed by electrophoresis in 1 % agarose gel with 0.002 % ethydium bromide staining.

Data were processed by dispersion analysis [37] with 95 % confidence interval. Tables provide means (*M*) and standard error of means (±SEM).

Results. After long-term storage, BtH_{14} 404, 87, and 7-1/23 strains were plated on FA in Petri dishes and selected for formation of crystalline endotoxin and spores and for spore formation only.

Variants BtH_{14} 404/14, 87/21, 7-1/23-4 (crystallogenic) and 404/19, 87/33, 7-1/23-8 (acrystallogenic) were used in further study.

Cultures did not differ morphologically. In all variants colonies were rough, flat, matt, and grayish-white, of undular edge, fine-grain structure, and viscous consistence. Light microscopy of 16-hour cultures also did not find any differences [Fig. 1, A]. Besides, 7-day cultures of BtH_{14} forms did not significantly differ by morphological properties (see Fig. 1, B).

Table 1 provides data on larvicidal properties in BtH_{14} variants. Findings

show that technological efficacy of crystallogenic and acrystallogenic variants of BtH_{14} strain was practically similar, accordingly $(3.36\pm0.25\text{-}4.02\pm0.15)\times10^9$ and $(3.74\pm0.19\text{-}4.13\pm0.15)\times10^9$ CFU/ml, respectively.

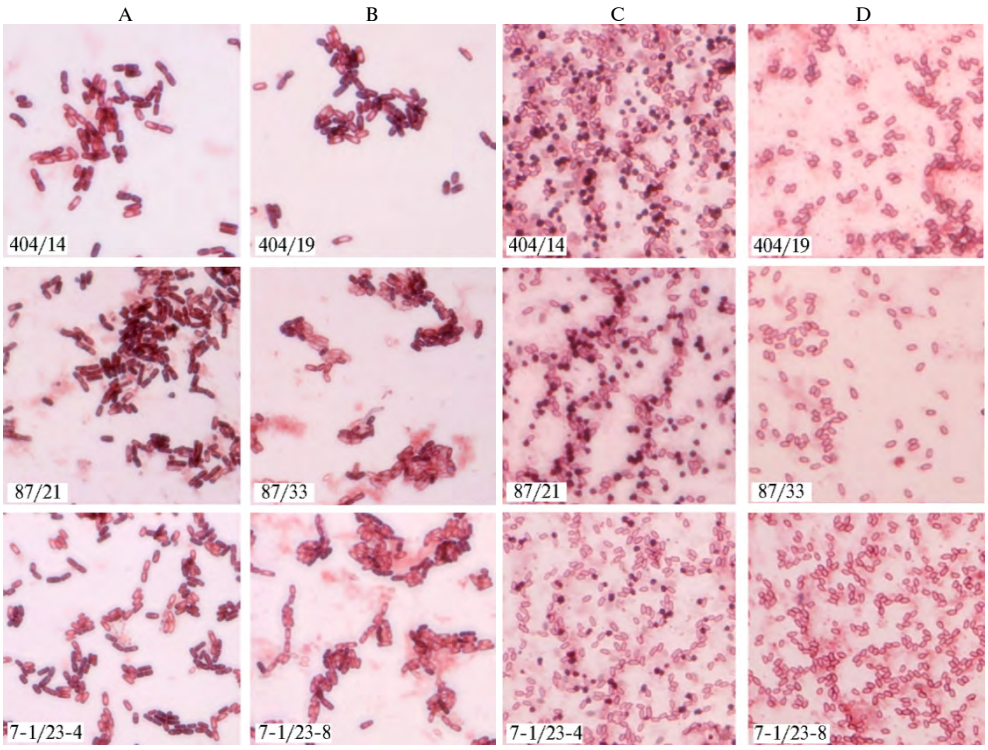

Fig. 1. Crystallogenic (A, C) **and acrystallogenic** (B, D) ***Bacillus thuringiensis* var. *israelensis* (BtH_{14}) variants after 16 hours** (A, B) **and 7 days** (C, D) **of culturing.** Light microscopy (model Zeiss Axio Imager A2, Carl Zeiss, Germany, immersion lens ×100) of preparations stained by aniline black (Lucar, Russia).

Larvicidal activity of crystallogenic variants of BtH_{14} (LK_{50} for L_4 *A. aegypti*) was practically similar within the range $(0.120\pm0.012\text{-}0.160\pm0.018)\times10^{-3}$ %. Acrystallogenic BtH_{14} variants were inactive to L_4 *A. aegypti* in commonly accepted dilutions $(1.0; 0.5; 0.25; 0.125; 0.06)\times10^{-3}$ % CF, and only 1 % suspension (concentration is 7000 times more) caused 22-39 % death in mosquito larvae in 24 hours, whereas in active variants the same concentration caused 100 % death in 15 minutes.

It was interesting that CF of acrystallogenic variants in 12 hours stratified into pellet and supernatant, whilst in crystallogenic variants it remained suspended (Fig. 2). According to our observations, CF of virulent BtH_{14} 7-1/23A strain in suspension preserved for up to 3 years.

1. Larvicidal properties of studied variants of *Bacillus thuringiensis* var *israelensis* (BtH_{14}) ($M\pm$SEM, lab test)

Variant	Results of light microscopy of 7-day culture	Spore titer, $\times10^9$/ml	LC_{50} for L_4 *Aedes aegypti*, $\times10^{-3}$ %
404/14	Spores, crystals	3.36±0.25	0.160±0.018
404/19	Spores	3.74±0.19	0
87/21	Spores, crystals	4.02±0.15	0.120±0.012
87/33	Spores	4.13±0.15	0
7-1/23-4	Spores, crystals	3.95±0.10	0.150±0.020
7-1/23-8	Spores	4.00±0.18	0

N o t e. Results were processed by ANOVA with confidence interval of 95 %. Differences in productive capacity of crystallogenic strains between each other and as compared to acrystallogenic were statistically insignificant. Crystallogenic strains statistically significantly differed from acrystallogenic strains in larvicidal activity.

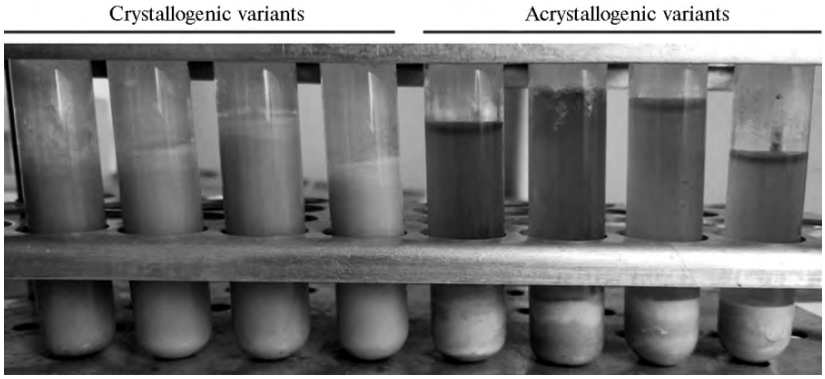

Fig. 2. Culture fluid of crystallogenic *Bacillus thuringiensis* var. *israelensis* (BtH_{14}) variants in 12 hours at room temperature remains a suspension, whereas acrystallogenic variants form pellet.

Crystallogenic (virulent) and acrystallogenic (avirulent) variants of BtH_{14} did not differ in analyzed complex of biological properties. Thus, they have formed acetyl methyl carbinol and lecithinase, hydrolyzed starch, have manifested proteolytic activity and did not have urease activity. Strains metabolized glycerin, glucose, laevulose, maltose, and mannose, did not utilize arabinose, galactose, dulcitol, xilose, salicine, saccharose, sorbitol, cellobiose, and esculite. Moreover, they did not form pigment, but form film on meat peptone broth.

2. Primers used for PCR analysis of crystallogenic and acrystallogenic variants

Primer	Gene	Oligos (5′→3′)	T_m, °C	Reference
Cry11	*cry11*	TTAGAAGATACGCCAGATCAAGC(f) CATTTGTACTTGAAGTTGTAATCCC (r)	45	[38]
Bti		CAAACATTTCATTCCAATAACA (f) ATACTGTGTGGGATGCTTATTA (r)	59	[39]
Cry4	*cry4*	GCATATGATGTAGCGAAACAAGCC(f) ACCTGGAACATCTGACAACCAATC (r)	62	[40] [35]

Since morphologically crystallogenic (virulent) and acrystallogenic (avirulent) variants of BtH_{14} did not demonstrate visual differences, and crystal formation occurs only in few days of incubation, we have characterized crystallogenic and acrystallogenic variants of BtH_{14} strain by PCR using 18-hour cultures. Used primers are presented in Table 2.

Bti primers amplify the nucleotide sequence which is specific for *B. thuringiensis* var. *israelensis* and located on chromosomal DNA. Positive result of amplification with Bti primers (Table 3, Fig. 3) confirmed that the studied variants belong to *B. thuringiensis* var. *israelensis*. PCR analysis revealed *cry4* and *cry11* genes in variants 404/14, 87/21 and 7-1/23-4. In case of variants 404/19, 87/33, 7-1/23-8, no amplification occurred, which confirms absence of crystalline endotoxin and larvicidal activity against *A. aegypti*.

3. Comparative characterization of crystallogenic and acrystallogenic variants of *Bacillus thuringiensis* var. *israelensis* by PCR test

Variant	Primers			Crystallogenicity
	Bti	Cry 4	Cry 11	
404/14	+	+	+	+
404/19	+	–	–	–
87/21	+	+	+	+
87/33	+	–	–	–
7-1/23-4	+	+	+	+
7-1/23-8	+	–	–	–

N o t e. «+» and «–» mean presence and absence of positive reaction or manifestation of the train.

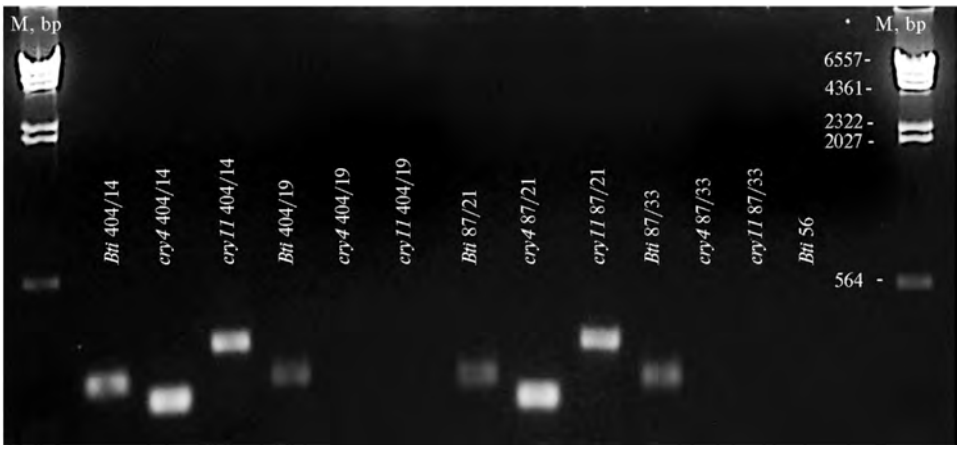

Fig. 3. Gel electrophoresis of PCR products from *Bacillus thuringiensis* var. *israelensis* DNA fragment amplification with Bti, Cry4 and Cry11 primers for crystallogenic and acrystallogenic strains. M — molecular weight marker λ DNA/HindIII (Thermo Fisher Scientific, USA). Genes (as per used primers) and strain numbers are specified.

Therefore, the obtained data draw us to conclude that virulent variants of *Bacillus thuringhighiensis var. israelensis* 404/14, 87/21, 7-1/23, as opposed to avirulent 404/19,87/21, 7-1/23-8, carry genes encoding insecticide toxins Cry4 and Cry11, form crystalline endotoxins, have higher larvicidal activity (1 % suspension of culture fluid of crystallogenic variants caused 100 % death of *A. aegypti* larvae in 15 minutes, whilst acrystallogenic variants caused only 22-39 % death in 24 hours) and remain suspended in culture fluid as opposed to avirulent variants forming pellet in 12 hours. Fast PCR screening to identify genes encoding main protein toxins of *B. thuringiensis* may be helpful in assessing production properties of *B. thuringiensis* var. *israelensis* strains. Since virulent and avirulent variants do not show significant morphological and biochemical differences during long-term culturing, presence or absence of *cry* genes is the most convenient indicator to select promising strains. Molecular genetic methods do not exclude the need for accounting the spore formation rate and ratio of spores to protein crystalline inclusions by light microscopy method, and to control strain titer as indicator of its technological efficacy.

REFERENCES

1. Ben-Dov E. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins*, 2014, 6(4): 1222-1243 (doi: 10.3390/toxins6041222).
2. Akbaev M.Sh., Vodyanov A.N., Kosminkov N.E., Yatusevich A.I., Pashkin P.I., Vasilevich F.I. *Parazitologiya i invazionnye bolezni zhivotnykh* [Parasitology and invasive animal diseases]. Moscow, 2000 (in Russ.).
3. Polanczyk R.A., Pires da Silva R.F., Fiuza L.M. Effectiveness of Bacillus thuringiensis against Spodoptera frugipera (*Lepidoptera*: *Noctuida*). *Brazilian Journal of Microbiology*, 2000, 31(3) 165-167 (doi: 10.1590/S1517-83822000000300003).
4. Sessitsch A., Reiter B., Berg G. Endophytic bacterial communities of field grown potato plants and their plant growth promoting abilities. *Canadian Journal of Microbiology*, 2004, 50: 239-249 (doi: 10.1139/w03-118).
5. Kandybin N.V., Patyka T.I., Ermolova V.P., Patyka V.F. *Mikrobiokontrol' chislennosti nasekomykh i ego dominanta* Bacillus thuringiensis [Insect microbiocontrol and its dominant *Bacillus thuringiensis*]. St. Petersburg—Pushkin, 2009 (in Russ.).
6. Scherwinski K., Wolf A., Berg G. Assessing the risk of biological control agents on the indigenous microbial communities: *Serratia plyuthica* HRO-C48 and *Streptomyces* sp. HRO-71 as model bacteria. *Biocontrol*, 2006, 52: 87-112 (doi: 10.1007/s10526-006-9006-8).
7. Lednev G.R., Novikova I.I. V sbornike: *Biologicheskie sredstva zashchity rastenii, tekhnologii ikh izgotovleniya i primeneniya* [In: Biological plant protection products, technologies for their manufacture and use]. St. Petersburg, 2005: 261-272 (in Russ.).

8. Danilov L.G. *Vestnik zashchity rastenii*, 2018, 3: 38-42 (in Russ.).
9. Lacey L.A., Grzywacz D., Shapiro-Ilan D.I., Frutos R., Brownbridge M., Goettel M.S. Insect pathogens as biological control agents: back to the future. *Journal of Invertebrate Pathology*, 2015, 132: 1-41 (doi: 10.1016/j.jip.2015.07.009).
10. Bravo A., Gill S.S., Soberon M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*, 2007, 49: 423-435 (doi: 10.1016/j.toxicon.2006.11.022).
11. Arglo-Filho R.C., Loguercio L.L. *Bacillus thuringiensis* in an environmental pathogen and host-specificity has developted as an adaptation to human-generated ecological nishes. *Insects*, 2014, 5(1): 62-91 (doi: 10.3390/insects5010062).
12. Mnif I., Ghribi D. Potential of bacterial derived biopesticides in pest management. *Crop Protection*, 2015, 77: 52-64 (doi: 10.1016/j.cropro.2015.07.017).
13. Naidar R., Deschamps A., Roudet J., Calvo-Garrido C., Bruez E., Rey P., Fermaud M. Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. *Biological Control*, 2016, 92: 55-65 (doi: 10.1016/j.biocontrol.2015.09.003).
14. Heydari A., Pessaraki M. A review on biological control of fungal plant pathogens using microbial antagonists. *Journal of Biological Sciences*, 2010, 1(4): 273-290 (doi: 10.3923/jbs.2010.273.290).
15. Akram W., Mahboob A., Jave d A. *Bacillus thuringiensis* strain 199 can induce systemic resistance in tomato against *Fusarium* wilt. *European Journal of Microbiology and Immunology*, 2013, 3: 275-280 (doi: 10.1556/EuJMI.3.2013.4.7).
16. Choudhary D.K., Johri B.N. Interactions of *Bacillus* spp. and plants — with special reference to induced systemic resistance. *Microbiological Research*, 2009, 164(5): 493-513 (doi: 10.1016/j.micres.2008.08.007).
17. Kumar P., Dubey R.C., Mahshwari D.K. *Bacillus* strain isolated from rhizosphere showed plant growth promoting and antagonistic activity against phythopathogens. *Microbiological Research*, 2012, 167(8): 493-499 (doi: 10.1016/j.micres.2012.05.002).
18. Raddadi N., Cherif A., Ouzari H., Marzorati M., Brusetti L., Boudabous A., Daffonchio D. *Bacillus thuringiensis* beyond insect biocontrol: plant growth promotion and biosafety of polyvalent strains. *Annals of Microbiology*, 2007, 57(4): 481-494 (doi: 10.1007/BF03175344).
19. Raymond B., Federici B.A. In defense of *Bacillus thuringiensis*, the safest and most successful microbial insecticide available to humanity — a response to EFSA. *FEMS Microbiology Ecology*, 2017, 93(7): fix084 (doi: 10.1093/femsec/fix084).
20. Al-Khamada A.D. *Vestnik zashchity rastenii*, 2009, 4: 54-62 (in Russ.).
21. Pane C., Villecco D., Campanile F., Zaccardelli M. Novel strains of *Bacillus* isolated from compost and compost-amended soils as biological control agents against soil-borne phytopathogenic fungi. *Biocontrol Science and Technology*, 2012, 22(12): 1373-1388 (doi: 10.1080/09583157.2012.729143).
22. Tao A., Pang F., Huang S., Yu G., Li B., Wang T. Characterization of endophytic *Bacillus thuringiensis* strains isolated from wheat plants as biocontrol agents against wheat flagsmut. *Biocontrol Science and Technology*, 2014, 24(8): 901-924 (doi: 10.1080/09583157.2014.904502).
23. *The manual of biocontrol agents.* British Protection Council Publication, Alton, V.K., 2014.
24. Al-Momani F., Obeidat M., Saasoun I., Mequam M. Serotyping of *Bacillus thuringiensis* isolates their distribution in different Jordanian habitats and pathogenecity in *Drosophila melanogaster*. *World Journal of Microbiology and Biotechnology*, 2004, 20: 749-753 (doi: 10.1007/s11274-004-4517-x).
25. Choi Y.S., Cho E.S., Je Y.H., Roh J.Y., Chang J.H., Li M.S., Seo S.J., Sohn H.D., Jin B.R. Isolation and characterization of a strain of *Bacillus thuringiensis* subsp. morrisoni PG-14 encoding δ-endotoxin Cry1Ac. *Current Microbiology*, 2004, 48: 47-50 (doi: 10.1007/s00284-003-4102-9).
26. Grishechkina S.D., Ermolova V.P., Kovalenko T.K., Antonets K.S., Belousova M.E., Yakhno V.V., Nizhnikov A.A. Polyfunctional properties of the *Bacillus thuringiensis* var. *thuringiensis* industrial strain 800/15. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2019, 54(3): 494-504 (doi: 10.15389/agrobiology.2019.3.494eng).
27. Grishechkina S.D., Ermolova V.P., Romanova T.A., Nizhnikov A.A. Search for natural isolates of *Bacillus thuringiensis* for development of ecologically friendly biologicals. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2018, 53(5): 1062-1069 (doi: 10.15389/agrobiology.2018.5.1062eng).
28. Shrestha A., Sultana R., Chae J.-C., Kim K., Lee K.-J. *Bacillus thuringiensis* C-25 which is rich in sell wall degrading enzymes efficiently control lettuce drop caused by *Sclerotinia minor*. *Eur. J. Plant Pathol.*, 2015, 142(3): 577-589 (doi: 10.1007/s10658-015-0636-5).
29. Saber W.I.A. Ghoneem K.V., Al-Askar A.A., Rashad Y.M., Ali A.A., Rashad E.M. Chitinase production by *Vacillus subtilis* ATCC 11774 and its effect on biocontrol of *Rhizoctonia* disease of potato. *Acta Biologica Hungarica*, 2015, 66(4): 436-448 (doi: 10.1556/018.66.2015.4.8).
30. Malovichko Y.V., Nizhnikov A.A., Antonets K.S. Repertoire of the *Bacillus thuringiensis* virulence factors unrelated to major classes of protein toxins and its role in specificity of host-pathogen interactions. *Toxins*, 2019, 11: e11060347 (doi: 10.3390/toxins11060347).
31. Zhang M.-Y., Lovgren A., Low M.G., Landen R. Characterization of an avirulent pleiotropic of the insect pathogen *Bacillus thuringiensis*: reduced expression of flagellin and phosphlipases. *Infection and Immunity*, 1993, 61(12): 4947-4954.
32. Lebenko E.V., Sekerina O.A., Chemerilova V.I. *Mikrobiologiya*, 2005, 74(1): 87-91 (in Russ.).

33. Ermolova V.P., Grishechkina S.D., Nizhnikov A.A. Activity of insecticidal *Bacillus thuringiensis* var. *israelensis* strains stored by various methods. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2018, 53(1): 201-208 (doi: 10.15389/agrobiology.2018.1.201eng).
34. Smirnoff U.A. The formation of crystals in *Bacillus thuringiensis* var. *thuringiensis* Berliner before sporulation of temperature inculcation. *J. Insect. Pathol.*, 1965, 2: 242-250.
35. Guidi V., Patocchi N., Lüthy, P., Tonolla M. Distribution *of Bacillus thuringiensis* subsp. *israelensis* in soil of a Swiss Wetland reserve after 22 years of mosquito control. *Applied and Environmental Microbiology*, 2011, 77(11): 3663-3668 (doi: 10.1128/AEM.00132-11).
36. Hansen B.M., Hendriksen N.B. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Applied and Environmental Microbiology*, 2001, 67(1): 185-189 (doi: 10.1128/AEM.67.1.185-189.2001).
37. Dospekhov B.A. *Metodika polevogo opyta* [Methods of field trials]. Moscow, 1973 (in Russ.).
38. Ben-Dov E., Zaritsky A., Dahan E., Barak Z., Sinai R., Manasherob R., Margalith Y. Extended screening by PCR for seven cry-group genes from field-collected strains of *Bacillus thuringiensis. Applied and Environmental Microbiology*, 1997, 63(12): 4883-4890.
39. Schneider S., Hendriksen N.B., Melin P., Lundstrцm J.O., Sundh I. Chromosome-directed PCR-based detection and quantification *of Bacillus cereus* group members with focus on *B. thuringiensis* serovar *israelensis* active against nematoceran larvae. *Applied and Environmental Microbiology*, 2015, 81(15), 4894-4903 (doi:10.1128/aem.00671-15).
40. Bravo A., Sarabia S., Lopez L., Ontiveros H., Abarca C., Ortiz A., Quintero R. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Applied and Environmental Microbiology*, 1998, 64(12): 4965-4972.

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1290-1305
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 579.6:577.15

doi: 10.15389/agrobiology.2019.6.1290eng
doi: 10.15389/agrobiology.2019.6.1290rus

UV-INDUCED YEAST LIPASE PRODUCER WITH A WIDE SUBSTRATE SPECIFICITY — SELECTION, CHARACTERIZATION AND PRODUCTION OF THE ENZYME

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The authors declare no conflict of interests
Acknowledgements:
The authors are grateful to the staff of the State Research Institute of Genetics and Breeding of Industrial Microorganisms of the National Research Center Kurchatov Institute (GosNIIgenetika) and personally to Prof. S.P. Sineokii, the Director of the All-Russian Collection of Industrial Microorganisms of the GosNIIgenetika.
The work was carried out within the project № 0710-2017-0010 "Search for living systems and substances of natural origin with an analysis of their biological activity for the creation of functional food and feed".
Received September 6, 2019

Abstract

Lipases are capable of changing the quantitative and/or qualitative characteristics of fat-containing raw materials and widely used for various tasks in modern food and agricultural industries. To meet the growing demand for these enzymes, highly effective producers are needed, especially those exhibiting multiple activity to lipids of different structure and origin. The aim of the study was to search for a new yeast strain with high production of lipase with broad substrate specificity, and to optimize its fermentation conditions. This work objectives also included obtaining an enzyme with a high grade of hydrolysis of various oils, and the study of its technological properties. Lipolytic characteristics were studied in 110 yeast isolates obtained from natural sources and the collections of Moscow State University of Food Production and State Research Institute of Genetics and Selection of Industrial Microorganisms. Qualitative assay of lipase activity was carried out using a differential nutrient medium with tributyrin and dye methyl red; quantitative analysis was carried out in accordance with the modified Y. Ota & K. Yamada method at pH 5.5. Of the 23 strains with lipolytic activity (LA), 12 had sufficiently high LA indices from 2.5 to 7.5 U/cm^3, of which M10 isolate with maximum activity was selected. For this isolate, morphological, cultural, physiological and biochemical properties were studied and molecular genetic identification was performed. The strain was identified as *Candida parapsilosis* (99 % of homology) using phylogenetic analysis and deposited in the All-Russian Collection of Industrial Microorganisms under the number Y-4055. After UV mutagenesis, a highly active mutant *C. parapsilosis* M10-10 was obtained. Using mathematical planning methods, the optimal nutrient medium for its growth and lipase production was determined as, %: mustard oil — 2.6, yeast extract — 1.8, soy flour — 1, glucose — 0.5, Tween 80 — 0.42, $CaCO_3$ — 0.3, KH_2PO_4 — 0.03, $MgSO_4 \cdot 7H_2O$ — 0.02. An enzyme with a purification grade of 20× and lipolytic activity of 30630 U/g was obtained after culturing the strain M10-10 at a 30-40 °C temperature and pH of the nutrient medium 5.5-6.5. It was found that by 48 hours of fermentation, lipase reaches the highest activity in the culture medium when inoculum M10-10 is in an amount of 5%. Optimal conditions for the enzyme were determined as 37 °C and pH 5.5. In terms of activity, the resulting product is not inferior to commercial domestic and foreign enzymes, including Novozym 435 (Sigma-Aldrich, USA) with a lipolytic activity of 24020 U/g. The fatty acid specificity of the new lipase was determined by enzymatic treatment of various vegetable oils and gas chromatography of lipid products using a Shimadzu GC 2010 (Shimadzu, Japan). Modification of vegetable oils with M10-10 lipase in an oil/water emulsion significantly reduced 2.4, 4.6, 2.9 and 1.5 times the saturated fatty acids fraction (including palmitic and stearic acids) and increased 1.5, 1.6, 1.1 and 12 times the polyunsaturated fatty acids fraction (including ω-3 linolenic and ω-6 linoleic acids) for olive, mustard, sunflower and coconut oils, respectively.

Keywords: *Candida parapsilosis* M10-10, lipase producer, enzyme preparation, UV mutagenesis, fermentation conditions, lipolytic activity, vegetable oils, modification, saturated fatty acids, unsaturated fatty acids

Modification of raw materials or alteration of the original raw material components is used in order to improve quality of food and agricultural products. One of the objectives of modern food industry is to obtain fats and oils with required physical and chemical properties (consistence, plasticity, hardness) by modification thereof [1, 2], which is achieved by changing the fatty acid composition of lipids. Transformation of vegetable oils by lipases is the most perspective method of its modification [3, 4].

In recent years, lipases (EC 3.1.1.3) are actively used in food industry, namely in bakery, fermentation, and milk enrichment products, in processing of vegetable oil, and in production of margarine [2, 5]. Lipase treatment fosters better taste and texture of food products, extended shelf life, and increased softness of several products. Besides, lipases are used for creation of new functional ingredients and functional food products such as equivalents of cacao oil or equivalents of human milk fat [5]. As additives or biocatalysts, for instance, in production of coffee additives, lipases are used to modify taste and to obtain aromatic notes due to synthesis of compound ethers of short-chain fatty acids and spirits [6]. At processing of eggs such enzymes improve emulsifying properties of egg yolk lipids [5]. While demand for improved quality of meat grows at the global market, use of lipase in production of feed is actualized [7]. In dairy industry such enzymes are used for lipolysis of fats, improvement of taste and acceleration of cheese maturing, and production of cheese-like products [8]. Various cheese types could be produced by using lipases from various sources, for instance, pregastric lamb enzyme for Romano cheeselipase from *Penicillium camemberti* for Camembert cheese, enzymes from *P. roqueforti* for blue cheese, and *Aspergillus niger* or *A. oryzae* enzymes for Cheddar cheese [9, 10].

The perspectives of lipase use in food and agricultural industries [11, 12] necessitate studying practically significant characteristics of such enzymes, i.e. substrate specificity, optimum activity, and stability at alteration of external parameters such as, for instance, acidity of environment and temperature.

Known lipolytic enzyme preparations (EP) of microbial origin in Russia and abroad usually have fungal origin (mainly, representatives of genus *Candida C. rugosa*, *C. lipolytica*, actinomycetes *Streptomyces* and *Thermoactinomyces*) and bacterial origin (members of *Pseudomonas*, *Bacillus* genus) and optimum activity at neutral and alkali pH. Food industry needs new producers of such enzymes preserving functionality at acid pH and having significant lipolytic activity (LA) [13, 14].

Although recombinant genetically engineered enzymes are recently often proposed to food productions, issues on the safety of use of such strains and their metabolites in foods are still unsolved. Thus, the use of induced mutagenesis method with further sampling by target traits remains relevant. The main advantage of such method is that it is relatively simple and practically does not require detailed information both on genetics and metabolism of producer strain, as well as on properties of desired product.

In this work we have isolated yeast strain secreting lipase with high activity and wide substrate specificity, having characterized its technological properties and properties of enzyme preparation as compared to known Russian and foreign analogues.

Our purpose was to get highly effective lipase producer by UV-mutagenesis and selection, to determine terms of cultivation required for maximum enzyme biosynthesis, and to create enzyme preparation on its basis ensur-

ing high level of hydrolysis of various oils.

Techniques. Lipolytic properties were analyzed in 110 yeast isolates of taxa *Candida interace*, *C. maltose*, *C. tropicalis*, *Debaryomyces huansenii*, *Rhodotorula rubra*, *Zygosaccharomyces bailii*, *Z. rouxii*, *Saccharamyces cerevisiae*, *Yarrowia lipolytica*. Part of strains was received from collection of microorganisms at the Faculty of biotechnology and technology of bioorganic synthesis products of Moscow State University of Food Productions and the GosNIIgenetica, while other cultures were isolated from products with high content of lipids (samples of oils, meat and dairy products, fruits), and natural habitats (soil).

Cultural and morphological traits of strains and isolates were described using agar and liquid Saburo mediums (a thermostat TSO-1/80 SPU, OJSC Smolensk Special Design and Technological Bureau of Programmed Control Systems, Russia); light microscopy was used to study morphological characteristics (MIK-MED 5, LOMO JSC, Russia).

The following basic nutrient media (NM, ingredients in percentage) were used. NM No. 1 (selective medium for strains capable of olive oil utilization as the only source of carbon) contained olive oil — 1, Na_2HPO_4 — 0.6, KH_2PO_4 — 0.3, NH_4Cl — 0.1, NaCl — 0.05, agar — 2 (pH = 7.2±0.1). NM No. 2 differential medium with tributyrin and methyl red contained Na_2HPO_4 — 0.6, KH_2PO_4 — 0.3, NH_4Cl — 0.1, tributyrin — 0.3, NaCl — 0.05, agar — 2, methyl red — 0.2 (pH = 6.5±0.1). NM No. 3, the Saburo medium, liquid or with 2% agar (HiMedia Laboratories Pvt., Ltd, India, included peptone — 1, glucose — 4, (pH = 5.6±0.1). NM No. 4 liquid medium for yeast fungi culture contained yeast extract — 2.0, glucose — 2.0, $CaCO_3$ — 0.5, KH_2PO_4 — 0.05 (pH = 5.4±0.1). NM No. 5 supplemented with lipase synthesis inducers included mustard oil — 2, yeast fungi extract — 2, soya flour — 1, glucose — 0.5, Tween 80 — 0.2, $CaCO_3$ — 0.3, KH_2PO_4 — 0.03, $MgSO_4 \cdot 7H_2O$ — 0.02 (pH = 5.5±0.1). For optimization of NM composition, we used the Gausse-Seidel method of successive displacements implying an experimental search for function extremum of many variables presupposing stepwise finding of private extremums of target function for each factor. Mediums were sterilized by autoclaving during 30-40 minutes at 0.25 MPa.

Samples taken for isolation of potential lipase producers were kept for at least 1 month at 4 °C. Afterwards, samples were suspended in sterile 0.9% sodium chloride (ratio 1:10), with addition of 4% gentamicin sulphate (0.1% final concentration) to exclude development of related bacteria, and incubated for 1 hour at 30 °C. Next, enriched cultures were obtained using Saburo medium (NM No. 3), then the cultures were re-plated on the selective NM No.1 and incubated for 72 hours at 30 °C. Obtained pure yeast cultures, after control microscopy, were plated on differential NM No. 2 with indicator to reveal lipolytic property. Lipase synthesis potential of strain was assessed after growing for 48 hours at 30 °C by transparent areas around colonies and by changing the color of solid NM from yellow to orange and in furtherance to red. Pure cultures of lipolytically active microorganisms were re-plated on Saburo agar for further storage.

To quantify LA in CF centrifugates and to select the most lipolytically active strains, east isolates were grown in Erlenmeyer 750 cm^3 flasks with 100 cm^3 NM No. 4 for 48 hours at 30±2 °C and shaking. Water suspension of yeasts (5×10^6 cells/cm^3) from NM No. 3 agar slants grown for 48 hours at 30 °C was an inoculum. The inoculum made 5 % of NM No. 4 volume per flask. At the end of culturing, CF was clarified for 15-20 min at 8000 rpm (laboratory centrifuge Dastan, OJSC TNK Dastan, Russia). LA was assessed by titrimetry using olive oil as a substrate as per modified method of Ota and Yamada [15] at pH 5.5. The method is based on the rate of olive oil fermentolysis quantified by titration

of resultant organic acids with 0.05 N NaOH. Lipase activity unit means quantity of enzyme hydrolyzing 40% emulsion of olive oil to produce 1 μmol oleic acid for 1 hour at 37 °C and pH 7.0.

Selected producer strain was identified by sequencing variable region of gene encoding 18S rRNA [16]. Sequences encoding 5.8S rRNA and internal transcribed spacers ITS1 and ITS2, as well as nucleotide sequences of D1/D2 domain of 26S rRNA were compared to establish phylogenic homology [17, 18]. Biomass for DNA extraction was grown on Saburo agar. Standard PCR kits (Applied Biosystems, Inc., USA) were used to extract chromosomal DNA according to producer's instruction. Conservative primers NS1 and NS4; ITS1 and ITS4; NL1 and NL4 were used for 18S rRNA, primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) for 5.8S-ITS region, and CTB6 (5′-GCATATCAATAAGCGGAGGAAAG-3′) with TW13 (5′-GGTCCGTGTTTCAAGACGG-3′) for D1/D2 domain [16]. Fragments were amplified as per the following protocol: 3 minutes at 95 °C (1 cycle); denaturation for 30 sec at 95 °C, primer annealing for 30 sec at 57 °C, elongation for 30 sec at 72 °C (35 cycles); 5 minutes at 72 °C (1 cycle) (a Mastercycler gradient, Eppendorf, Germany). Obtained fragments were subjected to gel electrophoresis (5 V/cm^3, 30 min; Bio-Rad Laboratories, Inc., USA). Amplicons stained with 10 mg/ml ethydium bromide were UV-visualized. For DNA sequencing (an automatic sequencer ABI 373A, Applied Biosystems, USA), fragments were purified using DNA Extraction kit (Thermo Scientific, Lithuania).

Specialized BLAST server software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and GenBank NCBI (https://www.ncbi.nlm.nih.gov/) were used to analyze sequencing data and to construct phylogenetic trees. Homology of nucleotide sequences of at least 97% served as criteria for taxonomic classification.

UV-mutagenesis was carried out in Petri dishes on NM No. 2 with tributyrin. Cell suspension was spread on plates in sterile conditions. Open Petri dishes were exposed for maximum 50 minutes to UV-lamp (Mineralight, USA; 30 W, 1 m distance from the lamp to plate surface). Samples were taken with 10 min interval and incubated in tubes on Saburo agar (NM No. 3) slants during 48 hours at 30 °C for culture enrichment.

Lipase-producing strain was cultured in semi-industrial conditions (OJSC Biohimmash, Moscow) in a 16 l lab fermenter, operating volume 11 l (Bioengineering AG, Switzerland) with NM No. 4 for 48 hours at 30±2 °C, pH 5.5 and airflow of 16 dm^3/min. Yeast suspension containing 5×10^6 cell/cm^3 was used as an inoculum in amount of 5 % (volume/vilume). Culture fluid was separated by centrifuging (SL 40, Thermo Scientific, USA). To purify supernatant from low-molecule admixtures and to concentrate EP, ultrafiltration (UVMT-5-20, polymer membrane with cut off 10 kDa, temperature 10-15 °C, input pressure 0.3 MPa, output pressure 0.2 MPa) was used followed by sterilizing filtration at 15-17 °C. The obtained residue was lyophilized. In pursuance of sterilizing filtration, stainless steel filter support of 142 mm in diameter (Milipore, USA) was used. Hydrophilic polyester-sulfone membrane filters with 0.22 μm pores (Pall Corp., USA) were used as filtering element. The assembled filter support was sterilized in autoclave for 30 min at 121 °C. Operating pressure at sterilizing filtration of EP concentrate comprised 0.1 MPa. Lyophilization (USP-20, FRG) was carried out at 1 cm layer depth, freezing temperature comprised –50 °C, coolant temperature was –70 °C in the first 1.5 h with temperature increase up to –20-30 °C during the next 1.5 h, and final drying during 3-4 hours at heat carrier temperature of 40 °C.

Experimental EP of lipase was compared to commercial Russian and foreign analogues, i.e. the preparations based on strains *Y. lipolytica* Pold, *S. cere-*

visiae FDS101, *Y. lipolytica* RNCIM Y-3600, *Candida* spp. L3170, *Pichia pastoris* DVSA-PLC-004, as well as with Pancreatin (PJSC Biosintez, Russia), Creon®10000 (Abbott Products GmbH, Germany), Mezym® forte 10000 (Berlin-Chemie AG, Germany), and Novozym 435 (Sigma-Aldrich, USA). In these tests, 1% solutions of studied preparations in distilled water were made and their LA was determined by modified method of Ota and Yamada [15].

To clarify fatty-acid specificity of the enzyme and to obtain lipid products rich in polyunsaturated fatty acids (PUFAs), olive, mustard, sunflower, and coconut oils were hydrolyzed [19]. To determine conditions of effective fermentolysis of vegetable oils, the effect of EP amount, oil to water ratio, temperature, and time of exposure were assessed. Free fatty acids (FA) were extracted by cold refining method. Hydrolysates were rinsed by water (1:3), and cooled up to 7±1 °C. NaOH water solution (2.5%) also cooled up to 7±1 °C was gradually added to a hydrolysate (10:1 volume/volume). The mixture was separated during 30 min at 7±1 °C. Resultant sodium salts of fatty acids were rinsed by water. The isolated neutral fat was neutralized with 5 % H_2SO_4, the remaining salts were separated. The obtained FA methyl ethers were analyzed by liquid gas chromatography (Shimadzu GC 2010, Shimadzu, Japan) with mass-detector GCMS-QP 2010 and column MDN-1 (l = 30 m, ∅ = 0.25 mmN; solid-phase-bound methyl silicone filler). Operating parameters: injector temperature 200 °C, interface temperature 210 °C, detector temperature 200 °C; helium carrier, flow rate of 1 cm^3/min, split ratio of 1:5. Mass-detector parameters: registration mode — TIC, with m/z range of mass analyzer 45-500. Sample components were identified using the mass-spectrum library NIST 02 (https://www.nist.gov/srd). Identification validity comprised over 93 %.

1. Lipolytic activity (LA) **in culture fluid centrifugates of strains and isolates** (nutrient medium No. 4)

Strain, isolates	LA, U/cm^3
Candida interace[1]	2.5±0.13
C. maltosa[1]	2.5±0.13
C. tropicalis[1]	5.0±0.25
Rhodotorula rubra[1]	2.5±0.13
Yarrowia lipolytica[2]	5.0±0.25
П-РОС[3] (P-ROS[3])	5.0±0.25
П-М[3] (P-M[3])	5.0±0.25
М10[3] (M10[3])	7.5±0.38
П5[3] (P5[3])	2.5±0.13
П8[3] (P8[3])	2.5±0.13
Ж1[3] (J1[3])	5.0±0.25
Ол[3] (Ol[3])	5.0±0.25

N o t e. [1] — collection of the Moscow State University of Food Productions, Moscow, [2] — RNCIM collection of GosNIIgenetica, Moscow; [3] — strains isolated from high-lipid products and natural sources.

Results are presented as means (*M*) at 3-fold replication with standard error of mean (±SEM). Confidence interval of arithmetic mean for p = 0.05 was determined using Statistica 6 software (StatSoft, Inc., USA) and Microsoft Excel 2010. The difference between two mean values was statistically significant if their confidence intervals were not overlapped.

Results. It was established that only 23 out of 110 yeast isolates plated on NM No. 1 can utilize olive oil as a single carbon sourcea. A total of 12 yeast culture isolates (Table 1) were selected by activity, where CF of M10 isolated from the surface of dairy butter brick had the highest LA. The M10 isolate was selected for further studies. As per morphological, cultural, physiological and biochemical traits (Table 2), the M10 strain was referred to *Candida* genus.

2. Morphocultural and physio-biochemical characterization of *Candida* sp. M10 isolate

Trait	Description
M o r p h o l o y	
Culture morphology on the 1st day	Round or oval cells, 2.5-4.5 rm in width and 2.5-9.0 rm in length
Culture morphology on the 2nd day	Budding of most cells; formation of pseudo mycelium.

Continued Table 2

Cultural traits	
Growth on Saburo agar	Round colonies with ruffled border; 1-7 mm in diameter; large-wrinkled surface, flat profile built in substrate; shiny and non-transparent colonies; light dingy-beige color; wave-shaped edges; coarse-grained structure; tight-leathery consistence
Growth in liquid Saburo medium	Opaqueness; residue and rink on tube walls; no medium pigmentation; no gas formation; yeast odor
Spore formation	Absent
Physio-biochemical traits	
Nitrate assimilation	Negative
Growth ability in vitamin free medium	Positive (biotin serving as a strong growth stimulator)
Gelatin liquefaction	Positive
Urea hydrolysis	Positive
NaCl concentration	Intensive growth at 8%, 10%, and 12% of NaCl
pH	Good growth at pH from 3 to 9
Temperature	Weak growth at 20 °C; intensive growth at 25-37 °C; weak growth at 50 °C

The nucleotide sequence of variable 18S rRNA gene region of M10 strain genome was as follows:

CATACATGTCTAAGTATAAGCAATTATACAGTGAAACTGCGAATGGCTCATAAA-
TCAGTTATCGTTTATTTGATAGTACCTTACTACTTGGATAACCGTGGTAATTCT-
AGAGCTAATACATGCTTAAAATCCCGACTGTTTGGAAGGGATGTATTTATAGA-
TAAAAAATCAATGCCTTCGGGCTCTTTGATGATTCATAATAACTGAATCACAT-
GGCCTTGTGCTGGCGATGGTTCATTCAAATTTCTGCCCTCGATGGTAGGATA-
GTGGCCTACCATGGTTTCAACGGGTAACGATAAGGGTTCGATTCCGGAGAG-
GGAGCCTGAGAAACGGCTACACATCCAAGGAAGGCACAGGCGCCAAATTAC-
CCAATCCCGACACGGGGAGGTAGTGACAATAAATAACGATCAGGGCCCTTTC-
GGGTCTTGTAATTGGAATGAGTACAATGTAAATACCTTAACGAGGAACAATT
GGAGGGCAAGTCTGGTGCGCAGCCGCGGTAATTCCAGCTCCAAAACGTATA-
TTAAAGTTGTTGCAGCAGTTAAAAAGCTCGTAGTTGAACCTTGGGCCTTGGC-
CTGCCCGG.

Initially, the primary screening of nucleotide sequence using GenBank databases and BLAST server had shown that M10 strain belongs to groups: *Eukaryota*, *Fungi*, *Ascomycota*, *Saccharomycotina*, *Saccharomycetes*, *Saccharomycetales*, *mitosporic Saccharomycetales*, *Candida*. Analysis of variable DNA region of 18S rRNA gene allowed this strain to be referred to several species of *Candida* genus with probability of less than 97 %. Thus, to specifically identify the strain, we carried out a comparative analysis of DNA encoding 5.8S rRNA and internal transcribed spacers ITS1 and ITS2, and also comparative analysis of 26S rRNA gene sequences encoding D1/D2 domain.

For DNA region of 5.8S rRNA gene and internal transcribed spacers ITS1 and ITS2 the following nucleotide sequence was obtained:

GCGGGGTAGTCTACCTGATTTGAGGTCGAATTTGGAAGAAGTTTTGGAGTTTG-
TACCAATGAGTGGAAAAAACCTATCCATTAGTTTATACTCCGCCTTTCTTTCAA-
GCAAACCCAGCGTATCGCTCAACACCAAACCCGAGGGTTTGAGGGAGAAATG-
ACGCTCAAACAGGCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAA-
AGATTCGATGATTCACGAATATCTGCAATTCATATTACTTATCGCATTTCGCTG-
CGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTGACTATT-
AAATAATCGGTTGACATTAAATAAAATTTGGTTGAGTTTATCTCTGGCAGGC-
CCCATATAGAAGGCCTACCAAAGCAAAGTTTTCAAAAAAAGAAAAACACATG-
TGTAAGAAAAAATGCAGTTAAGCACTTTTCATTCTGTAATGATCCTTCCGCAG-
GTTCACCAGGAAGAATATTAAGAATGAAAAGTGCTTACTGCATTTTTTCTAAC-
ATGTGTTTT.

The sequence for region of 26S rRNA gene was as follows:

GGATTGCCTTAGTAGCGGCGAGTGAAGCGCAAAAAGCTCAAATTTGAAATCT-
GGCACTTTCAGTGTCCCGAGTTGTAATTTGAAGAAGGTATCTTTGGGTCTTGC-
TCTTGTCTATGTNTCTTGGAACAGAACGTCACAGAGGGTGAGAATCCCGTGC-
GATGAGATGTCCCAGACCTATGTAAAGTTCCTTCGAAGAGTCGAGTTGTTGG-
GAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGA-

GAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAA-
GAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCAGA-
CTTGGTATTTTGTATGTTACTCTCTCGGGGGTGGCCTCTACAGTTTACCGG-
GCCAGCATCAGTTTGAGCGGTAGGATAAGTGCAAAGAAATGTGGGACTGCT-
TCGGTAGTGTGTTATAGTCTTTGTCGATACTGCCAGCTTAGACTGAGGACTG-
CGGCTTCGGCCTANGA.

Therefore, according to clarified data, M10 strain is close to *Candida parapsilosis* (homology level 99 %) [20]. Based on the results of molecular identification, strain *C. parapsilosis* M10 was deposited to All-Russia Collection of Industrial Microorganisms (GosNIIgenetica) under accession number RNCIM Y-4055.

Enzyme biosynthesis by a producer mainly depended on NM composition, physiological state of microorganism, terms of culturing, and the amount of inoculum [21, 22]. At submerged culturing of *C. parapsilosis* M10 strain, we have gradually varied concentrations and types of carbon, nitrogen, phosphor, and microelement sources in nutrient medium No. 5, since such factors may affect lipase production [23].

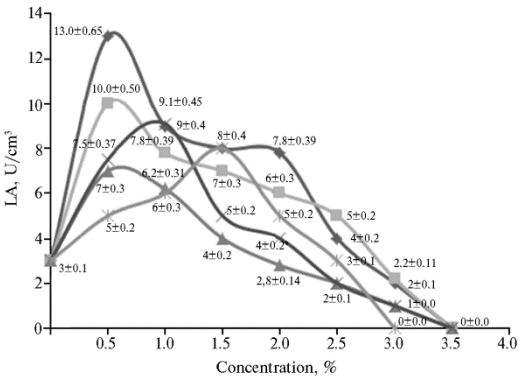

Fig. 1. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 at different concentrations of carbon sources in nutrient medium:** — glucose, — saccharine, — fructose, — maltose, — galactose.

Different sugars, which concentrations were changed at culturing of M10 strain on NM No. 5, were used as carbon source (Fig. 1). Maximum LA occurred in variant with 0.5% glucose concentration as yeast fungi prefer glucose out of sugars.

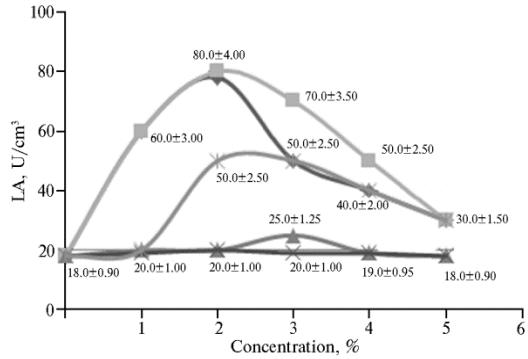

Fig. 2. Lipolytic activity (LA) **of producer strain C*andida parapsilosis* M10 depending on inducer (oil) type and concentration ////:** — olive, — mustard, — pure sunflower, — refined sunflower, — flax seed.

It is known that various vegetable oils are often used to induce lipase synthesis and as an additional carbon source. It has been reported that Y. *lipolytica* DSM 3286 strain produced 34.6±0.1 U/cm^3 lipase on medium with olive oil with addition of yeast extract [24].

It was established in previous work [25] that maximum LA in 48 hours of culturing *C. rugosa* (DSM 2031) strain comprised 4.43; 3.29; 2.86; 1.74; 1.23, and 1.03 U/cm^3 for sesame, peanut, sunflower, palm, coconut, and castor oils, respectfully. In our study we have also tested various oils and varied concentrations thereof (Fig. 2). Thus, upon addition of 2% mustard oil LA of M10 strain comprised 80 U/cm^3. According to results, mustard oil contains FA inducing lipase synthesis in *C. parapsilosis* M10.

Fickers et al. [26], having studied the ability of various mineral nitrogen

sources, for instance, NH_4Cl and $(NH_4)_2SO_4$, and organic substrates (casamino acids, trypton, urea, yeast extract, various peptones) to maintain lipase growth and production in *Y. lipolytica* LgX64 81 strain, had shown that enrichment of the medium by mineral nitrogen did not significantly affect cell growth or enzyme biosynthesis, whilst lipase production was notably increased at addition of several sources of organic nitrogen. The highest yield (166-fold yield increase up to 484.7±59.1 U/cm^3) was noted at presence of Hycase SF with trypton N1.

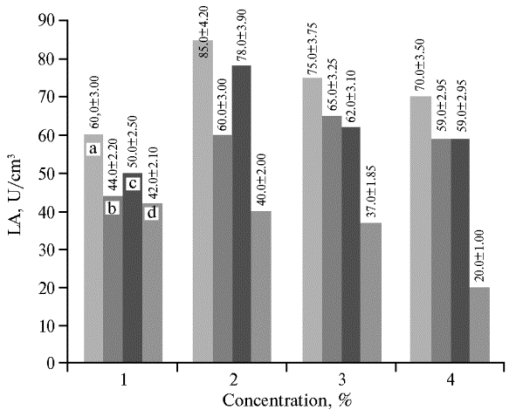

Fig. 3. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 depending on nitrogen type and concentration in nutrient medium:** a — yeast extract, b — soya flour, c — peptone, d — $(NH_4)H_2PO_4$.

As a nitrogen source, we used yeast extract, soya flour, peptone, and $NH_4H_2PO_4$ in different concentrations (Fig. 3). The highest lipase activity of producer strain was noted on nutrient medium No. 5 with 2 % yeast extract. Possibly, it was due to the fact that yeast extract contains essential amino acids and B group vitamins required for enzyme biosynthesis.

3. Lipolytic activity (LA) **of *Candida parapsilosis* M10 depending on soya flour concentration in nutrient medium**

Concentration, %	LA, U/cm^3
0.5	80±4.0
1.0	90±4.5
1.5	85±4.3
2.0	70±3.5

Soya flour containing up to 30% fat could render positive effect on lipase biosynthesis [27]. During the additional experiment on NM No. 5 with yeast extract (2.0 %), we have introduced different quantity of soya flour (Table 3). The most activity of producer was obtained on medium with 1.0% soya flour.

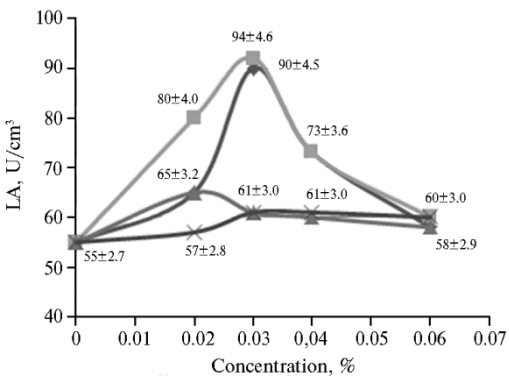

Fig. 3. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 depending on P type and concentration in nutrient medium:** —•— — K_2HPO_4, —■— — KH_2PO_4, —▲— — $(NH_4)_2HPO_4$, —×— — $(NH_4)H_2PO_4$.

Phosphorus is required for microorganism activity and enzyme biosynthesis [28]. We have studied the effect of K_2HPO_4, KH_2PO_4, $(NH_4)_2HPO_4$, and $(NH_4)H_2PO_4$ on LA of CF centrifugate of *C. parapsilosis* M10 strain (Fig. 4). The best indices of lipase activity were on medium No. 5 with 0.03% KH_2PO_4. The graph also allows tracing such fact that potassium ions are required for lipase biosynthesis.

Due to optimization by mathematical method of experiment design the following NM (No. 6), % was established: mustard oil — 2.6, yeast extract — 1.8, soya flour — 1, glucose — 0.5, Tween 80 — 0.42, $CaCO_3$ — 0.3, KH_2PO_4 — 0.03, $MgSO_4 \cdot 7H_2O$ — 0.02, pH 5.5±0.1. Due to optimized NM, LA of *C. par-*

apsilosis M10 strain CF increased up to 204 U/cm^3.

Enzyme concentration in microbial cell could be significantly increased by changing the growth conditions, by mutagenesis or genetic manipulations [22, 23, 32]. Mutant *C. parapsilosis* M10-10 with LA in CF of 235 U/cm^3 was obtained due to UV-mutagenesis of *C. parapsilosis* M10 strain (Fig. 5). UV exposure lasting over 10 min rendered negative effect on enzyme biosynthesis. Similarly, Chen et al. [29] had isolated *Trichosporon fermentans* WU-C12 strain from soil showing maximum yield (nearly 30 U/cm^3) at culturing during 4 days at 30 °C. Afterwards 2PU-18 strain with higher enzyme activity (up to 70 U/cm^3) was obtained by UV-induced mutagenesis and by changing the medium composition. Other authors, when working with *Aspergillus niger*, have used nitrogen acid in addition to UV-treatment as a mutagen (both methods used twice) and have obtained the 20.7 and 39.1 % increase in lipase production, respectively, after double UV and nitrogen acid treatment as compared to parent strain having yield of 34.8 U/ml [30].

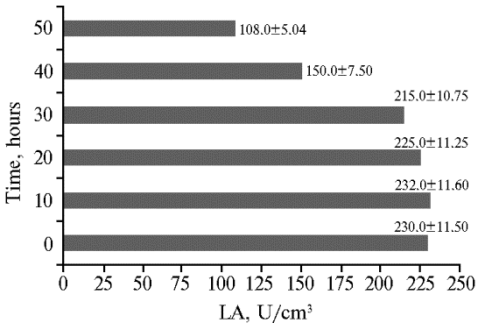

Fig. 5. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 CF depending on UV-exposure.**

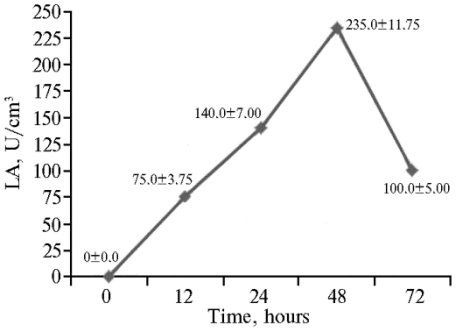

Fig. 6. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 CF depending on time of culturing.**

4. Lipolytic activity (LA) **of producer strain *Candida parapsilosis* M10 CF depending on pH**

pH	LA, U/cm^3
4.0	145±7.25
4.5	170±8.5
5.0	220±11.0
5.5	235±11.75
6.0	230±11.5
6.5	228±11.4
7.0	200±10.0
7.5	150±7.5
8.0	40±8.0

We have experimentally established that the highest lipase activity in CF is attained in 48-hour M10-10 culture, 5% inoculum volume to growth medium volume (Fig. 6), 30-40 °C and pH 5.5-6.5 (Table 4). Production of dry EP in fermenter culture comprised 7.92 g/cm^3 with LA 30630 U/g.

Comparison of LA in Russian and foreign lipase preparations and obtained EP implies (Table 5) that M10-10 G20× lipase was not inferior to other preparations. We have experimentally established the following optimal conditions for enzyme hydrolysis of oil using of M10-10 G20× lipase EP, the 0.1% enzyme (61.2 U/g lipids), oil to water ratio 1:1, 37 °C, pH 5.5, and 4-hour incubation.

5. Comparison of lipolytic activity (LA) **of the developed *Candida parapsilosis* M10-10-based lipase preparation and known commercial preparations**

Preparation	Collection, producer	LA, U/g
Lipase M10-10 G20×	Obtained in this work	30630±18.5
Preparation based on *Yarrowia lipolytica* Po1d strain [30]	Collection of Moscow State University of Food Productions	2400±8.5
Preparation based on *Saccharomyces cerevisiae* FDS101 strain [31]	Collection of Moscow State University of Food Productions	1800±6.7

Continued Table 5

Preparation based on *Y. lipolytica* strain RNCIM Y-3600 [32]	RNCIM of GosNIIgenetica, Russia	2700±6.2
Lipase from *Candida* spp. L3170	Sigma-Aldrich, USA	5000
PLC lipase BD16449 (based on *Pichia pastoris* DVSA-PLC-004 strain) [33]	Collection of Moscow State University of Food Productions	205±0.7
Pancreatin	Biosintez, Russia	4300
Kreon® (10000 U)	Abbott Products GmbH», Germany	10000
Mezym® forte 10000	Berlin-Chemie AG, Germany	10000
Novozym 435	Sigma-Aldrich, USA	24020±10.2

Identification of fatty-acid specificity of enzyme had shown that maximum yield of free FA in 4 hours comprised 30.0% for olive oil, 30.5% for mustard oil, 32.2% f sunflower oil, and 35.6% for coconut oil (of total acid content in oils). Thus, it was inferred on lipase enzyme specificity to the residues of saturated FA. Hydrolysis by lipase M10-10 G20× and further refining of vegetable oils allowed us validly to reduce the relative content of saturated FA 2.4; 4.6; 2.9 and 1.5 times and to increase PUFAs 1.5; 1.6; 1.1 and 12 times for olive, mustard, sunflower, and coconut oils, respectively (Table 6). Accordingly, modification of vegetable oils by lipase preparation M10-10 G20× in oil:water emulsion allows us to obtain lipid products enriched with ω-3 and ω-6 linoleic and linolenic PUFAs valuable for human health.

6. Fat components and fatty acid composition of vegetable oils and modified lipid products derived from M10-10 G20× lipase hydrolysis

Component	Olive		Mustard		Sunflower		Coconut	
	O	M	O	M	O	M	O	M
Mass content of fat components, %								
FA	20.0±4.00	8.3±1.66	14.3±2.86	3.1±0.62	12.0±2.40	4.1±0.82	89.1±17.82	60.8±12.16
PUFAs	11.4±2.28	17.5±3.50	37.3±7.46	58.1±11.62	58.5±11.70	65.4±13.08	1.6±0.32	19.4±3.88
PUFAs ω-6	6.9±1.38	7.9±1.58	24.0±4.80	27.9±5.58	58.5±11.70	65.4±13.08	1.6±0.32	19.4±3.88
PUFAs ω-3	4.5±0.90	9.7±1.94	13.3±2.66	19.0±3.80	–	–	–	–
Mass content of fatty acids, %								
Capric $C_{10:0}$	–	–	–	–	–	–	7.3±1.46	2.3±0.46
Lauric $C_{12:0}$	–	–	1.3±0.26	–	–	–	45.3±9.06	35.2±7.04
Myristic $C_{14:0}$	–	–	–	–	–	–	21.2±4.24	18.3±3.66
Palmalite $C_{16:0}$	15.5±3.10	6.8±1.36	9.4±1.88	1.4±0.28	7.7±1.54	1.3±0.26	12.3±2.46	10.7±2.14
Stearic $C_{18:0}$	4.2±0.84	1.6±0.32	3.1±0.62	1.8±0.36	4.0±0.80	2.7±0.54	3.0±0.60	2.8±0.56
Oleic $C_{18:1}$	61.3±12.26	64.7±12.94	34.5±6.90	38.3±7.66	26.3±5.26	27.6±5.52	9.3±1.86	19.9±3.98
Linoleic $C_{18:2}$	6.9±1.38	7.9±1.58	24.0±4.80	27.9±5.58	58.5±11.70	65.4±13.08	1.6±0.32	10.9±2.18
Linolenic $C_{18:3}$	4.5±0.90	9.7±1.94	13.3±2.66	19.0±3.80	–	–	–	–
Arachic $C_{20:0}$	0.3±0.06	–	0.6±0.12	–	0.4±0.08	–	–	–
Gondoinic $C_{20:1}$	0.3±0.06	–	5.5±1.10	2.8±0.56	–	–	–	–
Erucidic $C_{22:1}$	–	–	4.8±0.96	3.2±0.64	–	–	–	–

Note. FA — fatty acids, PUFA — polyunsaturated fatty acids; O — original, M — modified products. Dashes mean trace amounts or absence of components.

During the last years, yeast genus *Candida* (for instance, *C. antarctica*, *C. rugosa*, *C. tropicalis*, *C. curvata*, *C. parapsilosis* strains), as well as *Galactomyces geotricum*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Arxula adeninivorans*, *Trichosporon fermantans*, *T. asahii*, *Rhodotorula mucilaginosa*, *Aureobasidium pullulans* are deemed the most perspective microbial producers of lipase with high enzyme activity [35, 36]. However, only some of lipases are commercially used for mass production purposes [37]. Such enzymes have high activity and wide temperature and pH optimums.

Activity of new lipase M10-10 G20× is close to such in Novozym 435 preparation (Sigma-Aldrich, USA) obtained based on *C. antarctica* strain. Literature sources confirm that lipase synthesized by *C. antarctica* strains manifest high LA. Lipase CALB with activity of 117 U/g of dry weight [38], as well as genetically engineered strain of *Pichia pastoris* synthesizing lipase of *C. antarctica*

CALB with activity of 220 U/g of dry weight are known [39]. Few more commercially successful EP such as Chirazyme® L-2 (Boehringer Mannheim GmbH, Germany) and SP 525 (Novo-Nordisk A/S, Denmark) are obtained based on the same microorganism [37].

In 2004, *Y. lipolytica* Pold (CLIB 139) and *Y. lipolytica* Polf (ATCC MYA-2613) widely used for metabolic engineering were derived from *Y. lipolytica* W29 (ATCC 20460) strain [40]. Moreover, genetically engineered *Saccharomyces cerevisiae* strains producing lipases Lip7 and Lip8 of *Y. lipolytica* with activity of 283 and 121 U/g (per dry weight) are also known [41]. In experiments, optimal pH for hydrolysis of olive oil by *Y. lipolytica* lipase comprised nearly 8.0. At temperature below 37 °C and pH 4.5-8.0, these enzymes remain stable for 20 minutes, at 5 °C — for 22 hours. Lipases synthesized by *C. rugosa* are one of mostly used in the industry due to high enzyme activity. Indian researchers have extracted and characterized three different *C. rugosa* lipase forms [4]. Purified isoforms of extracellular lipase (lipA, lipB and lipC) had molecular weights of 64, 62, 60 kDa and temperature optimum within the range of 35-40 °C. *Geotrichum candidum* lipases are of commercial interest due to high specificity to long-chain cis-9-unsaturated fatty acids in substrates. *G. candidum* is known as producer of extracellular lipase stable at pH 6.5-8.5 [42]. Ciafardini et al. [43] had established that yeast strain *Williopsis californica* 1639 isolated from virgin olive oil produces extracellular lipase with activity optimum at pH 6. Böer et al. [44] had cloned AL1P1 gene encoding lipase of yeast *Arxula adeninivorans*. It was shown that the enzyme is a 100 kDa dimmer with amino acid sequence similar to such in lipases from *C. albicans* and *C. parapsilosis*. Its maximum activity is at 30 °C and pH 7.5.

Commercial lipase preparations often are a mix of various isoforms. Thence, low reproducibility of biocatalytic processes when using commercial lipases, for instance, *C. rugosa* (CRL), could be due to presence of different isoforms, which complicates the interpretation of findings [45].

Our study of thermal- and pH-stability of lipase M10-10 G20× EP had shown that preparation, unlike the abovementioned, completely preserves its high activity in the range of 30-40 °C and pH 4.0-7.0, which is the advantage for industry and agricultural application. In addition, the potential of modern metabolic engineering of lipase producers, which we discussed above, can be applied to strain M10-10.

Thus, the following conclusions could be drawn based on performed studies. A new yeast lipase producer *Candida parapsilosis* M10-10 with lipolytic activity (LA) in cultural fluid (CF) of 235 U/cm3 was derived from UV-exposure of an isolate from surface of butter brick. Culture conditions for the optimal enzyme biosynthesis have been developed. Production of enzyme preparation (EP) involves submerged culturing of *C. parapsilosis* M10-10 strain in fermenter with further separation of cultural fluid, ultrafiltration, sterilizing filtration, and lyophilization. The technology allows 7.92 g/dm^3 yield of dry EP with LA of 30630 U/g. The lipolytic activity of M10-10 G20× EP is enough to ensure high hydrolysis of various vegetable oils. The EP LA is inferior to that of domestic and foreign analogues, i.e. Novozym 435, Chirazyme® L-2, SP 525, CALB and *C. antarctica* CALB lipases. The developed preparation can be used for modification of raw material components in agricultural production.

REFERENCES

1. Ipatova L.V., Kochetkova A.A., Nechaev A.P., Tutel'yan V.A. *Zhirovye produkty dlya zdorovogo pitaniya. Sovremennyi vzglyad* [Fatty foods for a healthy diet. Modern look]. Moscow, 2009 (in

Russ.).
2. Raveendran S., Parameswaran B., Ummalyma S.B., Abraham A., Mathew A.K., Madhavan A., Rebello S., Pandey A. Applications of microbial enzymes in food industry. *Food Technology and Biotechnology*, 2018, 56(1): 16-30 (doi: 10.17113/ftb.56.01.18.5491).
3. Tutel'yan V.A., Nechaev A.P. *Pishchevye ingredienty v sozdanii sovremennykh produktov pitaniya* [Food ingredients in the creation of modern food]. Moscow, 2014 (in Russ.).
4. Benjamin S., Pandey A. Isolation and characterization of three distinct forms of lipases from *Candida rugosa* produced in solid state fermentation. *Brazilian Archives of Biology and Technology*, 2001, 44(2): 213-221 (doi: 10.1590/S1516-89132001000200016).
5. Olempska-Beer Z.S., Merker R.I., Ditto M.D., DiNovi M.J. Food-processing enzymes from recombinant microorganisms — a review. *Regulatory Toxicology and Pharmacology*, 2006, 45(2): 144-158 (doi: 10.1016/j.yrtph.2006.05.001).
6. Hasan F., Shah A.A., Hameed A. Industrial applications of microbial lipases. *Enzyme and Microbial Technology*, 2006, 39: 235-251 (doi: 10.1016/j.enzmictec.2005.10.016).
7. *Lipase market — global forecast to 2020.* Available: https://www.marketsandmarkets.com/Market-Reports/lipase-market-205981206.html. Accessed 15.06.2019.
8. Jooyandeh H., Kaur A., Minhas K.S. Lipases in dairy industry: a review. *Journal of Food Science and Technology-Mysore*, 2009, 46(3): 181-189.
9. Houde A., Kademi A., Leblank D. Lipases and their industrial applications. *Appl. Biochem. Biotechnol.*, 2004, 118(1-3): 155-170 (doi: 10.1385/abab:118:1-3:155).
10. Collins Y.F., McSweeney P.L., Wilkinson M.G. Evidence of a relationship between autolysis of starter bacteria and lipolysis in cheddar cheese during ripening. *Journal of Dairy Research*, 2003, 70(1): 105-113 (doi: 10.1017/s0022029902005915).
11. Guerrand D. Lipases industrial applications: focus on food and agroindustries. *Oilseeds and fats, Crops and Lipids*, 2017, 24(4): D403 (doi: 10.1051/ocl/2017031).
12. Sharma R., Chisti Y., Banerjee U.C. Production, purification, characterization, and applications of lipases. *Biotechnology Advances*, 2001, 19(8): 627-662 (doi: 10.1016/s0734-9750(01)00086-6).
13. Gracheva I.M., Krivova A.Yu. *Tekhnologiya fermentnykh preparatov* [Enzyme technology]. Moscow, 2000 (in Russ.).
14. Shukla P., Gupta K. Ecological screening for lipolytic molds and process optimization for lipase production *Rhizopus oryzae* KG-5. *Journal of Applied Sciences in Environmental Sanitation*, 2007, 2(2): 35-42.
15. Sambrook J., Russell D.W. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, NY, 2001.
16. Naumova E.S., Serpova E.V., Naumov G.I. *Biokhimiya*, 2007, 72(12): 1659-1667 (in Russ.).
17. Esteve-Zarzoso V., Belloch C., Uruburu F., Querol A. Identification of yeasts by RFLP analysis of the 5.8S rDNA gene and two ribosomal internal transcribed spacers. *International Journal of Systematic Bacteriology*, 1999, 49(1): 329-337 (doi: 10.1099/00207713-49-1-329).
18. Polygalina G.V., Cherednichenko B.C., Rimareva L.V. *Opredelenie aktivnosti fermentov* [Determination of enzyme activity]. Moscow, 2003 (in Russ.).
19. Ottavei B.P. *Obogashchenie pishchevykh produktov i biologicheski aktivnye dobavki* [Food fortification and dietary supplements]. Moscow, 2010 (in Russ.).
20. Kuhn D.M., Mikherjee P.K., Clark T.A., Pujol C., Chandra J., Hajjeh R.A., Warnock D.W., Soil D.R., Ghannoum M.A. *Candida parapsilosis* characterization in an outbreak setting. *Emerging Infectious Diseases*, 2004, 10(6): 1074-1081 (doi: 10.3201/eid1006.030873).
21. Ivanova L.A., Voino L.I., Ivanova I.S. *Pishchevaya biotekhnologiya. Kniga 2. Pererabotka rastitel'nogo syr'ya* /Pod redsktsiei I.M. Grachevoi [Food biotechnology. Vol. 2. Processing of plant materials. I.M. Grachevaya (ed.)]. Moscow, 2008 (in Russ.).
22. Treichel H., de Oliveira D., Mazutti M.A., di Luccio M., Oliveira J.V. A review on microbial lipases production. *Food Bioprocess Technol.*, 2010, 3(2): 182-196 (doi: 10.1007/s11947-009-0202-2).
23. Maldonado R.R., Macedo G.A., Rodrigues M.I. Lipase production using microorganism from different agro-industrial by-product. *International Journal of Applied Science and Technology*, 2014, 4: 108-115.
24. Darvishi F., Nahvi I., Zarkesh-Esfahani H., MomenbeikF. Effect of plant oils upon lipase and citric acid production in *Yarrowia lipolytica* yeast. *Journal of Biomedicine and Biotechnology*, 2009, 2009: article ID 562943 (doi: 10.1155/2009/562943).
25. Lakshmi B.S., Kangueane P., Abraham B., Pennathur G. Effect of vegetable oils in the secretion of lipase from *Candida rugosa* (DSM 2031). *Letters in Applied Microbiology*, 1999; 29: 66-70 (doi: 10.1046/j.1365-2672.1999.00578.x).
26. Fickers P., Nicaud J.M., Gaillardin C., Destain J., Thonart P. Carbon and nitrogen sources modulate lipase production in the yeast *Yarrowia lipolytica*. *Journal of Applied Microbiology*, 2004, 96(4): 742-749 (doi: 10.1111/j.1365-2672.2004.02190.x).
27. Vargas G.D.L.P., Treichel H., de Oliveira D., Beneti S.C., Freire D.M.G., Di Luccio M. Optimization of lipase production *by Penicillium simplicissimum* in soybean meal. *J. Chem. Technol.*

Biotechnol., 2008, 83(1): 47-54 (doi: 10.1002/jctb.1776).
28. Dobrev G., Strinska H., Zhekova B., Dobreva V., Delchev N. Influence of the fermentation medium composition on lipase production by *Rhizopus arrhizus*. *Bulgarian Journal of Agricultural Science*, 2014, 20(Suppl.1): 42-45.
29. Chen J., Shimura S., Kirimura K., Usami S. Enhancement of lipase production from hydrocarbons by mutation of *Trichosporon fermentans*. *Appl. Microbiol. Biotechnol.*, 1993, 38(6): 714-718 (doi: 10.1007/BF00167133).
30. Sandana Mala JG, Kamini NR, Puvanakrishnan R. Strain improvement of *Aspergillus niger* for enhanced lipase production. *The Journal of General and Applied Microbiology*, 2001, 47(4): 181-186 (doi: 10.2323/jgam.47.181).
31. Pignède G., Wang H., Fudalej F., Gaillardin C., Seman M., Nicaud J.M. Characterization of an extracellular lipase encoded by LIP2 in *Yarrowia lipolytica*. *Journal of Bacteriology*, 2000, 182(10): 2802-2810 (doi: 10.1128/jb.182.10.2802-2810.2000).
32. Darvishi F. Expression of native and mutant extracellular lipases from *Yarrowia lipolytica* in *Saccharomyces cerevisiae*. *Microbial Biotechnology*, 2012, 5(5): 634-641 (doi: 10.1111/j.1751-7915.2012.00354.x).
33. Yuzbasheva E.Yu., Yuzbashev T.V., Gordeeva T.L., Laptev I.A., Vybornaya T.V., Sineokii S.P. *Shtamm drozhzhei Yarrowia lipolytica — produtsent kletochno-svyazannoilipazy*. *Patent RF № RU 2475532 C1*. Zayavl. 09.02.2012. Opubl. 20.02.2013. Byul. № 5 [Yarrowia lipolytica Yarrowia lipolytica — producer of cell-bound lipase. Patent RF № RU 2475532 C1. Appl. 09.02.2012. Publ. 20.02.2013. Bul. № 5] (in Russ.).
34. Dayton C.L.G., Galhardo F. *Enzymatic degumming utilizing a mixture of PLA and PLC phospholipases*. *Patents US № US20080182322A1*. Priority date 2007-01-30. Pub. Date 2008-07-31.
35. Ciafardini G., Zullo B.A. Virgin olive oil yeasts: a review. *Food Microbiology*, 2018, 70: 245-253 (doi: 10.1016/j.fm.2017.10.010).
36. Gupta R., Kumari A., Syal P., Singh Y. Molecular and functional diversity of yeast and fungal lipases: their role in biotechnology and cellular physiology. *Progress in Lipid Research*, 2015, 57: 40-54 (doi: 10.1016/j.plipres.2014.12.001).
37. Faber K. *Biotransformations in organic chemistry: a textbook*. Springer Int. Publ., 2018 (doi: 10.1007/978-3-319-61590-5).
38. Tanino T., Aoki T., Chung W.Y., Watanabe Y., Ogino C., Fukuda H., Kondo A. Improvement of a *Candida antarctica* lipase B-displaying yeast whole-cell biocatalyst and its application to the polyester synthesis reaction. *Appl. Microbiol. Biotechnol.,* 2009, 82(1): 59-66 (doi: 10.1007/s00253-008-1764-z).
39. Su G.D., Zhang X., Lin Y. Surface display of active lipase in *Pichia pastoris* using Sed1 as an anchor protein. *Biotechnol. Lett.*, 2010, 32(8): 1131-1136 (doi: 10.1007/s10529-010-0270-4).
40. Madzak C., Gaillardin C., Beckerich J.M. Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. *Journal of Biotechnology*, 2004, 109(1-2): 63-81 (doi: 10.1016/j.jbiotec.2003.10.027).
41. Liu W.S., Pan X.X., Jia B., Zhao H.Y., Xu L., Liu Y., Yan Y.J. Surface display of active lipases Lip7 and Lip8 from *Yarrowial ipolytica* on *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol*, 2010, 88: 885-891 (doi: 10.1007/s00253-010-2782-1).
42. Gopinath S.C.B., Hilda A., Lakshmi priya T., Annadurai G., Anbu P. Purification of lipase from *Geotrichum candidum*: conditions optimized for enzyme production using Box-Behnken design. *World Journal of Microbiology and Biotechnology*, 2003, 19(7): 681-689 (doi: 10.1023/A:1025119222925).
43. Ciafardini G., Zullo B.A., Iride A. Lipase production by yeasts from extra virgin olive oil. *Food Microbiology*, 2006, 23(1): 60-67 (doi: 10.1016/j.fm.2005.01.009).
44. Böer E., Mock H.P., Bode R., Gellissen G., Kunze G. An extracellular lipase from the dimorphic yeast *Arxula adeninivorans*: molecular cloning of the ALIP1 gene and characterization of the purified recombinant enzyme. *Yeast*, 2005, 22(7): 523-535 (doi: 10.1002/yea.1230).
45. Akoh C.C., Lee G.C., Shaw J.F. Protein engineering and applications of *Candida rugosa* lipase isoforms. *Lipids*, 2004, 39: 513-526 (doi: 10.1007/s11745-004-1258-7).

AGRICULTURAL BIOLOGY,
2019, V. 54, № 6, pp. 1306-1323
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Fodder crops

UDC 636.2:631.52:631.461.52

doi: 10.15389/agrobiology.2019.6.1306eng
doi: 10.15389/agrobiology.2019.6.1306rus

HIGHLY EFFECTIVE ROOT NODULE INOCULANTS OF ALFALFA (*Medicago varia* L.): MOLECULAR-GENETIC ANALYSIS AND PRACTICAL USAGE IN VARIETY CREATION

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The authors declare no conflict of interests
Acknowledgements:
Supported financially by Ministry of Science and Higher Education of the Russian Federation (Agreement No. 14.607.21.0178, RFMEFI60717X0178)
Received October 5, 2019

Abstract

Among grass stands of perennial forage legume crops, alfalfa is a preferred leguminous plant for the creation of cultivated pastures and restoration of degraded soils. *Medicago sativa* L. nothosubsp. *varia* (Martyn) Arcang is a high-yielding crop and tolerant to adverse cultivation conditions, which is important for risky farming zones in Russia. Alfalfa productivity greatly depends on the success of the formation of a plant-microbial symbiotic system with root nodule bacteria (rhizobia), due to which it becomes able to fix atmospheric nitrogen. Modern symbiogenetics has shown that the effectiveness of symbiotic systems depends on the complementary interactions of the plant and microsymbiont genomes. Proceeding from this, the biological products that are used to treat legume seeds should also contain selected rhizobia strains with corresponding genotypic characteristics. In the present work, a comparative analysis of the grass yield of 73 variety-strain combinations formed by *Sinorhizobium meliloti* isolates from salt affected areas and by two strains 425a and 415b of commercial importance, with alfalfa varieties obtained by classical and symbiotically depended plant breeding approaches was performed. The prospects of selecting highly effective strains which are complementary to economically valuable varieties of alfalfa in model field trials experiments have been shown. The strains A1 and A2 were found to be symbiotically more active with tested alfalfa varieties than strains of commercial importance. It has been evaluated that variety-strain combinations based on varieties obtained by a symbiotically depended plant breeding approach are characterized by increased adaptability, and the potential to increase their yield significantly exceeds 50 %. It was revealed that productivity of variety-strain combinations formed by strain 425a are under the influence of uncontrolled factors as it was shown by two-factor analysis of variance (two-way ANOVA). A high complementarity of strains A1 and A2 to the variety Agnia, and symbiotic characteristics of the strain 425a, led us to study genomic characteristics of these strains. A comparative analysis of genomes done by using DNA biochips approach revealed significant differences between symbiotically highly effective strains. It was established that genes related to symbiotic activity and stress tolerance in strains recovered from salinized soils had mainly a divergent structure. The data of the first stages of the molecular genetic analysis of highly effective strains strongly indicate the need to con-

tinue research that will allow targeted selection of microsymbiont strains for modern varieties of alfalfa. Data produced through the research of a number of variety-strain combinations growth in various climatic conditions of the Russian Federation clearly demonstrate the benefit for symbiotically depended plant breeding approach in order to create new economically valuable varieties of legumes claimed by developing a sustainable forage base for farmers.

Keywords: *Medicago varia*, *Sinorhizobium meliloti*, alfalfa varieties, plant breeding approaches, symbiotic genes and stress tolerance genes, DNA biochip SM6kOligo, genomic islands, symbiotically effective variety-strain combinations, two-way ANOVA, sustainable forage base

Formation of sustainable feed supply in the field of livestock and poultry, including the improvement of feed quality, is a prerequisite for effective development of modern agricultural complexes. Cultivation of perennial forage grasses as pasture crops, and inclusion there in cereal and row crop rotation crops can greatly satisfy the meadow farming request in nitrogen and can significantly reduce the ecological risks associated with the use of mineral fertilizers.

Alfalfa is a forage crop providing high-protein feed for livestock and poultry enriched by all necessary vitamins, carbohydrates, mineral salts and microelements, with digestibility reaching up to 70-80 % [1, 2]. Alfalfa has long been cultivated in almost all main soil and climatic areas of Russia. Currently, 125 varieties of alfalfa of three species *Medicago sativa* L. nothosubsp. *varia* (Martyn) Arcang (variegated alfalfa), *Medicago sativa* L. *subsp. sativa* (dark blue alfalfa), and *Medicago sativa* L. subsp. *falcata* (L.) Arcang (yellow alfalfa) are approved for to use [3]. The practical preference is given to variegated alfalfa (hybrid of dark blue and yellow alfalfa), in which high-yielding crop typical for dark blue alfalfa with high quality of dry substance and tolerance to unfavorable cultivating conditions present in yellow alfalfa are successfully combined [1]. Modern winter-hardy varieties of *Medicago varia* could be cultivated in cold humid climate conditions on non-black soils of Russia. *M. varia* is widely used as a green manure to restore soil fertility, including degraded soils. Growing of this forage crop allows accumulation of ecologically safe biological nitrogen in roots and crops residues up to 120-200 kg/ha [4].

Alfalfa nitrogen accumulated in a biologically available form is a result of symbiotic synergy of plants with soil microorganisms – nodule bacteria. It is known that varieties of leguminous plants differ in response to inoculation by strains and even one and the same strain [5-8]. Nodule bacteria (rhizobia) strains used for treatment of plant seeds vary in symbiotic ability with one/several varieties/species of host plants (host specificity) [7, 9]. In many studies aimed to gain yield of leguminous plants, the priority is given to microsymbiont strains [10, 11]. At that, authors note that strains should successfully compete with local strains in rhizoplane of host plant roots both in standard conditions of alfalfa culturing, as well as under the effect of various abiotic factors (aridity, salinity, low or high pH of soil) [8, 12-17]. A number of studies show that strains tolerant to abiotic stress factors more often form effective symbiosis with host plants [15, 18-20]. However, research on selection of plants and microsymbiont strains are carried out independently from each other until present (11, 21), when publications discussing the need for joint studies are also in place [7, 14, 21].

According to the latest symbiogenetic achievements, genetic resources of plants and microorganisms are functionally integrated into one symbiotic system [23]. The fact that legume plant and microbial components and their integration play a key role in formation of symbiotic systems was considered in a number of publications [23-25]. Accordingly, host specificity of strains towards host plant species/varieties should be accounted for at directional obtainment of symbiotic systems, but the number of such studies at present is scarce [7, 21, 26, 27]. In practice, despite the fact that the economic feasibility of using biological prod-

ucts for legumes has been proven [28-32] and for certain types of legumes they use the appropriate biological products, strain-specificity is still not taken into account. As a result of inoculation of crops of different varieties of one type of legume by strain, which is part of the biological product, the formation of "random" variety-strain combinations characterized by significant variation of symbiotrophic indices like plant density, accumulation of plant green mass, protein content (protein nutritional value of the feed), root system development, size and a number of nodules on roots. Finally, it results in reduction of crop yield according to available data [6, 15, 33-35].

It is the principle of complementarity of genomes of legume-rhizobial symbionts that forms the basis of modern biotechnological method for creation of legumes varieties with improved their symbiotrophic indices. For the first time, positive results were obtained at selection of new clover varieties [36]. Works on creation of modern alfalfa varieties, such as Agnia and Taisia [37], were recently initiated. These varieties are already included into the State Register for Selection Achievements and admitted for use in the Russian Federation in 2012 and 2015, accordingly [3]. Due to the fact that new biotechnological approach allows reducing the duration of selection process for 5-7 years it became possible to create legume varieties of new generation with high-yield indices (increase of feed mass and crop yield) faster [37].

Present work resulted in a highly yield alfalfa variety - strain combinations with narrow and wide adaptability based on results of field plot trials carried out within the scope of Geographical Trials Network (GTN) of All-Russia Research Institute of Agricultural Microbiology (ARRIAM). It presents the first data of comparative analysis of complete-genome sequences of two highly effective and one commercial strain (425a) denoting the potential of future genome strain studies aimed at creation of high-yield plant -microbial symbiotic systems.

The purpose of this study was to assess the contributions of alfalfa and microbial components and their interaction to increase yield of symbiotic systems formed by different alfalfa varieties obtained by traditional method of selection and by selection method accounting symbiotrophic indices (joint symbiotic selection method) and highly effective *Sinorhizobium meliloti* strains from ecosystem of Aral Sea region in comparison with commercial strains 425a and 415b used in production of Rhizotorphin for alfalfa.

Techniques. Commercial strains 425a and 415b of *Sinorhizobium meliloti* are used for the bioproduct Rhizotorphin is made (development of ARRIAM); the 141 native strains (isolates) of *S. meliloti* were originally recovered from nodules of wild alfalfa growing on soils with sulfate-chloride type of salinization [38]. Salt tolerance of *S. meliloti* strains was determined by cell growth (OD_{600} = 0.005) in liquid rich medium TY [39] containing 3.5 % NaCl (w/v) during 96 hours at 28 °C and 180 rpm according to [38, 40]. Rm1021 *S. meliloti* strain was used as a reference.

Molecular genetic analysis of strains. Total DNA of bacteria was isolated by using NucleoSpin Tissue Kit (Macherey-Nagel GmbH, Germany). DNA was marked by Bio Prime kit with Klenow fragment of DNA-polymerase I, by accidental octamers and aminoalkyl-dUTP and fluorophores Cy3 and Cy5 (Thermo Fisher Scientific, USA). DNA-samples were cleaned in Microcon columns (Millipore, USA), quantitatively included mark was determined by Nanodrop spectrophotometer (Thermo Fisher Scientific, USA).

Genomes of the strains A1, A2 and 425a were sequenced by MiSeq method (Illumina, USA). From the 7 to the 15 contigs were obtained for each strain. Sequences corresponding to genome islands (GIs) in contigs were searched by using Mauve (http://darlinglab.org/mauve/mauve.html). Presence of

GIs in genomes wass tested by PCR-method according to [41]. DNA-microchips for hybridization done on full genome nucleotide sequence of the reference Rm1021 strain (NC_003037, NC_003047, NC_003078) were provided by the Center for Biotechnology, Bielefeld University, Germany. Each chip contained 6205 gene specific 70-dimensional oligonucleotide probes in three replications. Chips were hybridized according to standard terms [42], scanning was carried out by using Tecan HS 4800 Pro (Tecan, Switzerland), scans were analyzed by programs Genepix (Molecular Devices, USA) and EMMA (Bielefeld University, Germany). Probabilities of gene presence/absence and dynamic cutoff threshold for each trial were calculated by using programs GACK [43] and Statistica 6.0 (Dell Software Company, USA).

Assessment of symbiotic activity of strains. The variety Vega of *M. varia* inoculated by tested strains were grown in tubes 85 cm3 with vermiculite and Krasilnikov-Korenyako medium without nitrogen (standard conditions) or in tubes 60 cm3 with 0.7 % agar with the same medium but with adding 0.6 % NaCl (model salinity conditions) in 8-fold replicas in microvegetative plant tests [13]. Symbiotic effectiveness of strains was assessed by dry mass of inoculated plants (DMP) calculated by aerial-dry mass (each plant sample was dried for 7 days at 37 °C up to constant mass) as regards to mass of non-inoculated plants (Inoculation Free Control, (IFC).

Field plots trials were carried out in ARRIAM Geographical Trials Network (GNT) in four geographical locations on plots with area from 1.8 to 24 m^2 during 2003-2018. In total the 10 varieties of *M. varia* produced by traditional method of selection (further TS) or by joint symbiotic selection method (further JS) and variety Mira of *M. lupulina* (yellow clover) were used for vegetative tests. The varieties Vega 87, Lada, Lugovaya 67, Pastbishchnaya 88, Selena, Marusinskaya 425, Syulinskaya, and Kuzbasskaya were produced by traditional method of selection, whereas Agnia and Taisia, as well as varietal-sample L10/2 were obtained by joint symbiotic selection method. Alfalfa of varieties Marusinskaya 425 (originated by Morshanskaya Selection Station) and Kuzbasskaya (originated by Siberian Federal Research Center of Agrobiotechnologies RAS) were obtained by sampling of wild alfalfa ecotypes, with further selection of plants with high-yield on fertile soil (these varieties included into the State Register for Selection Achievements in 1938 and 1957, respectively) [3]. The variety Syulinskaya adapted to cold climate of central region of Yakutia, it do not include into the State Register. The varieties Vega 87, Lada, and Lugovaya 67 were created for planting on soil with high dosages of mineral fertilizers [3, 44]. The varieties Pastbishchnaya 88 and Selena were obtained by crossing of genotypes with high adaptability and were oriented for planting on weak-acid and moderately cultivated soils [3, 44]. Such varieties were included into the State Register in 1998 and 2000, correspondingly. The varieties Agnia and Taisia were created by using joint symbiotic selection biotechnology for planting on moderately cultured and non-cultured soils (included in the State Register in 2012 and 2015, accordingly [3, 44]. The variety Mira of *M. lupulina* was obtained by chemical mutagenesis of local wild-growing alfalfa plants from Moscow Region [3, 44].

Seeds passed preplanting treatment by powdering or working solution of the preparation; wide-row planting, with row spacing of 0.3-0.7 m, on depth of 2-3 cm; 3-fold or 4-fold replication of plots; seeding rate is 2.4 mil. seeds/ha; fertilizers were not applied (data of GTN) [29]. Statutory reporting on each trial included data of dry matter values (DM; plant mass dried at 105 °C until constant mass) and/or plant green mass (GM) obtained by separate mowing or total values of grass mowing for vegetation period. Experiments on planting of alfalfa

varieties could have significantly differed by schemes, but, usually, lasts at least 2-3 years, and number of independent experiments could have varied from 2 to 6. The value of DM and/or GM for each experiment replica and for IFC, as well as the mean value (average) and the errors of the mean value of DM and/or GM were calculated based on the values of DM and/or GM obtained for individual plants of each experiment replica and for IFC.

In addition, the least significant difference (LSD_{05}) showing the significance of differences between the experiment values and the IFC was provided for each field plot experiment [45]. Statistical significance between tested field plots variants and IFC was calculated by Lowest Significant Difference (LSD_{05}; [45]. Upon conduction of experiments with 5-6 years duration with such varieties as Agnia, Taisia, Selena, Marusinskaya 465, Vega 87, Lada, Pastbishchnaya 88 and varietal sample Lugovaya L10/2 the green mass data, mean values, standard error means and LSD_{05} were calculated for each year.

Statistical processing. Yield assessments for variety-strain combinations formed on the basis of the above mentioned alfalfa varieties and *Sinorhizobium* strains were obtained for each plot trial or plant test by evaluation the gain of yield of green (GR) or dry mass of inoculated plants (DPM) or by dry matter (DM), correspondingly, according to the following formula:

$$CY_i = [(PM_i - M_{IFCi})/ M_{IFCi}] \times 100\ \%,$$

where CY_i is crop yield in i-th experiment, PM_i is plant mass in i-th experiment, M_{IFCi} is IFC weight in the i-th experiment according to Geographical Trials Network. Mean crop yield (MCY) was calculated by GM and DM as

$$MCY = (\sum CY_i/n),$$

where i is trial serial number, n is total number of trials. Outlying yields determined based on relative deviation were ignored at calculation of MCY [45]. MCY error was calculated based on formulae:

$$x = S/\sqrt{n},$$

where x is MCY error, S is standard deviation calculated for CY values, n is total number of trials. For only one CY (result of one trial), error was 5%.

One-way ANOVA was used to assess the validity of dry mass yields of plants inoculated by nodule bacteria strains, at $\alpha = 0.05$ [46]. Two-way ANOVA [46] was carried out for quantitative assessment of contributions of host plant genotypes and genotypes of studied strains to crop yield evaluated by GM or DM.

Correlation coefficient was determined based on description [47], χ^2 with PAST software [48].

Results. Assessment of symbiotic properties of strains in microvegetative plant tests. Upon studying of 141 strains of *S. meliloti* in symbiosis with the variety Vega 87 of *M. varia* under standardized conditions of sterile microvegetative plant tests, we have found the 36 strains forming highly effective symbiosis (mean dispersion of DM yield in inoculated plants was within the range from 77.0 to 157.9 % regarding the reference strain).

The most perspective was given to the six strains (A1-A6) differing by saline tolerance (Table 1). Yields of DMP from inoculation by strains A2, A4 and A5, differed by phenotype, was in averaged 317 %, by strains A3 and A6 (saline sensitive phenotype) — over 180 % in comparison to inoculation free control (IFC). The most yields were established at using of the A1 strain of salt tolerant phenotype in comparison to IFC and reference strain, those were > 500 and 360 %, accordingly (see Table 1). The increase of yield mass in relation to the reference strain was similar in case of A6 strain, exceeding by 70 % in case of A3 strain and was more than twice higher the latter upon inoculation by A2, A4

and A5 strains (see Table 1). Under conditions of model salinization, inoculation of plants by tested strains also promoted the increase of green mass in plants. Statistically valid gain of yields in comparison to reference strain was obtained only in case of A2, A4 and A5 strains (in average over 180 %; $p < 0.05$). An exception was made to A1 strain formed highly effective symbiosis in plant tests under standard conditions, whereas under saline conditions the assessed yield of GM was by more than 20% lower than values obtained for A3 and A6 strains, but similar with data obtained for the reference strain (see Table 1).

1. Symbiotic activity of *Sinorhizobium meliloti* strains with the variety Vega 87 of *Medicago varia* L. in microvegetative plant tests

Strain	P	Standard conditions				Model saline conditions			
		DPM, mg	error MG, mg	DPM, %	error MG, %	DPM, mg	error MG, mg	DPM, %	error MG, %
A1	R	52,5	5,49	508,64	63,71	11,1	1,44	58,4	20,6
A2	S	35,7	2,41	314,23	27,94	17,8	1,41	155,0	20,2
A3	S	28,0	2,87	224,47	33,28	15,2	1,99	118,0	28,5
A4	R	36,0	3,72	317,89	43,17	23,2	1,85	232,7	26,6
A5	S	36,2	3,42	320,19	39,60	20,9	2,66	198,8	38,2
A6	S	21,2	2,50	146,27	28,93	12,6	0,91	81,2	13,1
Rm1021	S	21,2	0,88	146,26	10,26	10,5	0,79	50,4	11,3
IFC		8,63	0,57	0,00	6,58	6,98	0,46	0,0	6,6
LSD_{05}		11,76				4,64			

Note. P — phenotype (R — tolerant, S — sensitive to NaCl); DPM — dry mass of inoculated plants (mean value for 10 replications); error MG — error of mean yield of DMP; IFC — inoculation free control; Rm1021 — reference strain; LSD — the least significant difference.

Therefore, analysis of symbiotic properties of the six strains, two of which had salt tolerant, and four were of salt sensitive phenotype, does not yield the relationship between the own salt tolerance of strains and their possibility to form stress tolerant symbiotic systems, that data, does not inconsistent with our previous results [38]. At the same time, analysis of yields of DM obtained under non saline and under saline conditions had revealed a positive correlation: strains, inoculation by which promoted significant increase in crop yield without salinization, also formed highly productive symbiotic systems at saline conditions (correlation coefficient *r* comprised 0.89).

Assessment of symbiotic properties of strains in field plot trials. Symbiotic effectiveness and host specificity of the above strains were tested in symbiosis with 10 varieties of *M. varia* produced by traditional methods of selection or by modern joint symbiotic selection methods, and with the variety Mira of *M. lupulina* (Table 2). Commercial strains 425a and 415b were used as a reference group of strains. A total of 73 variety-strain combinations were tested, of which 32 were tested in 2-6 independent field plots trials within the scope of GTN (see Table 2).

2. Regions of field plots trials with alfalfa varieties (ARRIAM GTN)

Field plot trials	Soil characteristics	Alfalfa (*Medicago varia* L.) varieties
Bryansk Region[1], Bryansk State Agricultural Academy (Bryansk SAA; since 2014 — Bryansk State Agrarian University), 2002-2009	Grey forest weaky-loamy moderately cultured soil (pH 5.7)	Marusinskaya, Selena, Vega 87, Lada, Lugovaya 67
Moscow Region[1], Federal Scientific Center for Feed Production and Agroecology n/a V.R. Williams (FWRC FPA), 2002-2018	Sod-podzol, moderately loamy, weakly cultured soil (pH 4.49-5.08), uncultured soil	Pastbishchnaya, Agnia, Taisia, Selena, Lugovaya 67 (L10/2), Mira (yellow clover *M. lupulina* L.)
Federal State Novgorod Scientific Research and Design Technological Institute of Agriculture (NSRDTIA)[2], 2005-2009, 2018	Sod-podzol, weakly-loamy on clay, moderately cultured soil	Pastbishchnaya, Vega 87, Selena, Lada, Lugovaya 67
"RosAgro" LLC, Volosovskiy District of Leningrad Region[2], 2015	Sod-carbonated cultured soils (pH 6.5)	Agnia, Taisia
Tomsk State Pedagogic University (TSPU)[3], 2002-2006	Grey forest soil	Kuzbasskaya

Continued Table 2

Primorsk R&D Institute of Agriculture (Primorsk NIISH)[4], 2014	Meadow-brown, clay loam	Vega 87
Federal State Budgetary Scientific Institution Yakut R&D Institute of Agriculture n/a M.G. Safonov (Yakut NIISH), State Medical University[4], 2011	Taiga pale-yellow, cryomorphic soils, dark grey sod-podzol moderately loamy, on light-pale clay loam	Syulinskaya

Note. [1] — Central, [2] — North-Western, [3] — West-Siberian, [4] — Far East regions of performance of plot trials by Geographic Trials Network (ARRIAM GTN). Soil characteristics, period, and conditions of trials, and varieties of alfalfa are provided subject to reporting cards of GTN (see section Techniques).

3. Symbiotic parameters of plant-microbe symbiotic systems formed by *Sinorhizobium meliloti* strains and *Medicago varia* L. varieties in field plots trials (ARRIAM Geographic Trials Network)

Alfalfa variety	Y	The gain of plant yield after inoculation with strains (to IFC, %)							
		A1	A2	A3	A4	A5	A6	425a	4156
Central geographic region									
Agnia	1	132.1[3]	163.7[3]	nd	nd	nd	45.0[3]	11.4[3]	33.0[2]
	2	50.5±7.6 172.5[4]	43.5±10.5 109.3[4]	134.9[4]	198.9[4]	nd	nd	35.8[4]	nd
Taisia	1	nd	95.6±0.5	102±10.1	79.3±0.8	99.1±0.9	45.7±0.3	nd	82.3±0.7
	2	nd	62.5±11.4 198.2[9]	nd	nd	nd	nd	36±1.8 143.5[9]	33.0±1.6 87.0[9]
Pastbishchnaya 88	1	nd	nd	nd	nd	10.3[9]	nd	nd	17.1[9]
	2	nd	51.7±2.5	nd	nd	nd	nd	nd	nd
Selena	1	nd	47.4±16.3	nd	nd	nd	nd	nd	nd
	2	nd	115.2±5.8	nd	nd	nd	nd	26.3[1]	nd
Vega 87	1	nd	31.7[3]	nd	nd	nd	nd	nd	nd
Lada	1	nd	36.7[3]	nd	nd	nd	nd	nd	nd
Lugovaya 67	1	nd	6.0[3]	nd	nd	nd	nd	nd	nd
Lugovaya 67 (L10/2)	1	170.3±20.5 78.6[8]	136±14.3 40.2[8]	149±2.6 80.1[8]	171[7] 73.5[8]	80.1[8]	nd	nd	65.4±3.3
	2	133.8[8]	51.0[8]	148.1[8]	nd	nd	nd	nd	50.8[8]
Marusinskaya 425	1	9.9±1.6 27.9[4]	17.0±3.9	17.8±1.2	21.4±0.9	31.7[4]	nd	24.0±4.6	nd
Mira	2	95.7±4.8	nd	nd	nd	nd	nd	nd	nd
North-Western geographic region									
Agnia	1	nd	nd	nd	nd	nd	163.4[10]	nd	18.3[10]
	2	nd	nd	17.2±7.4	61.8±11.9 142.9[7]	149.3±27.4	143.5±20.1	9.8±0.5	22.9±5.0
Taisia	1	nd	nd	nd	nd	nd	176.6[10]	nd	14.1[10]
	2	nd	nd	nd	29.6[8] 62.8[7]	nd	98.0±7.3 150.0[10]	nd	15.0[10]
Pastbishchnaya 88	1	84.3±4.2	63.0±11.2 8.0[3]	nd	nd	nd	nd	17.1±5.9 39.2[5]	52.0[11]
	2	nd	7.4[3]	nd	nd	nd	nd	22.2[3]	nd
Selena	1	nd	63.7[3]	61.8[13]	25.9[13]	nd	nd	59.6[3]	50.5[13]
	2	nd	63.3[3]	nd	nd	nd	nd	58.3[3]	nd
Vega 87	1	120.1±6.0	81.3±18.3 6.4[5]	44.3[13]	nd	nd	nd	4.0[3]	25.5[13] 133[11]
	2	nd	31.9[3]	nd	nd	nd	nd	5.8[3]	nd
Lada	1	nd	45.4±8.7	nd	nd	nd	nd	27.3[5]	nd
	2	nd	37.3[3]	nd	nd	nd	nd	nd	nd
Lugovaya 67	1	nd	5.9[3]	nd	nd	nd	nd	12.6[3]	nd
	2	nd	6.4[3]	nd	nd	nd	nd	20.5[3]	nd
Mira	1	nd	77±3.9	nd	nd	nd	nd	nd	63±3.2
West-Siberian geographic region									
Kuzbasskaya	1	nd	14.5[2]	nd	nd	nd	nd	17.3 ± 0.3	nd
Far East geographic region									
Syulinskaya	1	42.3[9]	48.5[9]	nd	nd	nd	nd	38.1[9]	73.2[9]
Vega 87	1	15.3[6]	nd	nd	18.3[6]	nd	nd	12.2[6]	nd

Note. Variety Mira of *Medicago lupulina* L. Y — yield: 1 — green mass of plants (GM), 2 — dry matter (DM). Table provides mean yield gain and standard error of mean gain of plant mass calculated vs. the inoculation free control (IFC) and expressed in percentage (see section Techniques). Years of trials: [1] — 2003, [2] — 2006, [3] — 2007, [4] — 2008, [5] — 2009,([6] — 2011, [7] — 2012, [8] — 2013, [9] — 2014, [10] — 2015, [11] — 2016, [12] — 2017, [13] — 2018; na — no data available.

Since schemes of field plots trials varied (control strains, number of mowing and trials, years of trials, accounted parameters), comparative analysis of symbiotrophic effectiveness of variety-strain combinations formed based on the

above-mentioned varieties and nodule bacteria strains was conducted by gain of yields of green mass crops (GM) and/or dry matter (DM), the gain of yields are provided in Table 3. Yields obtained for trials lasting for 2-3-years are provided with indication of the result reporting year (see Table 3).

Assessment of crop yield of the variety Syulinskaya in symbiosis with strain A1 or A2 or with commercial strain 425a did not yield valid differences ($p > 0.05$) between such three variety-strain combinations in conditions of Far East Geographic region (see Table 3). At the same time, the variety Syulinskaya responded to inoculation by commercial strain 415b, since GM yield was approximately 2 times higher than in case of the above-indicated strains (see Table 3). It is interesting to note that statistically valid yields in GM for symbiotic systems of Syulinskaya-A2 and Selena-A2 planted in geographically distant regions (Far East and Central regions, accordingly) were similar (mean value 47.7±9.4 %). Inoculation of the variety Selena by strain A2 promoted 4-fold increase in DM of plants as compared to the use of commercial strain 425a in conditions of the Central region, as symbiotic systems formed by Selena variety with strains A2 or A3 or with commercial strains 425a or 415b did not validly differ by gain of yields in GM in conditions of North-Western geographical region ($p > 0.05$). Yield by DM for combination Selena-A2 was validly higher than for combination Pastbishchnaya 88-A2 (2 times higher) in conditions of the Central region (see Table 3).

High gains of yields (by GM) of the variety Pastbishchnaya 88 was achieved by using strains A1 or A2 in North-Western geographic region (mean value 70.4±8.3 %), that gain was by more than 3 times exceeded mean gain of yields when commercial strain 425a was applied ($p < 0.05$; see Table 3). Herewith, gain of yields by GM obtained for symbiotic systems Selena-A2 and Selena-A3 were similar to yields established for system Pastbishchnaya 88-A2, however by more than 3 times higher than for combination Pastbishchnaya 88-425a. It should be noted at the good response of Pastbishchnaya 88 to inoculation by strain 415b (yields by GM), whilst gain of yields from inoculation by strain 425a could significantly differ by years of trials (2009, see Table 3) in North-Western region. According to findings, symbiotic system Selena-A2 could be recommended for planting in Central and North-Western regions as highly-effective with a wide adaptive potential, and combinations Selena-A3, Pastbishchnaya 88-A1/A2 are — for North-Western region. Also, inoculation of varieties Selena and Pastbishchnaya 88 by commercial strain 415b or 425a could be of interests for last of mentioned geographic region, while the expected gain of crop yields could possibly be significantly lower (see Table 3).

The variety Marusinskaya 425 was tested in symbiosis with the five strains, the variety Kuzbasskaya — with the A2, and both varieties were assessed in symbiosis with commercial strain 425a. Gains of yields for symbiotic systems of the variety Kuzbasskaya with the strain A2 or with commercial strain 425a (the average yields gain was 14.5 and 17.3 %, accordingly, see table 3), and like for Marusinskaya 425 in symbiosis with strain A2 or A3 (mean value 17.9±2.7 %) did not validly differ. Slightly higher gains of yields were registered in case of inoculation of the same variety by strain A4 or commercial strain 425a (see table 3). We should note extremely low symbiotic response of the variety Marusinskaya 425 to inoculation by strain A1, along with gain of yield could have significantly varied by years of trials (2008, see Table 3). The highest gains of yields were obtained for Marusinskaya 425-A5 (yields by GM were in 1.3 times higher than for strain 425a) (see Table 3).

Vega 87 was mainly tested in North-Western regions with strains A1, A2, A3 and with both commercial strains, while with A2 only in the Central region.

Gain of crop yield of combination Vega 87-A2 assessed by GM was in 2.5 times higher in North-Western region than in the Central region, herewith we should note instable yields by years of trials (2009, see Table 3). Higher crop yields were obtained for combination Vega 87-A1 (gain of yield in GM 120.1±6.0 %) and in approximately 2 times lower — for combination Vega 87-A3 in the same geographic region (see Table 3). At the same time, inoculation of the variety Vega 87 by commercial strain 425a did not result in statistically valid gain of yields (4.0±0.2 %; see Table 3). Whereas the usage of commercial strain 415b allowed obtaining gain of mean yield of 25.5 %, the latter meaning could be also increased in few times in conditions of North-Western region depending on year of trials (2016, see Table 3). Combinations Vega-87-A1 and Vega 87-A2 could be considered as high-yield symbiotic systems for North-Western region.

Crop yield of the variety Lada from inoculation by A2 strain in conditions of the Central region, as well as in North-Western region, was similar (gain of mean value was 42.5±2.2 %; see Table 3). Inoculation of plants of the variety Lugovaya 67 was accompanied by formation of effective symbiotic system only when commercial strain 425a was used (DM yield comprised 20.5 %) in conditions of the North-Western region (see Table 3). Accordingly, combination Lada-A2 could be considered as effective with wide adaptability, whereas Lugovaya 67-425a symbiotic system provided statistically valid, but not high gain of yields at planting in the North-Western region.

Therefore, use of commercial strain 425a allows getting gain of crop yields (by GM), varying from 4 to 27.3 % for varieties Vega 87, Kuzbasskaya, Lada, Lugovaya 67, Marusinskaya 425 and Pastbishchnaya 88. Significant yields of ~~in~~ varieties Vega 87 and Pastbishchnaya 88 could be obtained at inoculation by commercial strain 415b. Statistically valid yields of the variety Selena (at least 50 %) was achieved when inoculation was done by any of two mentioned above strains, whereas responsiveness of the variety Syulinskaya to inoculation by strain 415b was in 2 times higher (over 70 %). Tested highly effective strains allowed getting gains of yields by GM from 40 to 80 % for 5 from 8 varieties obtained by traditional selection methods, herewith in case of varieties Pastbishchnaya 88 and Selena strain-varieties combinations were of high adaptive potential.

It should be noted that strain A1 selected as super effective in symbiosis with the variety Vega 87 done in microvegetative plant tests but it also produced the highest gain of yields with this variety in field trials too. Strains A1 and A2 were also tested in symbiosis with yellow clover of the variety Mira. The productivity of the corresponding variety-strain combinations was not inferior but even exceeded the increase in value obtained using commercial strain 415b. Strains A1 and A2 could be considered as perspective for use as a microbial bioproducts for the variety Mira cultivated as siderate on slightly acidic soils.

Thus, the presented results on the symbiotic activity of strains with alfalfa varieties which were produced by traditional selection methods proved that selection of highly effective strains in model plant tests is reasonable and an assessing of the genetic complementarity of micro- and macrosymbionts is important.

Agnia and Taisia are varieties of new generation which were by joint symbiotic selectin method. The productivity of the variety Agnia was evaluated with strain A1, which was used in the process of creating the variety. Crop yield of the variety Agnia was assessed with strain A1 used in process of creation of variety, and with strains A2-A6 in field plots trials in the Central and North-Western regions.

It should be noted the high responsiveness of the variety Agnia for inoculation by strains A1-A4 (average increase in SV of 153.9%; see Table 3) and

the variety Taisia towards strains A2-A5 in the Central region. However, the productivity of the same varieties when they were inoculated with strain A6 were comparable to or lower than those when strain 415b was used for inoculation (see Table 3). The variety Agnia was zoned in the Central region, while its planting in other region could provide different gain of yield values. High yields by DM were obtained at inoculating variety Agnia by strains A5 or A6 (average value 146.1%), as well as by strain A4 (more than 60%; see table 3). While strain A6 formed a highly effective symbiosis with the variety Taisia (gain of yields by GM and DM, accordingly 176.6 and 98.0%) in the North-West region. It should be noted that the yield (by DM) of varieties Agnia and Taisia could increase by 2.5–4 times in some years as it was observed in Central region (2008, 2014) and by 1.5–2 times in the North-West region (2012, 2015; see Table 3).

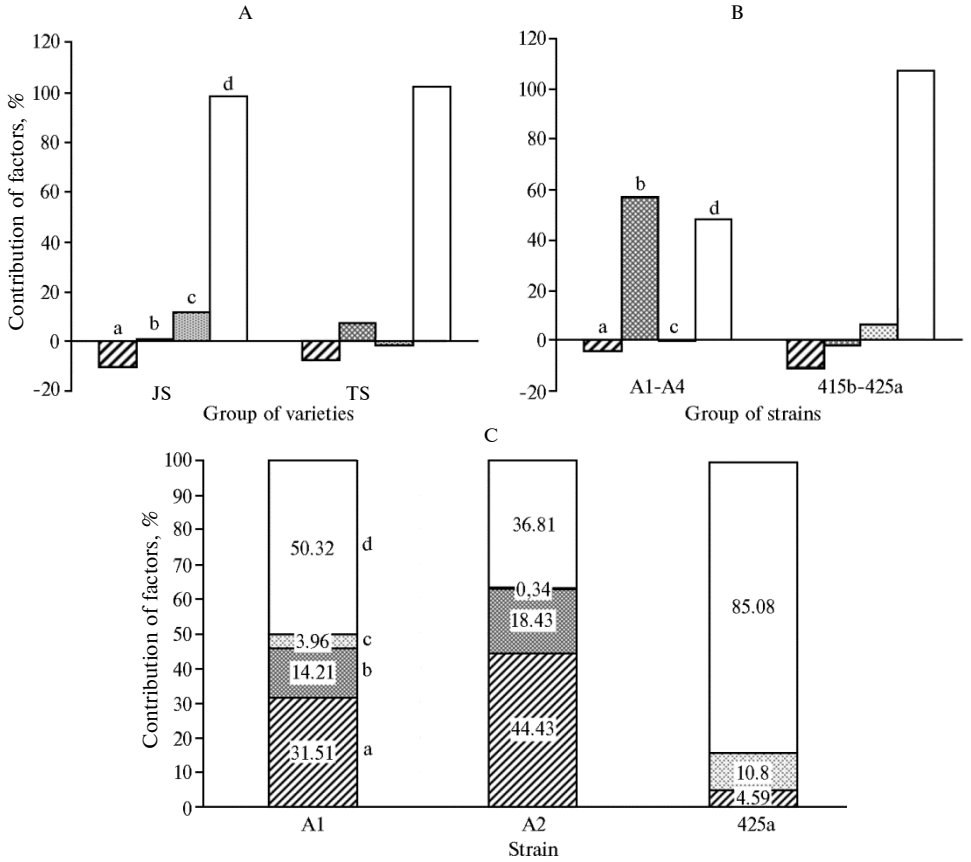
Similar high gains of yields were obtained in experiments with new varietal-sample L10/2 (Stepanova G.V., personal data) inoculated by strains A1-A5. Crop yields by GM for strains A1-A4 were similar and averaged to 154.3±9.0 %, which is 2-fold increased yields obtained when strain 415b was used. In case of strain A2, yield of the varietal-sample L10/2 was in 7 and 23 times higher (depending on year of trial; see Table 3) that yield obtained at inoculation of the original variety Lugovaya 67.

Thus, the increase in DM was no more than 10% and 23% when inoculation of crops of the variety Agnia was done by commercial strains 425a or 415b, respectively, in the North-West region, as well as less than an average of 35% in the case of the variety Taisia inoculated by strain 415b in the Central region. However, in different years, the yield gain was increased by 4.0 and 2.6 times, respectively (2014, see table 3). In case of inoculation of the variety Agnia or varietal-sample L10/2 by strains A1-A4, yields gain varied from 130 to nearly 200 % by years of trials (by DM or by GM). Yields gain in case of variety Taisia inoculated by strains A2-A5 comprised from 79 to 102 % in the Central region (see Table 3).

Two strains A6 and A3 formed highly effective symbiotic systems with varieties Agnia or Taisia in only one of the two geographical regions of the study. The strain A3 formed highly effective symbiosis in the Central region, and strain A6 in the Northwest region. High yield of combination Agnia-A6 was confirmed in commercial trial on plots of 0.1 ha done by "RosAgro" LLC located in Volosovskiy district, Leningrad Region [54]. Gain of crop yield by GM in such trial, comprised 163.4±8.7 % (see Table 3). Strains A4 and A5 (in contrast to the above-mentioned strains) formed high-effective symbiotic systems with both varieties in both geographical regions of study. Summarizing, our findings show that varieties created by joint symbiotic selection method are highly responsible to inoculation, which allows to increase yield 2-3 times due to high specificity of plant-microbe interaction in tested variety-strain combinations.

Two-way ANOVA analysis of plot trial data. Purpose of analysis was to determine the role (contribution) of variability factors from the side of plant symbiont, of microsymbionts, of their variety-strain combinations and of non-controlled factors in formation of crop productivity. An analysis of the gain yield values obtained for varieties Pastbishchnaya 88, Syulinskaya, Vega 87, Marusinskaya 425 (TS varieties) and for Agnia, Taisia, varietal-sample L10/2 (JS varieties) which were inoculated by studied highly effective strains allowed to show that productivity of variety-strain combinations based on JS varieties is largely depended on genetic characteristics of rhizobia strains (contribution value was 11.58%), while the productivity of combinations based on TS varieties, on the contrary, depended on genetic characteristics of corresponding varieties of alfalfa (6.99 %; Fig., A).

Uncontrolled factors in both cases had a similar effect on variety-strain combinations formed based on JS or TS varieties. Assessment of strain contribution had shown that highly effective native strains A1-A4 had actively formed symbiosis on studied alfalfa varieties (contribution was 56.6 %). In case of commercial strains 425a and 415b their genome characteristics had certain contribution (6 %) in gain of productivity of symbiotic systems with considered plant varieties (see Fig. B).



Two-way analysis of crop yield gain of plant-microbe systems formed by alfalfa varieties produced by traditional breeding or by joint symbiotic selection method with highly effective *Sinorhizobium meliloti* strains: A — contributions of varieties derived traditionally and by symbiotically depended selection, in symbiosis with studied highly effective strains; B — contributions of native and commercial strains to crop yield gain formation with studied alfalfa varieties; C — contributions of strains A1, A2 and 425a to crop yield gain formation with studied alfalfa varieties; a — variety-strain combination, b — plant varieties, c — strain, d — uncontrolled factors. JS — alfalfa varieties obtained by joint symbiotic selection (Agnia, Taisia, varietal L10/2); TS — alfalfa varieties obtained by traditional method of selection (Pastbishchnaya 88, Syulinskaya, Vega 87, Marusinskaya 425); A1, A2 and 425a — highly effective strains *S. meliloti*.

The same approach was applied to assess variation factors for formation of crop yields in variety-strain combinations formed by particular strains A1, A2 and 425a and by TS (Pastbishchnaya 88, Syulinskaya, Vega 87, Marusinskaya 425) and JS varieties. As a result, it was found that a significant contribution to productivity of variety-strain combinations was made by both host plant variety and the microsymbiont strain A1 or A2 (31.5 and 44.4% in yield gain, respectively). The contribution of genotype characteristics of host plant varieties to gain crop yield of variety-microbial combinations varied from 14.2 to 18.4 %, accordingly (see Fig., C). It should be noted that the contribution of genotype characteristics of strain A1 on yield gain was 3.96 %. By contrast, analysis of va-

riety-425a combinations showed that uncontrolled factors are main factors by their effect on yield production. The contribution of genotype characteristics of strain 425a was 10.80 % and of host plant was 4.59 % and contribution of both was at least 16 % in crop yield production.

Therefore, we for the first time shown that genotype characteristics of strain 425a more significantly affects formation symbiotic systems, whilst its host specificity regarding to this or other variety is not clear. These findings are giving some explanations why this strain used in bioproducts was so successful in different agrocenosis. At the same time, varieties Agnia, Taisia, and L10-2, created by the joint selection method, showed increased responsiveness to inoculation with strains of nodule bacteria. Further selection of complementary genotypes of plants and microsymbionts will increase their productivity by at least 50%. New variety-microbial systems tolerant to abiotic uncontrolled stress factors will have benefits for various agroclimatic conditions.

Since the high symbiotic complementarity of strains A1 and A2 to the variety Agnia created for planting on moderately cultured and uncultured soils, and the significance of genotypic characteristics of strain 425a was revealed, the study of the genomic characteristics of such highly effective strains became relevant.

Molecular genetic analysis of strains. We have conducted comparative analysis of genomes of highly effective strains A1, A2 and 425a by using DNA-biochip SM6kOligo, constructed on the bases of the reference strain Rm1021 [49]. It was established that genome of strain CXM1 (streptomycin-resistant mutant of the strain 425a) [49] lack 508 ORF (open reading frames), and genomes of the strains A1 and A2 lack 601 and 432 ORF, accordingly, while these sequences may have divergent structure. These ORFs, as established, are localized on chromosome, as well as on two megaplasmids, which are typical for *S. meliloti*. A group of 72 ORF, which are important for symbiotic activity was evaluated. The 17 *nod* genes from indicated group are involved in virulence, competitiveness, nodule formation, and host specificity, while other 31 fix and 8 *nif* genes are responsible for nitrogen fixation and effectiveness. All indicated *nod* genes are present in genome of CXM1, whereas sequences of *nod*D3 and *nod*N genes in genome of strain A1 have divergent structure. Analysis done for *fix* genes shown that *fix*L, *fix*T2 and *fix*M in genome of CXM1, *fix*O3, *fix*T2, *fix*P2, *fix*X in genome of strain A1 and, *fix*O3, *fix*L, *fix*N3, *fix*P3, *fix*I2, *fix*H in genome of strain A2 may have divergent structure or had been lost. Similar structural changes were found for *nif*D and *nif*N in genome of strain A1. Analysis of a group of six *nod* genes localized on megaplasmid II of the reference strain Rm1021 revealed structural changes in two genes (SMb20775 and SMb20825) in all tested native strains. Product of SMb20825 is predicted acetyltransferase of NodL family, localized on external cell membrane, which is related to signal receptor of nodule formation process. Blot hybridization of the sequence of six genes localized on chromosome (three *nod*, two *fix* and one *nif* gene) which are important for formation and functioning of symbiosis showed that their structure is conservative in the genomes of all the studied strains.

Besides genes related to symbiosis, the 23 genes responsible for to salt tolerance of rhizobia strains were analyzed. Six groups of genes: *kup*, *kdp*, *trk* and *kef*, responsible for intracellular concentration of potassium cations, *ots*/*tre* (encoding trehalose accumulation system) and *bet* genes (encoding betaine accumulation, biosynthesis, and catalysis systems) were studied. It was established that four groups of above mentioned genes are present in genomes of all studied strains. Significant structural changes with predicted functional importance were revealed for *bet* and *trk* genes in strains CXM1 and A1, while genes *betB2* and *trkD* are absent (or their sequences are divergent) in CXM1 and A1, correspond-

ingly. Therefore, genes from the considered groups that have divergent sequences (or absent) are most often found in strains adapted to soil saline conditions at which they form a symbiosis with alfalfa plants.

Genomes of strains A1 and A2 and 425a were screened for accessory elements of genome — for genomic islands (GIs), which are phage related sequences participating in horizontal gene transfer [49]. Analysis of contigs obtained for strains A1 and A2 and 425a allowed revealing the six GIs, which presence was confirmed by PCR-detection method (see Techniques section). It was found that, in contrast to the reference strain which harboring the three GIs, in highly effective native strains the three, the two and the one GIs were detected by PCR detection (see Techniques section). Lengths of GIs in the reference strain varied from 19 to 80 kbp, while in highly effective native strains from 10.6 to 44 kbp. Analysis of nucleotide sequences of GIs by sequencing data had shown that one of genomic islands (10.6 kbp) of strain A2 had homology with GI Sme80S (80 kbp) of the reference strain and with genomic island of 10 kbp in strain 425a (82 % homology). The second island of strain A2 is 44 kbp in size was homologous to GI (27 kbp) of strain A1 (85 % homology), while the other two GIs of the same strain did not have homologous sequences between each other and with the third island of the same strain. Presented data designated that GIs revealed in highly effective strains contain various genetic information, that fact confirm GIs participation in horizontal transfer of genes and transfer of new genetic information to recipient strains.

A significant structural and functional differences between gene pools of highly effective strains A1, A2 and commercial strain 425a according to the first data of done molecular genetic analysis are important for the formation of stress tolerant symbiotic plant-microbial systems. Finally, we would like to outline that we have carried out comparative analysis of highly effective strains from Aral Sea region exposed to salinization, and commercial strains 425a and 415b used for bioproduct Rhizotorphin for alfalfa by symbiotic activity and genome characteristics. For the first time it was shown that highly effective strains selected by symbioptophic characteristics in microvegetative laboratory tests are forming productive symbiosis with alfalfa varieties obtained by traditional and by joint symbiotic selection methods. At the same time, plant-microbial systems based on modern varieties are more adaptive and productive (yields gain by over 50 % as compared to varieties of traditional selection). Obtained data denoting that yield gain capacity of variety-microbial systems formed by commercial strain 425a mainly depends on uncontrolled factors (over 80 %). Genomic characteristics of this strain play more significant role in varying the yield increase than combination of this strain with varieties, that, apparently, determines the demand for biological products based on this strain for various alfalfa varieties cultivated in different geographical regions of Russia.

So, the yield (by green mass of plants and dry matter) of plant-microbial systems based on varieties obtained by joint symbiotic selection methods and highly effective strains of root nodule bacteria adapted to salinity significantly exceeded the similar indices of the “randomly formed” symbiotic system based on commercial strains 425a and 415b. Legume varieties of modern selection have increased susceptibility to inoculation by strains of nodule bacteria, and further selection of microsymbionts of complementary genotypes will contribute to a significant increase of yield and the creation of new variety-strain combinations with increased tolerance to abiotic stress factors and expand possibility to cultivate in various agroclimatic conditions. Varieties of modern selection have higher response to inoculation by root nodule bacteria strains, and further selection of strains with complementary genotypes would promote significant increase

of crop and creation of new variety-strain combinations with higher tolerance to abiotic stress factors, which could extend possibility of their planting in various agroclimatic conditions. Presented results of the assessment of crop yields gain obtained as a result of field plot trials carried out during many years in Russian regions differing by agroclimatic conditions clearly demonstrate, the need for the widespread introduction of the joint symbiotic selection method in order to create new varieties of alfalfa.

REFERENCES

1. Ivanov A.I. *Lyutserna* [Alfalfa]. Moscow, 1980 (in Russ.).
2. Dubovskii I.I. *Agroenergeticheskaya otsenka kul'tur i osnovnye napravleniya sovershenstvovaniya polevogo kormoproizvodstva v stepnykh raionakh Tsentral'no-Chernozemnoi polosy. Avtoreferat kandidatskoi dissertatsii* [Agro-energy crop assessment and the main directions for improving field feed production in the steppe regions of the Central Black Earth Strip. PhD Thesis]. Moscow, 2000 (in Russ.).
3. *Gosudarstvennyi reestr selektsionnykh dostizhenii, dopushchennykh k ispol'zovaniyu. Tom 1. Sorta rastenii (ofitsial'noe izdanie)* [The state register of selection achievements allowed for use. Vol. 1. Varieties of plants (official publication)]. Moscow, 2019 (in Russ.).
4. Shamsutdinov Z.Sh. Forage crops selection: progress and challenges. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2014, 6: 36-45 (doi: 10.15389/agrobiology.2014/6/36eng).
5. Bzheumykhov V.S., Kobozev I.V., Tokbaev M.M. *Izvestiya Timiryazevskoi sel'skokhozyaistvennoi akademii*, 2007, 2: 28-37 (in Russ.).
6. Provorov N.A., Simarov B.V. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 1986, 12: 37-42.
7. Fotev Yu.V., Sidorova K.K., Novikova T.I., Belousova V.P. *Vavilovskii zhurnal genetiki i selektsii,* 2016, 20(3) 348-354 (doi: 10.18699/VJ16.099) (in Russ.).
8. Shkarupa M.V. *Sbornik materialov X Vserossiiskoi konferentsii molodykh uchenykh i spetsialistov «Aktual'nye voprosy biologii, selektsii, tekhnologii vozdelyvaniya i pererabotki maslichnykh i drugikh tekhnicheskikh kul'tur»* [Proc. X All-Russian Conference of Young Scientists and Specialists «Actual issues of biology, breeding, technology for the cultivation and processing of oilseeds and other industrial crops»]. Krasnodar, 2019: 231-235 (in Russ.).
9. Ddelić D., Stajković-Srbinović O., Radović J., Kuzmanović D., Rasulić N., Simić A., Kneževi - Vukčević E. Differences in symbiotic N2 fixation of alfalfa, *Medicago sativa* L. cultivars and *Sinorhizobium* spp. strains in field conditions. *Romanian Biotechnological Letters*, 2013, 18(6): 8743-8750.
10. Shamseldin A., Youseif S.H., Abd El-Megeed F.H., Abdelkhalek A., Sadowsky M.J., Saleh S.A. Selection and use of effective, competitive, clover-nodulating rhizobium strains for use as commercial inoculants in alkaline and salt affected egyptian soils. *Asian Academic Research Journal of Multidisciplinary*, 2016, 3(7): 2319-2801.
11. Yadav J., Verma J.P., Rajak V.K., Tiwari K.N. Selection of effective indigenous rhizobia strain for seed inoculation on chickpea (*Cicer aritenium* L.) production. *Bacteriology Journal*, 2011, 1(1): 24-30 (doi: 10.3923/bj.2011.24.30).
12. Gasanov G.N., Usmanov R.Z., Musaev M.R., Abasov M.M. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2007, 1: 79-85.
13. Rumyantseva M.L., Stepanova G.V., Kurchak O.N., Onishchuk O.P., Muntyan V.S., Dzyubenko E.A., Dzyubenko N.I., Simarov B.V. Selection of salt tolerant alfalfa (*Medicago* L.) plants from different varieties and their morfo biological and symbiotic properties analysis. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2015, 50(5): 673-684 (doi: 10.15389/agrobiology.2015.5.673eng).
14. Aranjuelo I., Arrese-Igor C., Molero G. Nodule performance within a changing environmental context. *Journal of Plant Physiology*, 2014, 171(12): 1076-1090 (doi: 10.1016/j.jplph.2014.04.002).
15. Mahajan S., Tuteja N. Cold, salinity and drought stresses: an overview. *Archives of Biochemistry and Biophysics*, 2005, 444(2): 139-58 (doi: 10.1016/j.abb.2005.10.018).
16. O'Hara G., Yates R., Howieson J. Selection of strains of root nodule bacteria to improve inoculants performance and increase legume productivity in stressful environments. In: *Inoculants and nitrogen fixation of legumes in Vietnam* /D. Herridge (ed.). ACIAR Proceedings, 2002: 75-80.
17. Sańko-Sawczenko I., Łotocka B., Mielecki J., Rekosz-Burlaga H., Czarnocka W. Transcriptomic changes in *Medicago truncatula* and *Lotus japonicus* root nodules during drought stress. *International Journal of Molecular Sciences*, 2019, 20(5): 1204 (doi: 10.3390/ijms20051204).
18. Salim A., Cheloufi H., Attab S., Bouras N. Improvement of alfalfa growth under water stress by inoculation with *Sinorhizobium meliloti* strains from the Algerian Sahara. *International Journal of Sciences and Research.* 2019, 75(7/1): 35-40 (doi: 10.21506/j.ponte.2019.7.4).

19. Vriezen J.A., de Bruijn F.J., Nüsslein K. Responses of rhizobia to desiccation in relation to osmotic stress, oxygen, and temperature. *Applied and Environmental Microbiology*, 2007, 73(11): 3451-3459 (doi: 10.1128/AEM.02991-06).
20. Xu J., Li X.L., Luo L. Effects of engineered *Sinorhizobium meliloti* on cytokinin synthesis and tolerance of alfalfa to extreme drought stress. *Biotechnology*, 2012, 78(22): 8056-8061 (doi: 10.1128/AEM.01276-12).
21. Lazarev N.N. *Vestnik OrelGAU*, 2006, (2-3): 55-56 (in Russ.).
22. Bourion V., Heulin-Gotty K., Aubert V., Tisseyre P., Chabert-Martinello M., Pervent M., Delaitre C., Vile D., Siol M., Duc G., Brunel B., Burstin J., Lepetit M. Co-inoculation of a Pea Core-Collection with diverse rhizobial strains shows competitiveness for nodulation and efficiency of nitrogen fixation are distinct traits in the interaction. *Frontiers in Plant Science*, 2018, 8: 2249 (doi: 10.3389/fpls.2017.02249).
23. Tikhonovich I.A., Andronov E.E., Borisov A.Yu., Dolgikh E.A., Zhernakov A.I., Zhukov V.A., Provorov N.A., Rumyantseva M.L., Simarov B.V. *Genetika*, 2015, 51(9): 831-846 (doi: 10.7868/S001667581509012X) (in Russ.).
24. Gubry-Rangin C., Garcia M., Bena G. Partner choice in *Medicago truncatula-Sinorhizobium* symbiosis. *Proceedings. Biological sciences*, 2010, 277(1690): 1947-1951 (doi: 10.1098/rspb.2009.2072).
25. Wang D., Yang S., Tang F., Zhu H. Symbiosis specificity in the legume-rhizobial mutualism. *Cellular Microbiology*, 2012, 14(3): 334-342 (doi: 10.1111/j.1462-5822.2011.01736.x).
26. Khapchaeva S.A., Didovich S.V., Topunov A.F., Mulyukin A.L., Zotov V.S. *Ekologicheskaya genetika*, 2018, 16(4): 51-60 (doi: 10.17816/ecogen16451-60) (in Russ.).
27. Argaw A. Symbiotic effectiveness of inoculation with *Bradyrhizobium* isolates on soybean [*Glycine max* (L.) Merrill] genotypes with different maturities. *Springerplus*, 2014, 3: 753 (doi: 10.1186/2193-1801-3-753).
28. Vorobeikov G.A., Bredikhin V.N. *Mikroorganizmy v agrobiotekhnologiyakh i zashchite prirodnoi sredy*. SPb, 2018.
29. Kozhemyakov A.P., Laktionov Yu.V., Popova T.A., Orlova A.G., Kokorina A.L., Vaishlya O.B., Agafonov E.V., Guzhvin S.A., Churakov A.A., YAkovleva M.T. The scientific basis for the creation of new forms of microbial biochemicals. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2015, 50(3): 369-376 (doi: 10.15389/agrobiology.2015.3.369eng).
30. Arafa M.M., El-Batanony N.H., Nofal A.M. Inoculation effect of rhizobial strains on growth, yield and chemical composition of some legume crops in new reclaimed soil. *Middle East Journal of Agriculture Research*, 2018, 7(2): 352-363.
31. Buntic A.V., Stajkovic-Srbinovic O.S., Knezevic M.M., Kuzmanovic D.Z., Rasulic N.V., Delic D.I. Development of liquid rhizobial inoculants and pre-inoculation of alfalfa seeds. *Archives of Biological Sciences*, 2019, 71(2): 379-387 (doi: 10.2298/abs181008062b).
32. Solomon T., Pant L.M., Angaw T. Effects of Inoculation by *Bradyrhizobium japonicum* strains on nodulation, nitrogen fixation, and yield of soybean (*Glycine max* L. Merill) varieties on Nitisols of Bako, Western Ethiopia. *ISRN Agronomy*, 2012, Article ID 261475 (doi: 10.5402/2012/261475).
33. Atlasova L.G. *Izvestiya Samarskogo nauchnogo tsentra Rossiiskoi akademii nauk*, 2015, 17(5): 77-80 (in Russ.).
34. Tsotsieva V.P., Basieva L.Zh., Kozyrev A.Kh. *Izvestiya Gorskogo gosudarstvennogo agrarnogo universiteta*, 2015, 52(4): 57-62 (in Russ.).
35. Deaker R., Roughley R.J., Kennedy I.R. Legume seed inoculation technology — a review. *Soil Biology and Biochemistry*, 2004, 36(8): 1275-1288 (doi: 10.1016/j.soilbio.2004.04.009).
36. Drobysheva L.V., Zyatchina G.P. *Adaptivnoe kormoproizvodstvo*, 2016, 3: 94-108 (in Russ.).
37. Stepanova G.V., Zolotarev V.N. *Adaptivnoe kormoproizvodstvo*, 2015, 1: 28-38 (in Russ.).
38. Ibragimova M.V., Rumyantseva M.L., Onishchuk O.P., Belova V.S., Kurchak O.N., Andronov E.E., Dzyubenko N.I., Simarov B.V. *Mikrobiologiya*, 2006, 75(1): 94-100 (in Russ.).
39. Beringer J.E. R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology*, 1974, 84: 188-198 (doi: 10.1099/00221287-84-1-188).
40. Rumyantseva M.L., Simarov B.V., Onishchuk O.P., Andronov E.E., Chizhevskaya E.P., Belova V.S., Kurchak O.N., Muntyan A.N., Rumyantseva T.B., Zatovskaya T.V. *Biologicheskoe raznoobrazie kluben'kovykh bakterii v ekosistemakh i agrotsenozakh. Teoreticheskie osnovy i metody* /Pod redaktsiei M.L. Rumyantsevoi, B.V. Simarova [The biological diversity of nodule bacteria in ecosystems and agrocenoses. Theoretical foundations and methods. M.L. Rumyantseva, B.V. Simarov (eds.)]. St. Petersburg, 2011 (in Russ.).
41. Cherkasova M.E., Muntyan V.S., Saksaganskaya A.S., Simarov B.V., Rumyantseva M.L. *Ekologicheskaya genetika*, 2019, 17(3): 23-38 (doi: 10.17816/ecogen17323-38) (in Russ.).
42. Giuntini E., Mengoni A., De Filippo C., Cavalieri D., Aubin-Horth N., Landry C.R., Becker A., Bazzicalupo M. Large-scale genetic variation of the symbiosis-requiered megaplasmid pSymA revealed by comparative genomic analysis of *Sinorhizobium meliloti* natural strains. *BMC Genomics*, 2005, 6: Article number 158 (doi: 10.1186/1471-2164-6-158).
43. Kim C.C., Joyce E.A., Chan K., Falkow S. Improved analytical methods for microarray-based

genome-composition analysis. *Genome Biology*, 2002, 3(11): RESEARCH0065 (doi: 10.1186/gb-2002-3-11-research0065).
44. *Raionirovannye i perspektivnye sorta kormovykh kul'tur selektsii VNII kormov im. V.R. Vil'yamsa. Katalog* [Zoned and promising varieties of feed crops breeding VNII feed them. V.R. Williams. Catalog]. Moscow, 2007 (in Russ.).
45. Pustyl'nik E.I. *Statisticheskie metody analiza i obrabotki nablyudenii* [Statistical methods for the analysis and processing of observations]. Moscow, 1968 (in Russ.).
46. Lakin G.F. *Biometriya* [Biometrics]. Moscow, 1990 (in Russ.).
47. Gmurman V.E. *Teoriya veroyatnostei i matematicheskaya statistika* [Theory of Probability and Mathematical Statistics]. Moscow, 2004 (in Russ.).
48. Hammer O., Harper D.A.T., Ryan P.D. PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, 2001, 4(1):1-9.
49. Rumyantseva M.L., Muntyan V.S., Cherkasova M.E., Andronov E.E., Saksaganskaya A.S., Dzyubenko E.A., Dzyubenko N.I., Simarov B.V. A comparative analysis of genomic characters of reference *Sinorhizobium meliloti* strains, the alfalfa symbionts (review). *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2017, 52(5): 928-939 (doi: 10.15389/agrobiology.2017.5.928eng).