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. 56, Issue 6
November-December

2021 Moscow

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

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

Publisher: Agricultural Biology Editorial Office NPO

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

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

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

SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA
[AGRICULTURAL BIOLOGY], 2021, Vol. 56, № 6

CONTENTS

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1015-1030
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Reviews, challenges

UDC 636.5:573.6.086.83:577.21

doi: 10.15389/agrobiology.2021.6.1015eng
doi: 10.15389/agrobiology.2021.6.1015rus

GENOME EDITING: CURRENT STATE AND PROSPECTS FOR USE IN POULTRY

(review)

N.A. VOLKOVA ✉, A.N. VETOKH, N.A. ZINOVIEVA

Ernst Federal Research Center for Animal Husbandry, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail natavolkova@inbox.ru (✉ corresponding author), anastezuya@mail.ru, n_zinovieva@mail.ru
ORCID:
Volkova N.A. orcid.org/0000-0001-7191-3550
Zinovieva N.A. orcid.org/0000-0003-4017-6863
Vetokh A.N. orcid.org/0000-0002-2865-5960
The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation, grant No. 21-66-00007
Received September 27, 2021

Abstract

To date, significant progress has been made in the poultry's genetic modification. A sufficiently large number of methods and methodological approaches have been developed for the introduction of recombinant genes into bird cells. The efficiency of using these approaches for genetic modification of bird cells varies depending on the object of research, the selected target cells for the introduction of recombinant DNA and the method of their transformation. Blastoderm cells, primordial germ cells, spermatogonia, sperm cells, and oviduct cells can serve as target cells for gene modifications. Using retroviral, lentiviral and adenoviral vectors, electroporation and lipofection, genetic transformation of these target cells can be carried out. In general, three main strategies for creating a genetically modified bird can be distinguished: i) the introduction of genetic constructs directly into the embryo (J. Love et al., 1994; Z. Zhang et al., 2012) or into individual organs and tissues of adults (D.V. Beloglazov et al., 2015; S. Min et al., 2011), ii) transfection of target cells in vitro and their subsequent transplantation into the embryo or target organs (M.-C. van de Lavoir et al., 2006; B. Benesova et al., 2014), and iii) sperm transformation in vitro and insemination of females with transformed sperm (E. Harel-Markowitz et al., 2009). These approaches were used to develop methods for editing the avian cell genome. A number of papers have studied the possibility of modifying bird cells using various editing systems, in particular, ZFN (zinc finger nuclease), TALEN (transcription activator-like effector nucleases), and CRISPR/Cas9 (clustered regularly interspaced palindromic repeats). Promising areas of using this technology in poultry farming are the following: studying the genes functions (N. Véron et al., 2015), obtaining recombinant proteins in the egg white composition (I. Oishi et al., 2018), improving economically useful and productive qualities (J. Ahn et al., 2017), and increasing resistance to infectious diseases (A. Koslová et al., 2020; R. Hellmich et al., 2020). Chickens with knockout of genes of the heavy chain of immunoglobulin (B. Schusser et al., 2013; L. Dimitrov et al., 2016), ovomucin (I. Oishi et al., 2016), myostatin (G.-D. Kim et al., 2020), as well as an integrated human interferon beta gene (I. Oishi et al., 2018) were obtained using genome editing technology. Quail with knockout of myostatin genes (J. Lee et al., 2020) and melanophilin (J. Lee et al., 2019) were also obtained. A number of studies have shown the simplicity, safety and availability of using the CRISPR/Cas9 editing system for modifying the poultry genome. This allows us to consider this system as an effective tool for the creation and commercial use of breeds and lines of birds with improved qualities in the framework of the implementation of large-scale breeding programs aimed at improving the quality of the resulting poultry products.

Keywords: poultry, quail, chicken, transgenesis, genome editing, CRISPR/Cas9, primordial germ cells, germ cells.

Farm poultry, in particular chickens and quails, is a convenient and accessible object for conducting various studies and solving problems in the field of developmental biology, medicine, and veterinary medicine [1, 2]. Unlike large farm animals, the bird has a short generation interval, which significantly reduces

the time to breed lines or populations of individuals with certain traits that are of interest both within the framework of individual studies and for solving larger problems. The similarity of the structure of protein glycosylation in birds and humans, as well as high egg productivity, sterility and availability of eggs, allow us to consider birds as an effective productive platform for the production of recombinant proteins [3]. This is especially true in the case of recombinant products that cannot be obtained using transgenic mammals (if such products are toxic to them).

It should be noted that the methods used to modify the mammalian genome are in most cases ineffective for the transgenesis of poultry. This is primarily due to the peculiarities of the physiology, reproduction and developmental biology of birds (4). Unlike mammals, in birds, the development of embryos in the reproductive organs of the female proceeds only at the early stages of embryogenesis. By the time of laying the egg immediately after laying, the embryo consists of approximately 60,000 morphologically undifferentiated pluripotent cells (5). Further development of the embryo occurs outside the body of the female when appropriate environmental conditions appear. Features of the embryonic development of birds significantly complicate the use of the traditional method of breeding transgenic animals - DNA microinjection into the pronucleus of zygotes. Limiting factors also become difficulties in accurately determining ovulation, a large amount of yolk in the egg, and a strong compaction of the cytoplasm. At the same time, the long period of embryonic development of birds outside the body of the female facilitates access to embryos for genetic engineering manipulations.

To date, there are a fairly large number of methodological approaches for the genetic modification of avian cells for development and optimization of particular stages of the genome editing technology using various systems, e.g., ZFN (zinc finger nuclease), TALEN (transcription activator-like effector nucleases) and CRISPR/Cas9 (clustered regularly interspaced palindromic repeats) [6, 7]. This technology is used in poultry farming to create cell lines and individuals with a knockout or insertion of individual genes when studying their functions [8], obtaining recombinant proteins in the composition of egg white, improving economically useful traits and the quality of poultry products [9, 10], increasing resistance to infectious diseases [11, 12].

The purpose of this review is to summarize data on the main achievements in the field of editing the genome of poultry and the prospects for their use in poultry farming.

Genome editing systems. Genome editing technology involves making targeted changes to the target genome region using site-specific nucleases [6, 13]. The most common are zinc finger nucleases (ZFN), TALE-associated nucleases (TALEN), and CRISPR/Cas9 [14, 15]. The principle of their action is based on the introduction of double-strand breaks into the genome region of interest, which are subsequently repaired by means of non-homologous end joining or homologous recombination [16, 17].

In the first case, the repair of double-strand breaks leads to the formation of insertions or deletions at the break site; in the second, an artificially introduced genetic construct imitating the sister chromatid is used to repair DNA [18]. Deletions and insertions lead to gene knockout (knockout), which is of interest in studying their functions, as well as in the production of animal products with improved qualities (for example, low-allergenic eggs). The introduction of donor DNA (genetic constructs) through homologous recombination makes it possible to introduce additional information into the genome.

ZFN and TALEN editing systems are more costly and time consuming

compared to CRISPR/Cas9. With the use of ZFN and TALEN nucleases, off-target effects are more often noted [7]. The possibility of automated selection of individual components of the CRISPR/Cas9 system using various online services makes it possible to increase the specificity of introducing genetic changes in the target gene and significantly reduce the likelihood of off-target mutations. In addition, the components of the system can be designed to virtually any target genomic DNA sequence. The CRISPR/Cas9 genome editing system is based on the natural defense mechanism (adaptive immunity) of bacteria and archaea against phages [19, 20]. This editing system includes two main components — Cas9 nuclease and guide (guide) RNA (gRNA, guide RNA). The guide RNA binds specifically to the target DNA region, which is subsequently cleaved by Cas9 [21-23]. The resulting DNA double-strand breaks are further repaired through homologous or non-homologous recombination, depending on the goals of the experiment [24, 25]. To introduce small deletions or insertions into the target DNA to knock out the gene, one guide RNA specific for this DNA region and Cas9 are used. If it is necessary to switch off several genes, a mixture of guide RNAs and Cas9 nuclease is used. To include donor DNA in a certain region of the genome (for example, to obtain producers of recombinant proteins), along with a guide RNA and a nuclease, a genetic construct for homologous recombination is introduced into the cell, which is a fragment of inserted DNA flanked by sequences homologous to the break [18, 26].

CRISPR/Cas9-based genome editing systems allow the introduction of site-specific mutations in target genes similar to naturally occurring genetic variants (editing without trace). With editing the genome of target cells using this system, expression of its main components Cas9 and guide RNA occurs from a single vector or introduced as a mixture. The most common is the first approach based on the use of a plasmid encoding Cas9 and a guide RNA. This eliminates the need for multiple transfection components, which simplifies the editing procedure and increases the stability of the results.

Methods of genetic modification of bird cells. A set of methods and methodological approaches used to obtain genetically modified individuals depends on the object of research, the choice of target cells for the introduction of recombinant DNA, and the method of genetic transformation of target cells. The main strategies for creating a genetically modified bird are the introduction of genetic constructs directly into the embryo [27, 28)] or the organs and tissues of adults [29, 30], the transfection of target cells in in vitro culture and their transplantation into an embryo or target organs [31, 32], and the sperm transformation in vitro to inseminate females with transformed sperm [33].

An effective tool for targeted delivery of recombinant DNA into cells of an embryo or organs and tissues of adults is the use of vectors based on recombinant viruses, which is associated with their natural ability to independently penetrate into target cells and integrate into a foreign genome with high efficiency.

With the use of viral vectors, the first successful experiments on the creation of a transgenic bird were carried out. In 1987 Salter et al. [34] obtained transgenic chickens by introducing a retroviral vector based on the avian leukosis virus (ALV) into the subembryonic cavity of stage X embryos. The efficiency of transgene transfer to offspring was 1-11%. Subsequently, the possibility of creating a transgenic bird using retroviral vectors based on the Rous sarcoma virus [35], reticuloendotheliosis virus (REV) [36], avian spleen necrosis [37], Moloney murine leukemia virus (MoMLV) was shown [38, 39]. To date, transgenic chickens have been created with integrated genes encoding -galactosidase, LacZ [37], β-lactamase [40, 41], green fluorescent protein (GFP) [42], bispecific antibodies

[43], hormone growth [44], human granulocyte colony stimulating factor [39], interferon α-2b [45].

The use of lentiviral vectors made it possible to increase the efficiency of poultry transgenesis [46]. McGrew et al. [47] obtained transgenic chickens with integrated *LacZ* and *eGFP* genes by lentiviral transfection of blastoderm cells of stage X embryos. The efficiency of transgene transfer to offspring was 4-45%. Using lentiviral vectors, transgenic chickens and quails were created that produce recombinant proteins, in particular, human β-interferon hIFNβ1 [48], bispecific antibodies [28, 48], GFP [49, 50], interleukin 1 receptor antagonist (rhIL1RN) [51], human lysozyme [52], α-defensin HNP4 (human neutrophil defensin 4) [53].

It should be noted that when viral vectors are introduced into the subembryonic cavity of embryos at stage X, the transgenic bird turns out to be a mosaic, and further crossings are required to create a generative individual. In this regard, the key point is the effectiveness of the transformation of cells of the reproductive organs of males and females. This problem can be solved by targeted modification of germ cells, which makes it possible to purposefully act on specific target cells, completely leveling the risks associated with the creation of transgenic mosaic individuals, from which it is impossible to obtain transgenic offspring in the future.

By culturing embryonic and spermatogenic cells in vitro, a variety of techniques can be used to introduce recombinant DNA into target cells using safe gene delivery systems. The use of genetically modified germ cells guarantees the presence in the oocyte after fertilization of one copy of the construct built into a certain locus. Recombinant DNA integrated into the genome of target cells can be stably transmitted over several generations. Manipulations on adult individuals significantly reduce the time and material costs for obtaining genetically modified offspring.

When creating a genetically modified poultry, both mature germ cells [33] and their precursors, primordial germ cells (PGCs) [54, 55] and spermatogonia [56, 57], can serve as target cells. The use of primary and early germ cells is of the greatest interest [58, 59]. With further development, they can form a significant population of transformed mature germ cells [60].

PGCs in the process of embryogenesis can differentiate into both male and female germ cells, which significantly expands the possibilities for realizing the potential of PGCs when creating genetically modified and chimeric individuals with desired properties. In avian embryos, primordial germ cells form in the epiblast and migrate through the hypoblast into the blood, then into the gonads [61]. With the introduction of donor PGCs into the dorsal aorta of recipient embryos during the period of migration of own PGCs from the blood into the gonads, colonization of the recipient gonads by donor cells is possible.

Spermatogonia serve as precursors of male reproductive cells (56). Of greatest interest are type A spermatogonia, which are classified as testicular stem cells. The unique property of self-renewal opens up wide opportunities for realizing the potential of these cells when breeding genetically modified poultry. Spermatogonia form a small population of cells located on the basement membrane of the seminiferous tubules. The process of their repeated self-renewal and further differentiation ensures the continuity of spermatogenesis with the formation of sperm, the highly specialized germ cells. Spermatogonia are the most resistant to various damaging factors (often only these cells survive, while the rest of the cells of the spermatogenic epithelium die) and undergo constant replication, maintaining their numbers during a process called renewal of the composition of stem cells.

Currently, approaches have been developed and optimized for obtaining and cultivating embryonic [62, 63] and spermatogenic [64, 65] avian cells. The

efficiency of genetic transformation of these target cells using various gene delivery systems, such as electroporation [66, 67], nucleofection [68], liposomal transfection [69, 70], the use of retroviral [71, 72] and lentiviral vectors [28, 73, 74], cationic polymers [30, 57], transposons [68, 75, 76].

PGCs can be transformed in two ways: in culture in vitro and in vivo by introducing genetic constructs into the dorsal aorta of embryos during the period of migration of their own PGCs into the gonads. Along with the traditional methods of transfection of cells in culture in vitro, the electroporation and lipofection, a number of works present the results of genetic modification of PGCs using other methods of gene delivery. Macdonald et al. [75] used Tol2 and piggyBac transposons to transfect chicken PGCs in vitro. The efficiency of transfection of target cells was 5.4 and 25.5%, respectively. The formation of functional gametes from transformed donor cells was shown, and transgenic progeny were obtained from primary germline chimeras. Naito et al. [68] obtained and transformed in vitro by nucleofection a culture of chicken PGCs with an efficiency of 10%. The transformed PZK culture was introduced into recipient embryos. From the birds bred after these manipulations, offspring were obtained. The presence of *GFP* was found in 1 out of 270 individuals.

There are a number of reports on the efficiency of transformation of PGCs in vivo to obtain germline chimeras. Zhang et al. [28] proposed a simple and effective way to create transgenic quails by injecting a lentiviral vector containing the *eGFP* reporter gene into the dorsal aorta of embryos. Out of 80 embryos, the authors obtained 48 G_0 chimeras (60%). The presence of *eGFP* was confirmed in most organs and tissues of the chimeric bird, including the germ cells of males. The efficiency of obtaining transgenic offspring from chimeric males reached 13%. Tyack et al. [69] and Lambeth et al. [76] for the genetic transformation of chicken PGCs in vivo, recombinant DNA was injected in combination with Lipofectamine 2000 and the Tol2 transposon directly into the dorsal aorta of chicken embryos. Germline F0 chimeras and transgenic progeny expressing integrated recombinant genes were obtained.

Jiang et al. [73] used a lentiviral vector conjugated with antibodies to SSEA4 (stage-specific embryonic antigen-4) specific to PGC membrane proteins to increase the efficiency of PGC transfection in vitro and in vivo. The proposed approach made it possible to increase the target efficiency of transduction of avian cells by 30.0-46.7%. In 50.0-66.7% of embryos, GFP expression occurred in the gonads.

Transformation of avian spermatogenic cells, as well as PGC, can be carried out in culture in vitro and in vivo by introducing genetic constructs into the parenchyma of the testes of males. In the latter case, as a rule, viral vectors are used. A number of studies have considered the possibility of using non-viral gene delivery systems. Min et al. [30] and Li et al. [57] studied the efficacy of the cationic polymer SofastTM in transforming rooster spermatogenic cells in vivo. This drug, in combination with the genetic construct, was injected directly into the parenchyma of the testis. Min et al. [30] bred avian influenza resistant chickens. The efficiency of transformation of spermatogenic cells was 72.2%. The transgene was present in 10% of the spermatozoa and in the blood of 7.8% of the F_1 offspring. Li et al. [57] used a genetic construct encoding the *GFP* reporter gene for the genetic transformation of spermatogenic cells. With its introduction in combination with a cationic polymer into the testes of roosters, the efficiency of target cell transformation reached 19.1%.

Thus, the technology for creating genetically modified individuals using PGCs and spermatogonia as donor cells involves their isolation, transformation,

and transplantation into recipient gonads, followed by the production of offspring with introduced traits [77, 78]. The efficiency of colonization of donor cells into recipient gonads has been shown in a number of studies using both donor PGCs [78, 79] and spermatogonia [32, 81, 82]. The preliminary treatment of recipients aimed at eliminating their own germ or spermatogenic cells in the gonads under the influence of gamma radiation The efficiency of transplantation of donor PGCs and spermatogonia can be increased by [83, 84] or chemical sterilization [85, 86]. In the latter case, busulfan is effective, which is an alkylating agent that causes DNA damage in target cells, which leads to the shutdown of all cellular mechanisms and cell destruction.

Table 1 summarizes the main methodological approaches currently used for the genetic modification of avian cells. Below, their effectiveness in editing the genome of poultry in in vitro and in vivo systems is considered.

1. Main methods to genetically modify poultry cells

Gene construct introduction	Target cells	Trasfection method	References
Direct introduction into an embryo or into organs or tissues of adults	Blastodermal cell	Viral vectors, lipofection	[27, 34, 47]
	Primordial germ cells	Viral vectors, transposons, lipofection	[28, 69, 76]
	Oviduct cells, spermatogenic testis cells	Viral vectors	[29, 74, 30]
Transfection of target donor cells in vitro followed by transplantation to recipients	Blastodermal cells, primordial germ cells, spermatogonia	Viral vectors, lipofection, electroporation, nucleofection, transposons	[31, 32, 87, 88]
Transformation of spermatozoa followed by female insemination	Sperm	Lipofection, electroporation	[33]

Genome editing of poultry. A number of successful experiments reported on the modification of avian cells using various editing systems to knockout of individual genes [89]. Functions of a number of genes associated with the biology of embryonic development and the pathogenesis of embryonic diseases [90], gametogenesis [91], and resistance to infectious diseases [92] have been studied on chicken cell lines DF-1 and DT-40. Methodological approaches to introducing mutations (knockout) into target genes [93], including those related to growth, development, and productive qualities, have been developed and optimized [94].

Abu-Bonsrah et al. [90] obtained two chicken cell lines with a knockout of the *HIRA*, *TYRP1*, *DICER*, *MBD3*, *EZH2*, and *RET* genes using the CRISPR/Cas9 system. It has been shown that using this editing system, it is possible to introduce a deletion larger than 75 kb into the target gene sequence. Through in vivo electroporation of chicken embryos, genetic changes were made to the *DGCR8* gene sequence in nerve cells. In genetically modified cells, there was a decrease in the expression of DGCR8 and the associated genes *Drosha*, *YPEL1*, and *Ngn2*. Morphological differences in the structure of the nervous tissue and cardiac muscle in transfected embryos were noted.

Zhang et al. [91] studied the effect of the *Stra8* gene on the differentiation of embryonic stem cells in spermatogonia. For this purpose, the Cas9/gRNA plasmid was introduced into DF-1 cells and embryonic stem cells. The efficiency of introducing mutations into the target gene was 25% in DF-1 cells and 23% in embryonic stem cells. It has been shown that Stra8 gene knockout blocks the differentiation of embryonic stem cells in spermatogonia in vitro. Y. Bai et al. (93) used the CRISPR/Cas9 system to introduce genetic changes in the *PPARG* and *ATP5F1E* ovalbumin gene sequences in the DF-1 chicken cell line. The mutation frequency varied from 0.5 to 3.0%. Cultivation of cells after transfection on a selective medium containing puromycin increased the efficiency of selection of genetically modified cells up to 95%. Lee et al. [94] on the DF-1 cell line considered

the possibility of using the Cas9-D10A nickase to introduce site-specific mutations in the target region of the target DNA. The myostatin gene was chosen as the target. Genotyping of the transfected cells confirmed the presence of mutations at the target site of the target DNA. The size of the introduced deletions varied from 2 to 39 nucleotides. At the same time, the analysis of six non-target sites did not reveal the presence of any non-specific mutations in them. In addition, there were no phenotypic differences between normal and modified cells. Western blotting did not show the presence of myostatin protein in the modified cells.

Along with reports on editing the genome of cell lines, there are a number of publications on the production of poultry with a knockout or gene insertion. The studies were carried out on chickens and quails. Schusser et al. [95], using PZK, bred chickens with a knockout of the immunoglobulin heavy chain gene by homologous recombination. Birds homozygous for the knockout of this gene did not synthesize antibodies and did not develop B-cells. At the same time, the migration of B-cell precursors into the bursa of Fabricius was preserved, while the formation of mature B-cells and their migration from the bursa of Fabricius were blocked. The development and functional activity of other types of cells of the immune system remained normal. Chickens with a knockout of the immunoglobulin heavy chain gene due to the lack of a peripheral population of B-cells serve as a unique experimental model for studying the immune response of birds to infectious diseases, and are also of interest for solving a number of problems in the field of virology and biology development and biotechnology. Dimitrov et al. [96] showed the possibility of modifying the chicken immunoglobulin heavy chain gene by in vitro PGC modification using the CRISPR/Cas9 system. As a result, four PGC lines were obtained, which were injected into the embryos. The efficiency of the transfer of the introduced modifications from the chimeric bird of the germ line to the offspring varied from 0 to 96%.

Using the TALEN editing system, Taylor et al. (97) bred chickens with a knockout of the *DDX4* locus on the Z sex chromosome to study the role of this gene in the formation of germ cells. The *DDX4* gene is a key determinant of germ cells in many animal species. It is supposed to control the formation of germ cells in birds. The effectiveness of his knockout in the PZK of chickens was 8.1%. Large deletions of 30 kb were introduced spanning the entire *DDX4* locus. After in vitro editing, PGCs were injected into recipient embryos and a chimeric germline bird was obtained. The offspring from this bird were homozygous for the knockout of the *DDX4* gene. In individuals, the initiation and development of PGCs in the gonads of embryos was noted, however, with the onset of meiosis, the development of reproductive cells was blocked, leading to infertility in females.

Knockout of egg protein genes is considered as an opportunity to reduce the allergenicity of chicken eggs. This is especially true in the production of products for persons sensitive to egg white. In 2014, Park et al. [98[obtained ovalbumin knockout chickens by genetically modifying PGCs with the TALEN editing system. Deletions were introduced into the target gene, which led to a shift in the reading frame and, as a result, to the shutdown of the function of the ovalbumin gene. Oishi et al. [99] created the ovomucin (*OVM*) gene knockout chickens. PGCs transfected in in vitro culture and transplanted into recipient embryos were used as target cells for genome editing with the CRISPR/Cas9 system. G_0 chimeras were used for subsequent crosses with the selection of G_2 chickens homozygous for the *OVM* gene knockout. Two out of three chimeric G_0 roosters produced offspring with a deletion in the *OVM* gene.

Later, the same scientific group, using a similar approach, bred chickens producing human beta-interferon (hIFN-β) by incorporating the *hIFN-β* gene into

the ovalbumin gene locus [100]. Such a bird produced 3.5 mg/ml hIFN-β in egg white. Females (unlike males) turned out to be infertile. The bioactivity and production of the recombinant hIFN-β protein in the offspring remained at the level of previous generations, which confirms the prospects of including the target genes in the ovalbumin gene locus of chickens to create individuals producing recombinant proteins in egg white for industrial use.

Qin et al. [101] evaluated the effectiveness of using an adenoviral vector to deliver the CRISPR/Cas9 system to chicken cells to knock out the ovalbumin (*OV*) gene and integrate the human epidermal growth factor (*hEGF*) gene into this locus. The efficiency of the *OV* gene knockout and the expression of the integrated *hEGF* gene was shown in a culture of primary chicken oviduct cells. The biological activity of the secreted hEGF protein was confirmed on Hela cells: cell proliferation when this protein was included in the cultivation medium corresponded to those established for the commercial hEGF preparation. The *OV* gene knockout was also carried out with the integration of the *hEGF* gene in blastoderm cells in vitro and in vivo. Chicken embryos with introduced genetic changes in the cells of the gonads were obtained. The efficiency of obtaining such embryos was higher with transplantation of in vitro modified blastoderm cells into the germinal disc of recipient embryos than with direct injection of the adenovirus vector into embryos in vivo. The proportion of modified germ cells in the gonads of embryos was also higher when using in vitro modified blastodermal cells.

A number of papers report on the successful editing of the genome of chickens and quails with myostatin gene knockout (*MSTN*). The protein myostatin inhibits the growth and development of muscle tissue. Knockout of the *MSTN* gene is of interest in creating lines with an increased growth rate of muscle tissue. G.-D. Kim et al. [102] obtained chickens with *MSTN* gene knockout by inserting an editing system into the PGC. The D10A-Cas9 nickase was used to introduce deletions into the target region of the target DNA. After the introduction of in vitro modified PGCs into embryos, deletions from 5 to 39 nucleotides in the MSTN gene locus were identified in 7 out of 52 chickens. This bird was further crossed in order to breed chickens homozygous for MSTN gene knockout. The features of growth and development of muscle tissue were studied. MSTN knockout birds showed a continuous increase in body weight up to 18 weeks of age, while in unmodified birds, the growth rate decreased after 13 weeks. A comparative assessment of meat productivity indicators revealed an increase in the mass of legs by 55.3% in individuals with a knockout of the *MSTN* gene compared to the control. At the same time, the mass of abdominal fat was 77.1% lower. Comparison of the mass of internal organs, including the heart, spleen, stomach and liver, did not reveal significant differences between genetically modified and unmodified chickens.

Lee et al. [103] produced myostatin gene *MSTN* knockout quails by injecting a recombinant adenovirus containing CRISPR/Cas9 into the germinal disc (blastoderm cells). In the birds, 3 bp deletions were identified. The mutation did not cause a frameshift and resulted in a cysteine deletion in the *MSTN* propeptide region. In quails homozygous for *MSTN* gene knockout, there was a significant increase in body weight and muscle tissue with muscle hyperplasia compared with quails heterozygous for *MSTN* gene knockout and wild type. In addition, in individuals with a knockout of the *MSTN* gene, the proportion of abdominal fat decreased and the mass of the heart increased compared to wild-type quails. The same scientific group bred quails with a knockout of the melanophilin *MLPH* gene associated with feather pigmentation (104). An adenovirus vector containing components of the CRISPR/Cas9 system was introduced into the subembryonic cavity

of the blastoderm of the embryos. Of the 100 injected embryos, 11 quails were obtained, of which five carried a mutation in the *MLPH* gene in reproductive cells. The efficiency of mutation transfer to offspring varied from 2.4 to 10.0%. In the offspring of one modified F0 bird, two different mutations were identified at the *MLPH* locus. Differences in the phenotype of modified quails with *MLPH* gene knockout were established. Quails homozygous for the *MLPH* gene knockout had gray plumage, while quails heterozygous for the introduced mutation and the wild type had dark brown plumage.

Along with the use of genomic editing technology to improve economically useful traits in agricultural poultry, it is of interest to create individuals resistant to infectious diseases, such as avian leukosis virus (ALV). This disease is difficult to control and prevent due to the lack of effective vaccines. There are several subgroups of ALVs. Hellmich et al. [12] attempted to develop chickens resistant to subgroup J of the avian leukemia virus (ALV-J), which causes myeloid leukemia and tumor formation. For this purpose, a deletion for tryptophan 38 (W38) was introduced into the c*hNHE1* locus using the CRISPR/Cas9 system. The W38 amino acid in chNHE1 is critical for virus entry into the cell, making it a preferred knockout target to increase pathogen resistance. The genetic modification introduced into the chicken genome completely protected the cells from infection with the ALV-J virus. The W38 deletion did not have a significant negative impact on the development or general functional state of genetically modified individuals. In general, the creation of ALV-J resistant individuals through precise gene editing allows this approach to be considered as an alternative strategy for controlling poultry diseases.

Table 2 summarizes the main achievements in editing the genomes of different poultry species.

2. The main achievements in editing the genomes of different poultry species

Poultry species	Target gene	Target cells	Target cell transfection	Editing system	References
Chickens	*IgH*	PGCs	Electroporation	CRISPR/Cas9	[96]
	DDX4	ПЗК	Electroporation	TALEN	[97]
	OVM	ПЗК	Lipofection	CRISPR/Cas9	[99]
	hIFN-β	ПЗК	Lipofection	CRISPR/Cas9	[100]
	MSTN	ПЗК	Lipofection	D10A-Cas9B	[102]
	OV	ПЗК	Lipofection	TALEN	[98]
	chNHE1	ПЗК	Electroporation	CRISPR/Cas9	[12]
Quail	*MSTN*	Blastodermal cells	Adenoviral vector	CRISPR/Cas9	[103]
	MLPH	Blastodermal cells	Adenoviral vector	CRISPR/Cas9	[104]

N o t e. PGCs — primordial germ cells.

Thus, at present, some progress has been made in editing the genome of poultry. Methodological approaches and techniques for modifying avian cells using various gene editing systems, in particular ZFN, TALEN, CRISPR/Cas9, have been developed and optimized. Chickens and quails have been bred with a knockout of a number of genes in order to study their functions, improve the productive qualities of poultry, increase resistance to infectious diseases, and obtain recombinant proteins in egg protein. A number of studies have shown the simplicity, safety, and availability of the CRISPR/Cas9 editing system for modifying the poultry genome, which makes it possible to consider this system as an effective tool for the creation and commercial use of bird breeds and lines with improved qualities.

REFERENCES

1. Ivarie R. Avian transgenesis: progress towards the promise. *Trends in Biotechnology*, 2003, 21(1): 14-19 (doi: 10.1016/S0167-7799(02)00009-4).
2. Amro W.A., Al-Qaisi W., Al-Razem F. Production and purification of IgY antibodies from

chicken egg yolk. *Journal of Genetic Engineering and Biotechnology*, 2018, 16(1): 99-103 (doi: 10.1016/j.jgeb.2017.10.003).
3. Lillico S.G., McGrew M.J., Sherman A., Sang H.M. Transgenic chickens as bioreactors for protein-based drugs. *Drug Discovery Today*, 2005, 10(3): 191-196 (doi: 10.1016/S1359-6446(04)03317-3).
4. Stern C.D. The marginal zone and its contribution to the hypoblast and primitive streak of the chick embryo. *Development*, 1990, 109: 667-682.
5. Mozdziak P.E., Petitte J.N. Status of transgenic chicken models for developmental biology. *Developmental Dynamics*, 2004, 229(3): 414-421 (doi: 10.1002/dvdy.10461).
6. Cooper C.A., Doran T.J., Challagulla A., Tizard M.L.V., Jenkins K.A. Innovative approaches to genome editing in avian species. *Journal of Animal Science and Biotechnology*, 2018, 9: 15 (doi: 10.1186/s40104-018-0231-7).
7. Bahrami S., Amiri-Yekta A., Daneshipour A., Jazayeri S.H., Mozdziak P.E., Sanati M.H., Gourabi H. Designing a transgenic chicken: applying new approaches toward a promising bioreactor. *Cell Journal*, 2020, 22(2): 133-139 (doi: 10.22074/cellj.2020.6738).
8. 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).
9. Ahn J., Lee J., Park J.Y., Oh K.B., Hwang S., Lee C.-W., Lee K. Targeted genome editing in a quail cell line using a customized CRISPR/Cas9 system. *Poultry Science*, 2017, 96(1): 1445-1450 (doi: 10.3382/ps/pew435).
10. Bhattacharya T.K., Shukla R., Chatterjee R.N., Bhanja S.K. Comparative analysis of silencing expression of myostatin (*MSTN*) and its two receptors (*ACVR2A* and *ACVR2B*) genes affecting growth traits in knock down chicken. *Scientific Reports*, 2019, 9: 7789 (doi: 10.1038/s41598-019-44217-z).
11. Koslová A., Trefil P., Mucksová J., Reinišová M., Plachý J., Kalina J., Kučerová D., Geryk J., Krchlíková V., Lejčková B., Hejnar J. Precise CRISPR/Cas9 editing of the *NHE1* gene renders chickens resistant to the J subgroup of avian leukosis virus. *Proceedings of the National Academy of Sciences*, 2020, 117(4): 2108-2112 (doi: 10.1073/pnas.1913827117).
12. Hellmich R., Sid H., Lengyel K., Flisikowski K., Schlickenrieder A., Bartsch D., Thoma T., Bertzbach L.D., Kaufer B.B., Nair V., Preisinger R., Schusser B. Acquiring resistance against a retroviral infection via CRISPR/Cas9 targeted genome editing in a commercial chicken line. *Frontiers in Genome Editing*, 2020, 2: 3 (doi: 10.3389/fgeed.2020.00003).
13. Petersen B. Basics of genome editing technology and its application in livestock species. *Reproduction in Domestic Animals*, 2017, 52(S3): 4-13 (doi: 10.1111/rda.13012).
14. Sander J.D., Joung J.K. CRISPR-Cas systems for genome editing, regulation and targeting. *Nature Biotechnology,* 2014, 32(4): 347-355 (doi: 10.1038/nbt.2842).
15. Panda S.K., Wefers B., Ortiz O., Floss T., Schmid B., Haass C., Wurst W., Kühn R. Highly efficient targeted mutagenesis in mice using TALENs. *Genetics,* 2013, 195(3): 703-713 (doi: 10.1534/genetics.113.156570).
16. Chu V.T., Weber T., Wefers B., Wurst W., Sander S., Rajewsky K., Kühn R. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nature Biotechnology*, 2015, 33: 543-548 (doi: 10.1038/nbt.3198).
17. Wefers B., Panda S.K., Ortiz O., Brandl C., Hensler S., Hansen J., Wurst W., Kühn R. Generation of targeted mouse mutants by embryo microinjection of TALEN mRNA. *Nature Protocols*, 2013, 8: 2355-2379 (doi: 10.1038/nprot.2013.142).
18. Menzorov A.G., Luk'yanchikova V.A., Korablev A.N., Serova I.A., Fishman V.S. *Vavilovskii zhurnal genetiki i selektsii*, 2016, 20(6): 930-944 (doi: 10.18699/VJ16.214) (in Russ.).
19. Barrangou R., Fremaux C., Deveau H., Richards M., Boyaval P., Moineau S., Romero D., Horvath P. CRISPR provides acquired resistance against viruses in prokaryotes. *Science*, 2007, 315(5819): 1709-1712 (doi: 10.1126/science.1138140).
20. Cong L., Ran F. A., Cox D., Lin S., Barretto R., Habib N., Hsu P.D., Wu X., Jiang W., Marraffini L. A., Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science*, 2013, 339(6121): 819-823 (doi: 10.1126/science.1231143).
21. Anders C., Niewoehner O., Duerst A., Jinek M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature*, 2014, 513: 569-573 (doi: 10.1038/nature13579).
22. Gonatopoulos-Pournatzis T., Aregger M., Brown K.R., Farhangmehr S., Braunschweig U., Ward H.N., Ward H.N., Ha K.C.H., Weiss A., Billmann M., Durbic T., Myers C.L., Blencowe B.J., Moffat J. Genetic interaction mapping and exon-resolution functional genomics with a hybrid Cas9-Cas12a platform. *Nature Biotechnology*, 2020, 38: 638-648 (doi: 10.1038/s41587-020-0437-z).
23. Najm F.J., Strand C., Donovan K.F., Hegde M., Sanson K.R., Vaimberg E.W., Sullender M.E., Hartenian E., Kalani Z., Fusi N., Listgarten J., Younger S.T., Bernstein B.E., Root D.E., Doen J.G. Orthologous CRISPR-Cas9 enzymes for combinatorial genetic screens. *Nature Biotechnology*, 2018, 36(2): 179-189 (doi: 10.1038/nbt.4048).
24. Pennisi E. The CRISPR craze. *Science*, 2013, 341(6148): 833-836 (doi: 10.1126/science.341.6148.833).

25. Wilson L.O.W., Reti D., O'Brien A.R., Dunne R.A., Bauer D.C. High activity target-site identification using phenotypic independent CRISPR-Cas9 core functionality. *The CRISPR Journal*, 2018, 1(2): 182-190 (doi: 10.1089/crispr.2017.0021).
26. Salsman J., Dellaire G. Precision genome editing in the CRISPR era. *Biochemistry and Cell Biology*, 2017, 95: 187-201 (doi: 10.1139/bcb-2016-0137).
27. Love J., Gribbin C., Mather C., Sang H. Transgenic birds by DNA microinjection. *Nat. Biotechnol.*, 1994, 12(1): 60-63 (doi: 10.1038/nbt0194-60).
28. Zhang Z., Sun P., Yu F., Yan L., Yuan F., Zhang W., Wang T., Wan Z., Shao Q., Li Z. Transgenic quail production by microinjection of lentiviral vector into the early embryo blood vessels. *PLoS ONE,* 2012, 7(12): e50817 (doi: 10.1371/journal.pone.0050817).
29. Beloglazov D.V., Volkova N.A., Volkova L.A., Zinov'eva N.A. Efficiency of local transgenesis of the oviductal cells in chicken as influenced by hormonal stimulation. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2015, 50(6): 729-735 (doi: 10.15389/agrobiology.2015.6.729eng).
30. Min S., Qing S.Q., Hui Y.Y., Zhi F.D., Rong Q.Y., Feng X., Chun L.B. Generation of antiviral transgenic chicken using spermatogonial stem cell transfected in vivo. *African Journal of Biotechnology*, 2011, 10(70): 15678-15683 (doi: 10.5897/AJB11.040).
31. van de Lavoir M.-C., Diamond J.H., Leighton P.A., Mather-Love C., Heyer B.S., Bradshaw R., Kerchner A., Hooi L.T., Gessaro T.M., Swanberg S.E., Delany M.E., Etches R.J. Germline transmission of genetically modified primordial germ cells. *Nature*, 2006, 441: 766-769 (doi: 10.1038/nature04831).
32. Benesova B., Mucksova J., Kalina J., Trefil P. Restoration of spermatogenesis in infertile male chickens after transplantation of cryopreserved testicular cells. *British Poultry Science*, 2014, 55(6): 837-845 (doi: 10.1080/00071668.2014.974506).
33. 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).
34. 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).
35. Chen H.Y., Garber E.A., Mills E., Smith J., Kopchick J.J., Dilella A.G., Smith R.G. Vectors, promoters, and expression of genes in chick embryos. *Journal of Reproduction and Fertility. Supplement*, 1990, 41: 173-182.
36. Bosselman R.A., Hsu R.Y., Boggs T., Hu S., Bruszewski J., Ou S., Kozar L., Martin F., Green C., Jacobsen F., Nicolson 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).
37. Mozdziak P.E., Borwornpinyo S., McCoy D.W., Petitte J.N. Development of transgenic chickens expressing bacterial beta-galactosidase. *Developmental Dynamics*, 2003, 226(3): 439-445 (doi: 10.1002/dvdy.10234).
38. Mizuarai S., Ono K., Yamaguchi K., Nishijima M., Kamihira M., Iijima S. Production of transgenic quails with high frequency of germ-line transmission using VSV-G pseudotyped retroviral vector. *Biochemical and Biophysical Research Communications*, 2001, 286(3): 456-463 (doi: 10.1006/bbrc.2001.5422).
39. 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).
40. Harvey A.J., Ivarie R. Validating the hen as a bioreactor for the production of exogenous proteins in egg white. *Poultry Science*, 2003, 82(6): 927-930 (doi: 10.1093/ps/82.6.927).
41. Harvey A.J., Speksnijder G., Baugh L.R., Morris J.A., Ivarie R. Expression of exogenous protein in the egg white of transgenic chickens. *Nature Biotechnology*, 2002, 20(4): 396 (doi: 10.1038/nbt0402-396).
42. Smith C.A., Roeszler K.N., Sinclair A.H. Robust and ubiquitous GFP expression in a single generation of chicken embryos using the avian retroviral vector, RCASBP. *Differentiation*, 2009, 77(5): 473-482 (doi: 10.1016/j.diff.2009.02.001).
43. Kamihira M., Ono K., Esaka K., Nishijima K., Kigaku R., Komatsu H., Yamashita T., Kyogoku K., Iijima S. High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector. *Journal of Virology*, 2005, 79(17): 10864-10874 (doi: 10.1128/JVI.79.17.10864-10874.2005).
44. Kodama D., Nishimiya D., Nishijima K., Okino Y., Inayoshi Y., Kojima Y., Ono K., Motono M., Miyake K., Kawabe Y., Kyogoku K., Yamashita T., Kamihira M., Iijima S. Chicken oviduct-specific expression of transgene by a hybrid ovalbumin enhancer and the Tet expression system. *Journal of Bioscience and Bioengineering*, 2012, 113(2): 146-153 (doi: 10.1016/j.jbiosc.2011.10.006).

45. Rapp J.C., Harvey A.J., Speksnijder G.L., Hu W., Ivarie R. Biologically active human interferon a-2b produced in the egg white of transgenic hens. *Transgenic Research*, 2003, 12(5): 569-575 (doi: 10.1023/A:1025854217349).
46. Scott B.B., Velho T.A., Sim S., Lois C. Applications of avian transgenesis. *ILAR Journal*, 2010, 51(4): 353-361 (doi: 10.1093/ilar.51.4.353).
47. McGrew M.J., Sherman A., Ellard F.M., Lillico S.G., Gilhooley H.J., Kingsman A.J., Mitrophanous K.A., Sang H. Efficient production of germline transgenic chickens using lentiviral vectors. *EMBO Reproduction*, 2004, 5(7): 728-733 (doi: 10.1038/sj.embor.7400171).
48. Lillico S.G., Sherman A., McGrew M.J., Robertson C.D., Smith J., Haslam C., Barnard P., Radcliffe P.A., Mitrophanous K.A., Elliot E.A., Sang H.M. Oviduct-specific expression of two therapeutic proteins in transgenic hens. *Proceedings of the National Academy of Sciences*, 2007, 104(6): 1771-1776 (doi: 10.1073/pnas.0610401104).
49. Chapman S.C., Lawson A., Macarthur W.C., Wiese R.J., Loechel R.H., Burgos-Trinidad M., Wakefield J.K., Ramabhadran R., Mauch T.J., Schoenwolf G.C. Ubiquitous GFP expression in transgenic chickens using a lentiviral vector. *Development*, 2005, 132(5): 935-940 (doi: 10.1242/dev.01652).
50. Byun S.J., Kim S.W., Kim K.W., Kim J.S., Hwang I.S., Chung H.K., Kan I.S., Jeon I.S., Chang W.K., Park S.B., Yoo J.G. Oviduct-specific enhanced green fluorescent protein expression in transgenic chickens. *Bioscience, Biotechnology, and Biochemistry*, 2011, 75(4): 646-649 (doi: 10.1271/bbb.100721).
51. Kwon S.C., Choi J.W., Jang H.J., Shin S.S., Lee S.K., Park T.S., Choi I.Y., Lee G.S., Song G., Han J.Y. Production of biofunctional recombinant human interleukin 1 receptor antagonist (rhIL1RN) from transgenic quail egg white. *Biology of Reproduction*, 2010, 82(6): 1057-1064 (doi: 10.1095/biolreprod.109.081687).
52. Cao D.N., Wu H.Y., Li Q.Y., Sun Y.M., Liu T.X., Fei J., Zhao Y.F., Wu S., Hu X.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).
53. Liu T.X., Wu H.Y., Cao D.N., Li Q.Y., Zhang Y.Q., Li N., Hu X.X. Oviduct-specific expression of human neutrophil defensin 4 in lentivirally generated transgenic chickens. *PLoS ONE*, 2015, 10(5): e0127922 (doi: 10.1371/journal.pone.0127922).
54. Chojnacka-Puchta L., Kasperczyk K., Płucienniczak G., Sawicka D., Bednarczyk M. Primordial germ cells (PGCs) as a tool for creating transgenic chickens. *Polish Journal of Veterinary Sciences*, 2012, 15(1): 181-188 (doi: 10.2478/v10181-011-0132-6).
55. Macdonald J., Glover J.D., Taylor L., Sang H.M., McGrew M.J. Characterisation and germline transmission of cultured avian primordial germ cells. *PLoS ONE*, 2010, 5(11): e15518 (doi: 10.1371/journal.pone.0015518).
56. Zheng Y., Zhang Y., Qu R., He Y., Tian X., Zeng W. Spermatogonial stem cells from domestic animals: progress and prospects. *Reproduction*, 2014, 147(3): 65-74 (doi: 10.1530/REP-13-0466).
57. Li B., Sun G., Sun H., Xu Q., Gao B., Zhou G., Zhau W., Wu X., Bao W., Yu F., Wang K., Chen G. Efficient generation of transgenic chickens using the SSCs in vivo and ex vivo transfection. *Science China Life Sciences*, 2008, 51: 734-742 (doi: 10.1007/s11427-008-0100-2).
58. Han J.Y. Germ cells and transgenesis in chickens. *Comparative Immunology, Microbiology & Infectious Diseases*, 2009, 32(2): 61-80 (doi: 10.1016/j.cimid.2007.11.010).
59. Nakamura Y., Usui F., Miyahara D., Mori T., Ono T., Takeda K., Nira-sawa K., Kagami H., Tagami T. Efficient system for preservation and regeneration of genetic resources in chicken: concurrent storage of primordial germ cells and live animals from early embryos of a rare indigenous fowl (Gifujidori). *Reproduction, Fertility and Development*, 2010, 22(8): 1237-1246 (doi: 10.1071/RD10056).
60. Takashima S. Biology and manipulation technologies of male germline stem cells in mammals. *Reproductive Medicine and Biology*, 2018, 17(4): 398-406 (doi: 10.1002/rmb2.12220).
61. Eyal-Giladi H., Kochav S. From cleavage to primitive streak formation: a com-plementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Developmental Biology*, 1976, 49(2): 321-337 (doi: 10.1016/0012-1606(76)90178-0).
62. Zhao D.-F., Kuwana T. Purification of avian circulating primordial germ cells by nycodenz density gradient centrifugation. *British Poultry Science*, 2003, 44(1): 30-35 (doi: 10.1080/0007166031000085382).
63. Mozdziak P.E., Angerman-Stewart J., Rushton B., Pardue S.L., Petitte J.N. Isolation of chicken primordial germ cells using fluorescence-activated cell sorting. *Poultry Science*, 2005, 84(4): 594-600 (doi: 10.1093/ps/84.4.594).
64. Sisakhtnezhad S., Bahrami A.R., Matin M.M., Dehghani H., Momeni-Moghaddam M., Boozarpour S., Farshchian M., Dastpak M. The molecular sig-nature and spermatogenesis potential of newborn chicken spermatogonial stem cells in vitro. *In Vitro Cellular & Developmental Biology — Animal*, 2015, 51: 415-425 (doi: 10.1007/s11626-014-9843-1).
65. Li B., Wang X.-Y., Tian Z., Xiao X.-J., Xu Q., Wei C.-X., Sun H.-C., Chen G.-H. Directional differentiation of chicken spermatogonial stem cells in vitro. *Cytotherapy*, 2010, 12(3): 326-331 (doi: 10.3109/14653240903518155).

66. Yu F., Ding L.-J., Sun G.-B., Sun P.-X., He X.-H., Ni L.-G., Li B.-C. Transgenic sperm produced by electrotransfection and allogeneic transplantation of chicken fetal spermatogonial stem cells. *Molecular Reproduction and Development*, 2010, 77(4): 340-347 (doi: 10.1002/mrd.21147).
67. Hong Y.H., Moon Y.K., Jeong D.K., Han J.Y. Improved transfection efficiency of chicken gonadal primordial germ cells for the production of transgenic poultry. *Transgenic Research*, 1998, 7(4): 247-252 (doi: 10.1023/A:1008861826681).
68. Naito M., Harumi T., Kuwana T. Long term in vitro culture of chicken primordial germ cells isolated from embryonic blood and incorporation into germline of recipient embryo. *Poultry Science*, 2010, 47(1): 57-64 (doi: 10.2141/jpsa.009058).
69. Tyack S.G., Jenkins K.A., O'Neil T.E., Wise T.G., Morris K.R., Bruce M.P., McLeod S., Wade A.J., McKay J., Moore R.J., Schat K.A., Lowenthal J.W., Doran T.J. A new method for producing transgenic birds via direct in vivo transfection of primordial germ cells. *Transgenic Research*, 2013, 22(6): 1257-1264 (doi: 10.1007/s11248-013-9727-2).
70. Sawicka D., Chojnacka-Puchta L. Effective transfection of chicken primordial germ cells (PGCs) using transposon vectors and lipofection. *Folia Biologica*, 2019, 67(1): 45-52 (doi: 10.3409/fb_67-1.04).
71. Kalina J., Senigl F., Micáková A., Mucksová J., Blazková J., Yan H., Poplstein M., Hejnar J., Trefil P. Retrovirus-mediated in vitro gene transfer into chicken male germ line cells. *Reproduction*, 2007, 134(3): 445-453 (doi: 10.1530/rep-06-0233).
72. Allioli N., Thomas J.-L., Chebloune Y., Nigon V.-M., Verdier G., Legras C. Use of retroviral vectors to introduce and express the β-galactosidase marker gene in cultured chicken primordial germ cell. *Developmental Biology*, 1994, 165(1): 30-37 (doi: 10.1006/dbio.1994.1231).
73. Jiang Z.-Q., Wu H.-Y., Tian J., Li N., Hu X.-X. Targeting lentiviral vectors to primordial germ cells (PGCs): An efficient strategy for generating transgenic chickens. *Zoological Research*, 2020, 41(3): 281-291 (doi: 10.24272/j.issn.2095-8137.2020.032).
74. Vetokh A.N., Volkova L.A., Iolchiev B.S., Tomgorova E.K., Volkova N.A. Genetic modification of roosters' germ cells using various methodological approaches. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2020, 55(2): 306-314 (doi: 10.15389/agrobiology.2020.2.306eng).
75. Macdonald J., Taylor L., Sherman A., Kawakami K., Takahashi Y., Sang H.M., McGrew M.J. Efficient genetic modification and germ-line transmission of primordial germ cells using piggyBac and Tol2 transposons. *Proceedings of the National Academy of Sciences*, 2012, 109(23): E1466-E1472 (doi: 10.1073/pnas.1118715109).
76. Lambeth L.S., Morris K.R., Wise T.G., Cummins D.M., O'Neil T.E., Cao Y., Sinclair A.H., Doran T.J., Smith C.A. Transgenic chickens overexpressing aromatase have high estrogen levels but maintain a predominantly male phenotype. *Endocrinology*, 2016, 157(1): 83-90 (doi: 10.1210/en.2015-1697).
77. Zhao R., Zuo Q., Yuan X. Jin K., Jin J., Ding Y., Zhang C., Li T., Jiang J., Li J., Zhang M., Shi X., Sun H., Zhang Y., Xu Q., Chang G., Zhao Z., Li B., Wu X., Zhang Y., Song J., Chen G., Li B. Production of viable chicken by allogeneic transplantation of primordial germ cells induced from somatic cells. *Nature Communications*, 2021, 12: 2989 (doi: 10.1038/s41467-021-23242-5).
78. Woodcock M.E., Gheyas A.A., Mason A.S., Nandi S., Taylor L., Sherman A., Smith J., Burt D.W., Hawken R., McGrew M.J. Reviving rare chicken breeds using genetically engineered sterility in surrogate host birds. *Proceedings of the National Academy of Sciences*, 2019, 116(42): 20930-20937 (doi: 10.1073/pnas.190631611678).
79. Kang S.J., Choi J.W., Kim S.Y., Park K.J., Kim T.M., Lee Y.M., Kim H., Lim J.M., Han J.Y. Reproduction of wild birds via interspecies germ cell transplantation. *Biology of Reproduction*, 2008, 79(5): 931-937 (doi: 10.1095/biolreprod.108.069989).
80. Wernery U., Liu C., Baskar V., Guerineche Z., Khazanehdari K.A., Saleem S., Kinne J., Wernery R., Griffin D.K., Chang I.-K. Primordial germ cell-mediated chimera technology produces viable pure-line Houbara bustard off-spring: potential for repopulating an endangered species. *PLoS ONE*, 2010, 5(12): e15824 (doi: 10.1371/journal.pone.0015824).
81. Trefil P., Bakst M.R., Yan H., Hejnar J., Kalina J., Mucksová J. Restoration of spermatogenesis after transplantation of c-Kit positive testicular cells in the fowl. *Theriogenology*, 2010, 74(9): 1670-1676 (doi: 10.1016/j.theriogenology.2010.07.002).
82. Kim Y.M., Park J.S., Yoon J.W., Choi H.J., Park K.J., Ono T., Han J.Y. Production of germline chimeric quails following spermatogonial cell transplantation in busulfan-treated testis. *Asian Journal of Andrology*, 2018, 20(4): 414-416 (doi: 10.4103/aja.aja_79_17).
83. 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 Cellular & Developmental Biology*, 2010, 46: 828-833 (doi: 10.1007/s11626-010-9361-8).
84. Trefil P., Polak J., Poplstein M., Mikus T., Kotrbová A., Rozinek J. Preparation of fowl testes as recipient organs to germ-line chimeras by means of gamma-radiation. *British Poultry Science*, 2003, 44(4): 643-650 (doi: 10.1080/00071660310001616246).
85. Tagirov M., Golovan S. The effect of busulfan treatment on endogenous spermatogonial stem cells in immature roosters. *Poultry Science*, 2012, 91(7): 1680-1685 (doi: 10.3382/ps.2011-02014).

86. 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).
87. 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).
88. van de Lavoir M.C., Collarini E.J., Leighton P.A., Fesler J., Lu D.R., Harriman W.D., Thiyagasundaram T.S., Etches R.J. Interspecific germline transmission of cultured primordial germ cells. *PLoS ONE*, 2012, 7(5): e35664 (doi: 10.1371/journal.pone.0035664).
89. Han J.Y., Park Y.H. Primordial germ cell-mediated transgenesis and genome editing in birds. *Journal of Animal Science and Biotechnology*, 2018, 9: 19 (doi: 10.1186/s40104-018-0234-4).
90. 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 Report*, 2016, 6: 34524 (doi: 10.1038/srep34524).
91. Zhang Y., Wang Y., Zuo Q., Li D., Zhang W., Wang F., Ji Y., Jin J., Lu Z., Wang M., Zhang C., Li B. CRISPR/Cas9 mediated chicken Stra8 gene knockout and inhibition of male germ cell differentiation. *PLoS ONE*, 2017, 12(2): e0172207 (doi: 10.1371/journal.pone.0172207).
92. Koslová A., Kučerová D., Reinišová M., Geryk J., Trefil P., Hejnar J. Genetic resistance to avian leukosis viruses induced by CRISPR/Cas9 editing of specific receptor genes in chicken cells. *Viruses*, 2018, 10(11): 605 (doi: 10.3390/v10110605).
93. Bai Y., He L., Li P., Xu K., Shao S., Ren C., Liu Z., Wei Z., Zhang Z. Efficient genome editing in chicken DF-1 cells using the CRISPR/Cas9 system. *G3 Genes|Genomes|Genetics*, 2016, 6(4): 917-923 (doi: 10.1534/g3.116.027706).
94. Lee J.H., Kim S.W., Park T.S. Myostatin gene knockout mediated by Cas9-D10A nickase in chicken DF1 cells without off-target effect. *Asian-Australasian Journal of Animal Sciences*, 2017, 30(5): 743-748 (doi: 10.5713/ajas.16.0695).
95. Schusser B., Collarini E.J., Yi H., Izquierdo S. M., Fesler J., Pedersen D., Klasing, K. C. Kaspers B., Harriman W. D., van de Lavoir M.-C., Etches R.J., Leighton P.A. Immunoglobulin knockout chickens via efficient homologous recombination in primordial germ cells. *Proceedings of the National Academy of Sciences,* 2013, 110(50): 20170-20175 (doi: 10.1073/pnas.1317106110).
96. Dimitrov L., Pedersen D., Ching K.H., Yi H., Collarini E.J., Izquierdo S., van de Lavoir M.-C., Leighton P.A. Germline gene editing in chickens by efficient CRISPR-mediated homologous recombination in primordial germ cells. *PLoS ONE*, 2016, 11(4): e0154303 (doi: 10.1371/journal.pone.0154303).
97. Taylor L., Carlson D.F., Nandi S., Sherman A., Fahrenkrug S.C., McGrew M.J. Efficient TALEN-mediated gene targeting of chicken primordial germ cells. *Development*, 2017, 144(5): 928-934 (doi: 10.1242/dev.145367).
98. 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).
99. 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).
100. Oishi I., Yoshii K., Miyahara D., Tagami T. Efficient production of human interferon beta in the white of eggs from ovalbumin gene—targeted hens. *Scientific Reports*, 2018, 8: 10203 (doi: 10.1038/s41598-018-28438-2).
101. Qin X., Xiao N., Xu Y., Yang F., Wang X., Hu H., Liu Q., Cui K., Tang X. Efficient knock-in at the chicken ovalbumin locus using adenovirus as a CRISPR/Cas9 delivery system. *3 Biotech*, 2019, 9: 454 (doi: 10.1007/s13205-019-1966-3).
102. Kim G.-D., Lee J.H., Song S., Kim S.W., Han J.S., Shin S.P., Park B.-C., Park T.S. Generation of myostatin-knockout chickens mediated by D10A-Cas9 nickase. *FASEB*, 2020, 34(4): 5688-5696 (doi: 10.1096/fj.201903035R).
103. Lee J., Kim D.-H., Lee K. Muscle hyperplasia in Japanese quail by single amino acid deletion in MSTN propeptide. *International Journal of Molecular Sciences*, 2020, 21(4): 1504 (doi: 10.3390/ijms21041504).
104. Lee J., Ma J., Lee K. Direct delivery of adenoviral CRISPR/Cas9 vector into the blastoderm for generation of targeted gene knockout in quail. *Proceedings of the National Academy of Sciences*, 2019, 116(27): 13288-13292 (doi: 10.1073/pnas.1903230116).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1031-1048
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 636.39:575.17

doi: 10.15389/agrobiology.2021.6.1031eng
doi: 10.15389/agrobiology.2021.6.1031rus

GENETIC MARKERS OF GOATS
(review)

M.I. SELIONOVA[1] ✉, V.I. TRUKHACHEV[1], A-M.M. AYBAZOV[1], Yu.A. STOLPOVSKY[2], N.A. ZINOVIEVA[3]

[1]*Russian State Agrarian University — Timiryazev Moscow Agricultural Academy*, 49, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail m_selin@mail.ru (✉ corresponding author), rector@rgau-msha.ru, velikii-1@yandex.ru;
[2]*Vavilov Institute of General Genetics RAS*, 3, ul. Gubkina, Moscow, 119333 Russia, e-mail stolpovsky@mail.ru;
[3]*Ernst Federal Science Center for Animal Husbandry*, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail n_zinovieva@mail.ru
ORCID:
Selionova M.I. orcid.org/0000-0002-9501-8080
Trukhachev V.I. orcid.org/0000-0002-4650-1893
Aybazov A-M.M. orcid.org/0000-0002-3704-3210
Stolpovsky Yu.A. orcid.org/0000-0003-2537-1900
Zinovieva N.A. orcid.org/0000-0002-6926-2055
The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation (project No. 19-76-20006, analysis of SNP-markers and search for loci under selection pressure in the Karachaev goat genome; No. 21-76-20008, analysis of microsatellite and DNA markers of goat productivity)
Received October 1, 2021

Abstract

Goat biodiversity comprises 635 breeds from in 170 countries (https://www.fao.org/dad-is). Wide geographical distribution and positive dynamics of goat populations in recent decades are due to high adaptability to various climatic conditions and the uniqueness of goat products (I.N. Skidan et al., 2015; A.I. Erokhin et al., 2020). DNA microsatellite markers have been widely used to study genetic differentiation of goat breeds and populations in many countries (C. Wei et al., 2014; G. Mekuriaw et al., 2016). Insignificant genetic distances (F_{ST} 0.033-0.069) between goat breeds bred in Europe confirm the frequent exchange of the gene pool between them. A more significant genetic differentiation (F_{ST} 0.134-0.183) is characteristic of breeds from East and Southeast Asia due to the ecological and geographical features and the remoteness of their habitats (K. Nomura et al., 2012; G. Wang et al., 2017; P. Azhar et al., 2018). The *CSN1S1*, *CSN1S2*, *CSN2*, and *BLG* gene polymorphisms are of most interest in dairy goat breeding (N. Silanikove et al., 2010; Vorozhko I.V. et al., 2016). Eighteen allelic variants have been described in the *CSN1S1* gene, eight in *CSN2*, and 16 in *CSN3* (S. Ollier et al., 2008; T.G. Devold et al., 2010). The $CSN1S1^{AA}$ association with more protein in milk and less total lipids and medium chain fatty acids has been found (Y. Chilliard et al., 2006; D. Marletta et al., 2007). Goats with BLG^{AB} genotype have longer lactation period, produce more milk with higher fat and protein contents (A.S. Shuvarikov et al., 2019). The sequencing of the goat genome (the AdaptMap project) and the development of the 52K SNP BeadChipGoat chip has expanded the search for genome regions involved in breeding (G. Tosser-Klopp et al., 2014; A. Stella et al., 2018). There is evidence that the *RARA*, *STAT*, *PTX3*, *IL6*, *IL8*, and *DGAT1* genes are linked to dairy performance traits (P. Martin et al., 2018; D. Ilie et al., 2018). At the genomic level, the *MC1R*, *ASIP* and *KIT* are associated with wool fiber coloration, *FGF5*, *EPAS1* and *NOXA1* with wool productivity of goats and their high-altitude adaptation (X. Wang et al., 2016; S. Song et al., 2016; Guo J. et al., 2018). Thus, the evaluation of genetic relationships between breeds, the search for genes associated with economically important traits are promising for use in breeding programs and further development of goat breeding (L.F. Brito et al., 2016; S. Desire, 2016; A. Molina et al., 2018; T.E. Deniskova et al., 2020). However, despite certain achievements, until now, loci associated with economically important traits in goats, such as breeding characteristics, the level of down, wool and milk productivity, as well as determining resistance to diseases, remain largely unknown.

Keywords: goats, microsatellites, breeds, productivity, genetic differentiation, genetic markers, GWAS

Goat raising is a dynamically developing branch of animal husbandry. According to the FAO (Food and Agriculture Organization of the United Nations), in 30 years the world's goat population has almost doubled, from 589 million in

1991 to 1 billion 200 million by the beginning of 2020. Today, there are 635 goat breeds in the world, bred in 170 countries, with only 38 breeds classified as transboundary (DAD-IS, Domestic Animal Diversity Information System, http://www.fao.org/dad-is).

The purpose of our review is to summarize and analyze data on modern genetic markers for the study of biodiversity, genetic structure, determination of the degree of inbreeding, purity of breeds and populations of goats, genome-wide association studies (GWAS) in order to identify genes associated with economically important indicators of productivity.

The domestic goat (*Capra hircus*) is propagated worldwide and comprises a large variety of breeds due to their biological peculiarities, including high adaptability to various climatic conditions. Goat raising cover mountain, high-mountain, steppe and semi-desert zones with a sparse grass vegetation. Other animal species (cattle, horses, and sheep) cannot make up for the need for nutrients and energy using such limited food resources. The widespread breeding of goats and the growth of their numbers are also associated with a global trend of increasing demand for products with unique properties, which include goat down, moger, goat milk, and goat meat [1].

Since ancient times, goat down has been a row material for warm products of special lightness, softness and elasticity, which is still relevant today. Herds of fiber goats are widespread in Turkey, India, Mongolia, China, Afghanistan, Kyrgyzstan, Uzbekistan, and Russia (https://www.fao.org/faostat/en).

1. Abundance (heads) **of breeds and populations of goats (*Capra hircus*) bred in Russia in 2000-2019** [5-7]

Breed (population)	Year				
	2000	2005	2010	2015	2019
Altai belaya pukhovaya (down goats)					8300
Alpine goats				900	5230
Gornoaltayskaya pukhocaya (down goats)	15700	11300	27300	22200	10800
Dagestanskaya pukhocaya (down goats)	5700	16600	19500	No data	5000[a]
Dagestan sherstnaya (wool goats)	5800	16700	19600	No data	11000[a]
Donskaya (Pridonskaya) goats	2000	1600	No data	No data	No data
Saanen goats		1100	6900	19900	29770
Karachaevskaya goats	No data	No data	No data	No data	8000[a]
Murciano-Granadina goats					470
Nubian goats					330
Orenburg goats	16900	22800	20500	17200	6500
Russkaya belaya goats	No data	No data	No data	No data	170[a]
Sovetskaya sherstnaya (wool goats)	31700	88700	83300	89900	28600
Tuvinskaya grubosherstbaya (Tuvan coarse-haired goats)	No data	No data	No data	No data	7200[a]
Total	77800	158800	177100	150100	97370
Not identified	2800	28500	7100	63200	41130

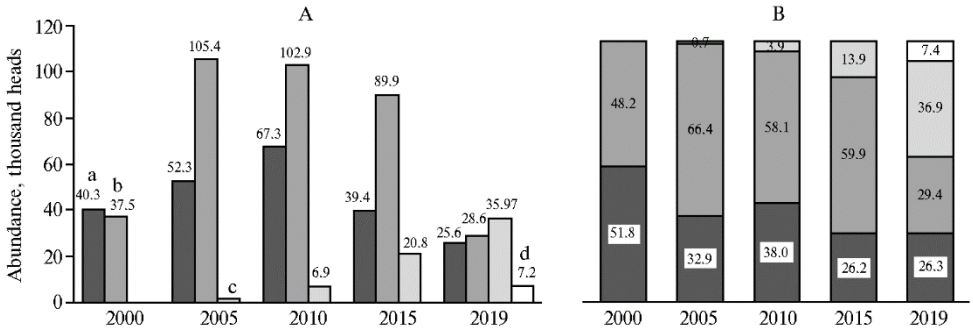
N o t e. Altai belaya pukhovaya (Altai white down goat breed) was officially approved in 2016. Saanen goats were brought to the Russian Federation in 2001, Alpine, Murciano Granadina and Nubian goats in 2015-2018. The total number of goats is calculated from official data provided by livestock breeding organizations; [a] — the number of goats based on the veterinary control records of the regional administrations of farm locations).

Over the past 10 years, goat milk production in Asia, Africa, North and South America has increased by 21.3, 18.4 and 9.5% on average. In France, Greece, Italy, Spain and the Netherlands, the share of goat milk consumption (including cheese production) is 15-20% of the total dairy production [2]. Goat milk is increasingly considered as a raw material for products with high biological and, in some cases, therapeutic value and for baby food. One of the features of goat's milk is a significantly greater dispersion of fat globules compared to cow's (average diameter of 3.19 μm and total area 21.78 cm^2/ml vs. 3.51 μm and 17.11 cm^2/ml), which provides its high digestibility due to availability for lipolytic enzymes. Goat milk contains 54.6-80.2% more unsaturated short-chain fatty acids ($C_{4:0}$-$C_{10:0}$) [3]. In addition, the high content of β-casein and a

negligible amount (virtually absent) of α_{s1}-casein, which causes allergic reactions, bring goat milk closer to human breast milk composition. Goat milk is also distinguished by the physicochemical properties of casein micelles, which contain more calcium and inorganic phosphorus, are less solvated and more resistant to heat, therefore, compared to the milk of other animal species, casein is more easily lost, which determines the high cheese suitability [4].

In Russia, 10 breeds and populations of goats for various use are currently raised. The livestock at the end of 2019 amounted to 97,370 animals (Table 1).

It should be noted that over the past 20 years there has been a significant change in the breed composition of goats in terms of productivity. Since 2015, there has been a significant decrease in the number of wool goats in terms of productivity and, accordingly, their share in the total livestock from 59.9 to 29.4%. The number of dairy goats has noticeably increased. In 2005, dairy goats were absent in the structure of Russian goat breeding, and by the end of 2019, they already accounted for 36.9% (Fig.).



Abundance of goat (*Capra hircus*) breeds for various use (A) **and their distribution** (%) (B) (the Russian Federation, 2005-2019): f — down goats, b — wool goats, c — milk goats, d — coarse-haired goats [5-7].

Currently, to accelerate goat breeding, it is not enough to use only traditional methods, and therefore there is an increasing need to integrate modern DNA technologies into the breeding process, since they can increase the efficiency of breeding through the selection of carriers of alleles associated with economically valuable traits [8, 9]. The following types of DNA markers can be distinguished, which are most widely used in the study of animal genomes, including goats: RFLP (restriction fragment length polymorphism), RAPD (randomly amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), MS (microsatellites, STR, short tandem repasts), SNP (single nucleotide polymorphism), CNV (copy number variation). Microsatellites, also known as STR markers, and markers based on single nucleotide polymorphisms (SNPs) have received the greatest distribution in studies of the goat genome.

Microsatellites (STR markers). Due to their availability, low cost, and information content, microsatellites remain one of the most common markers in phylogenetic and taxonomic studies and are used in programs for the conservation of agricultural animal genetic resources. This is especially true for aboriginal animal husbandry in general and goat breeding in particular, since there are about 600 aboriginal goat breeds in the world [10, 11].

In the study of genetic processes in populations, Wright's F-statistics, or fixation indices, are most often used, which characterize individual (F_{IS}), subpopulation (F_{ST}) and population (F_{IT}) levels of the genetic structure of a population: $F_{IS} = (H_S - H_I)/H_S$, $F_{ST} = (H_T - H_S)/H_T$, $F_{IT} = (H_T - H_I)/H_T$, where H_I is the observed heterozygosity, H_S is the expected heterozygosity in subpopulations, H_T

is the expected heterozygosity in the entire population during panmixia. F_{IS} indicates a decrease in heterozygosity due to non-random mating, F_{IT} indicates the degree of inbreeding of individuals in the whole population. At $F_{IS, IT} > 0$, there is a deficit of heterozygous individuals, at $F_{IS, IT} < 0$, there is an excess. F_{ST} indicates a decrease in heterozygosity due to gene flow restriction and genetic drift between subpopulations. The F_{ST} for the two populations serves as the genetic distance value. At $F_{ST} < 0.05$, population differentiation is insignificant, at $F_{ST} > 0.25$ it is significant [12]. Nei expressed fixation indices through allelic frequencies, observed and expected heterozygosity for any populations, and proposed the use of genetic distances [13, 14)].

With the development of genetic methods, the number of microsatellite loci increased, which were used in the study of the biodiversity of goats with wool and down productivity. Thus, six populations of Kashmir goats from China were studied using 11 microsatellite loci, which formed three separate clusters: Tibetan goat of Plateau type and Tibetan goat of Valley type, Sichuan type (black goats, Meigu, Jianchang, Baiyu) and Xinjiang goats [15]. In another study, 14 microsatellite loci were used to study the genetic differentiation of nine Kashmiri breeds from China. The obtained F_{ST} values indicated their high genetic isolation, with the Hegu breed bred in Tibet showing the greatest remoteness [16].

Kharzinova et al. [17], in a comparative study of the Sovetskaya sherstnaya (wool goats), Tajik sherstnaya (wool goats), Orenburg pukhovaya (down goats), Alpine and Saanen dairy breeds for 10 microsatellite loci, revealed that each of these breeds has its own population genetic structure and determined the degree of genetic differentiation of breeds.

Selionova et al. (18) assessed the genetic diversity and genetic distances between wool and down breeds of goats bred in the North Caucasus (Karachaev, Dagestan pukhovaya down goats, Dagestan sherstnaya wool goats), in Siberia (Sobetskaya sherstnaya wool goats), and in the South Urals (Orenburg goats), as well as between three species of mountain goats, the Siberian ibex (*C. sibirica*), bezoar ibex (*C. aegagrus*), and tur (*C. caucasica*) using 16 microsatellite loci. Karachay goats exhibits the greatest genetic diversity, i.e., the average number of alleles per locus was 9.1 vs. 6.5-7.5 for other breeds. Subspecies of the Caucasian tur formed the first cluster, Siberian ibex formed the second cluster, and breeds of domestic goats formed the third cluster. Groups of the bezoar goat were located at the root of the third cluster, which indirectly confirms their participation as an ancestral form of domestic goats [18].

Microsatellite markers were used to study the genetic diversity of five populations of native Mongolian goats (Gurvan egch, Darhatskaya, Burakh zavkhan, Ulgiy uulan, Altay uulan), two populations of local Tuvan goats, and three breeds (Sovetskaya sherstnaya, Tajik sherstnaya wool goats and Orenburg pukhovaya down goats). Two main groups have been identified, one group includes predominantly Mongolian aboriginal populations, and the other group includes Central Asian goat breeds. Populations of the local Tuvan goat were divided between the respective groups. At the same time, Mongolian goats were characterized by high intrapopulation diversity and a low degree of genetic differences between populations [19].

A number of studies are devoted to the study of the genetic diversity of dairy goats. Wang et al. [20] used 15 microsatellite markers to study breeds bred in China, i.e., the breeds of own selection (Guanzhong, Laoshan, and Wendeng), those bred using the Saanen breed (Xinong Saanen) and imported from Europe (Nubian). The average number of alleles per locus was 4.9, F_{IS} values ranged from 0.09 to –0.08, F_{ST} was 0.08. Between breeds Wendeng and Laoshan as well as Guanzhong and Xinong there are the closest genetic links that reflected the history

of formation and geography of breeding. It has been established that all four Chinese breeds had a common ancestor, the Saanen breed, which was imported to China from Europe in the 18th century [20].

Araujo et al. [21] compared the local dairy breed Moxoto with the Alpine and Saanen goats for 11 microsatellite loci. The F_{ST} value between the Moxoto and introduced breeds was 0.08, while between the latter it was 0.03, indicating their greater genetic similarity [21].

The genetic differentiation of dairy goat breeds from Thailand (Jamunapari, Alpine, Nubian, Saanen, and Toggenburg) was studied using 12 microsatellite markers. The Alpine, Saanen, and Toggenburg breeds were assigned to one phylogenetic cluster, while the Jamunapari and Nubian breeds formed two others. The average number of alleles per population per microsatellite locus was 7.4. F_{IS} values ranged from 0.18 to 0.04, F_{ST} was 0.07 [22].

Microsatellite markers were used to identify the breed of goats with maintained status in the production of dairy products. Thus, the Girgentana goats are bred on the island of Sicily, its distinctive feature is the unique quality of milk, but due to the small number of Girgentana goats are endangered, so measures are being taken to preserve the breed [23]. A panel of 20 microsatellite markers was used to genetically identify Girgentana, Maltese, and Derivata di Siria goats. Eight alleles of microsatellite markers were present in the Girgentana and Derivata di Siria breeds, but were absent in Maltese goats. Three microsatellite markers (FCB20, SRCRSP5, TGLA122), recognized as the most informative, were proposed for use in genetic monitoring of dairy products obtained from goats of the Girgentana breed and when mixed with milk from animals of other breeds [24].

In a large-scale study performed in China using 30 microsatellite loci and covering more than 2 thousand goats of 40 breeds and populations of various productivity directions, it was found that their genetic structure is mainly determined by geographical origin and periods of human migration across the country. More clearly, the genetic differentiation of goats was traced in Western China, for whose populations two clusters were established, the southwestern and northwestern. These clusters coincided with separation by natural barriers (mountain ranges, river basins) [25].

Dixit et al. [26] used 25 microsatellite markers to study genetic diversity and relationship between 20 breeds from India. Most of the loci were heterozygous, F_{IS} values ranged from 0.61 to 0.73. The Kanniadu breed showed the greatest diversity, and Osmanabadi the least. The overall F_{ST} value was 0.183, with 83.5% of the genetic variability found to be due to differences between individuals within a breed and only 16.5% between breeds. The smallest genetic distance was determined between the Ganjam and Malabari breeds (0.22), the largest between the Kanniadu and Malabari breeds (0.83) [26].

In a study of 18 native goat breeds and populations from seven East Asian countries, 26 microsatellite loci were involved. The average number of alleles per locus ranged from 2.5 to 7.6m being 5.8 on average for the studied breeds, while there was a deficit of heterozygotes and general inbreeding ($F_{IS} = 0.054$, $F_{IT} = 0.181$, $p < 0.01$). In Mongolia and Bangladesh, there was more genetic diversity in goat populations than in Japan, Korea and Indonesia. All breeds formed three clusters, the East Asian, Southeast Asian and Mongolian, which correlated with the use that the breeds are intended for, geographical origin and migration routes [27].

Cañón et al. [28] used 30 microsatellite markers to genotype 45 goat breeds from 15 European and Middle Eastern countries. In all breeds, a heterozygosity deficiency ($F_{IS} = 0.10$) and an average genetic differentiation between them was revealed. Multivariate analysis of allele frequencies revealed four clusters: the

breeds of the Eastern Mediterranean (Near East) (F_{ST} = 0.033) were the first, of the Central Mediterranean (F_{ST} = 0.040) the second, of the Western Mediterranean (F_{ST} = 0.051) the third, and of Northern and Central Europe (F_{ST} = 0.069) the fourth. The decrease in the genetic diversity of goats from the southeast to the northwest was accompanied by an increase in differentiation at the breed level. Approximately 41% of the genetic variability was associated with the geographical origin of the breeds. The data obtained were considered by the authors as confirmation of the hypothesis that livestock migrated from the Middle East to Western and Northern Europe, while the formation of breeds was more systematic in Northern and Central Europe than in the Middle East.

In a number of sources, it is proposed to consider the F_{ST} value equal to or greater than 0.25 as a significant genetic distance between breeds, from 0.05 to 0.25 as an average, less than 0.05 as insignificant [12, 13, 29, 30]. Analysis of the above data draws to the conclusion that, in general, small genetic distances (F_{ST} 0.033-0.069) have been established between breeds and populations of goats bred in Europe, which can be considered as confirmation of the frequent exchange of genes due to crossings to improve productivity. For breeds and populations of goats living in East and Southeast Asia, genetic differentiation is more significant (F_{ST} 0.134-0.183), which, apparently, is due to ecological and geographical features and remoteness of habitats.

The study of the origin of goats, the routs of their migration, genetic differentiation and features of the genetic structure as a result of adaptation to the breeding environment does not lose relevance. To obtain new data, single nucleotide polymorphisms (SNPs) are now increasingly used [31-33].

Single nucleotide polymorphisms (SNP markers). SNPs are the most common type of polymorphism in both nuclear and mitochondrial DNA. The main advantage of using SNPs as markers compared to microsatellites is their wide distribution in the genome, a clear mutational mechanism with low homoplasia and mutability.

In addition, SNPs in goats, unlike multi-allelic microsatellites, are presented as bivalent variants. The methodological advantages of SNP analysis include the absence of special requirements for DNA quality (SNP analysis is usually carried out by obtaining short fragments less than 100 bp long), a lower degree of erroneous genotyping, the possibility of automating the process and standardizing the data obtained. The study of SNP became widespread even at the early stages of the development of DNA diagnostics of farm animals, since it is this type of variability that underlies the polymorphism of genes associated with economically valuable traits. The development of high-throughput genotyping technologies has made SNPs the dominant type of DNA markers in the study of farm animal genomes.

Currently, SNPs are considered the preferred type of marker for genomic evaluation, including genome-wide association studies, to determine the relationship between individuals, determine the degree of inbreeding and hybridization, high-resolution genetic mapping and more complete characterization of genetic resources [34].

Polymorphism of goat productivity genes. Along with phylogenetic studies, the identification of genes and their allelic variants associated with economically valuable traits is important for the selection improvement of goat productivity. For milk goats, these are primarily indicators that characterize the quantitative parameters of milk yield, namely, the milk fat and protein content [35]. The main part of milk proteins is casein, containing four fractions (α_{s1}-,

α_{s2}-, β- and κ-casein), and whey proteins (β-lactoglobulin, α-lactalbumin) [36, 37]. The influence of these proteins on the technological properties of milk and the possibility of obtaining products with specified quality parameters have been comprehensively studied, which determined the interest in studying the genes that control their synthesis [38, 39].

The gene *CSN1S1* for α_{s1}-casein whose polymorphism is determined by a set of allelic variants is the most studied. They are defined as strong for the content of α_{s1}-casein at ~ 3.5 g/l (*A*, *A'*, *B1*, *B2*, *B3*, *B4*, *C*, *H*, *L*), medium at ~ 1.1 g/l (*E*, *I*), weak at ~ 0.45 g/l (*D*, *F*, *G*), and zero-variant (*O1*, *O2*, *N*) (no α_{s1}-casein in milk) [37-39]. The so-called strong alleles are more common in breeds from Spain, Italy, France, and Greece while medium and weak alleles are widely represented in goats in New Zealand and Brazil [43, 44]. Nine allelic variants (*A*, *B*, *C*, *D*, *E*, *F*, *0*, *sub A* and *sub E*) for the *CSN1S2* gene (α_{s2}-casein), eight variants (*A*, *A1*, *O'*, *O*, *B*, *C*, *D*, *E*) for the *CSN2* gene (β-casein), and 16 (*A*, *B*, *B'*, *B''*, *C*, *C'*, *D*, *E*, *F*, *G*, *H*, *I*, *J*, *K*, *L*, *M*) for the *CSN3* gene (κ-casein) [37, 40]. The main types of caseins are encoded by genes located on the chromosome 6 and closely linked in a single cluster of 250-350 thousand bp [45].

A number of works are devoted to the influence of polymorphism of the genes of the main milk proteins on coagulation properties, nutritional value indicators, and the formation of goat productivity. It was found that in products from the milk of goats with the *AA* genotype for the *CSN1S1* gene, the protein content was 4.5% higher than from the milk of animals with the *FF* genotype, which justifies the selection of carriers of the *A* allele [40].

In goats producing milk with a low content of α_{s1}-casein, there was a significant decrease in the amount of total lipids and medium-chain fatty acids C_8-C_{12} (caprylic, capric, lauric), as well as palmitic, stearic, linoleic and conjugated linoleic acids. That is, the polymorphism of the *CSN1S1* gene affects the intensity of lipogenesis in the secretory cells of the mammary gland [46, 47].

Investigations of five Chinese goat breeds (more than 4 thousand animals), including the most common breed Shaanbei White Cashmere, sequencing of the α_{s1}-casein gene revealed only one indel mutation of 11 bp, designated as genotype *II*, which was associated with the number of kids at the first lambing. Individuals with genotype *II* had a significantly larger number of offspring compared to *ID* and *DD* genotypes, which allowed the authors to recommend this indel mutation for inclusion in breeding programs to increase multiple pregnancy [48].

A number of studies have focused on the effect of the β-lactoglobulin (*BLG*) gene on goat productivity. Shuvarikova et al. [49] found that Saanen goats with the *AB* genotype were characterized by longer lactation and produced more milk (on average by 110.2 kg, $p < 0.01$) and more milk fat and protein (by 3.7 kg and 3.5 kg, $p < 0.05$, respectively) compared to *AA* and *BB* genotypes. Similar data were obtained by Fatikhov et al. [50]. The best indicators of nutritional and biological values of yogurt and cottage cheese were noted for the milk of Nubian and Alpine goat breeds with the *BB* genotype [49].

Kravtsova et al. [51] concluded that it is desirable to include genotyping for the *weaver*, *BLG* and pituitary transcription factor (*POU1F1*) genes in breeding programs to improve milk goats. It was found that individuals carrying the complex genotypes *T2T2/S1S2/D1D2* and *T2T2/S2S2/D1D1* for the *weaver/BLG/POU1F1* genes had a higher content of fat and protein in milk (5.64 and 3.63%) than goats of other genotypes (4.08 and 3.32%). Goncharenko et al. [52] reported that the bodyweight of Belaya pukhovaya breed of down goats heterozygous for *BLG* was 0.30-0.61 kg ($p < 0.05$) higher compared to goats of other genotypes.

Study of genomes using DNA chips and sequencing. The development of genetic analysis methods based on the study of complete genomes by hundreds or thousands of single nucleotide polymorphisms distributed throughout the genome has significantly expanded the possibilities of identifying genome regions that control physiological and biochemical processes that determine the phenotypic differences of animals [53, 54].

Genome-wide association studies (GWAS) with productivity traits in goats are currently being conducted in many countries around the world [55]. The use of GWAS was preceded by the large-scale work of the International Goat Genome Consortium (IGGC; http://www.goatgenome.org) on the implementation of several research projects of the complete sequencing of the genome of these animals. The AdaptMap project has genotyped 4653 animals in 148 populations from 35 countries on five continents [56]. The developed version of the SNP panel was based on the analysis of differences in 12 million SNP variants identified in the genomes of the Saanen, Alpine, Creole, Boer, Katjang, and Savanna goat breeds. Further validation of the SNP distribution was carried out on 10 other goat breeds. As a result, 52295 SNPs were selected, which were successfully used in the 52K SNP BeadChipGoat chip (Illumina, Inc., USA) [57]. Whole genome sequencing of bird and pig genomes from different countries suggested that intense artificial selection contributed to rapid phenotypic evolution in domestic animals [58, 59]. The development of DNA chips has significantly expanded the ability to identify loci under selection pressure in pigs and cattle [60-62], as well as sheep [63, 64]. These results demonstrated how positive selection has altered the genome of domestic animals. However, it should be noted that certain restrictions on the number of individuals for SNP genotyping on chips can lead to a change in the frequency distributions of alleles, which affects the accuracy of population genetic analysis [65]. For example, almost all SNPs included in the GoatSNP50 BeadChip (Illumina, Inc., USA) were selected from six Saanen, seven Alpine, and three Creole goat populations. At the same time, it turned out that the distribution density of the detected SNPs on genomic DNA was insufficient to obtain an accurate result when assessing loci under selection pressure [57].

GWAS of British milk goats covered a set of traits, including milk yield, milk fat and protein content, somatic cell counts, exterior indicators (i.e., the udder depth, the place of its attachment, the teat shape, the angle of the teat attachment, the size and shape of the fore and hind legs, the strength of the fore and hind hooves). The total phenotypic database included 137235 records for 4563 goats examined. Association analysis revealed SNPs on chromosome 19 that were significantly associated with the amount of milk. In addition, several more SNPs were found on chromosomes 4, 8, 14 and 29, the relationship of which with milk production turned out to be less significant. Three SNPs identified on chromosome 19 were associated with attachment site and udder depth and foreleg features. SNPs with a lesser statistical relationship were found on chromosomes 4-6, 10-18, 21, 23, and 27. However, the influence level on the total variance of the trait associated with significant SNPs was low and varied from 0.4 to 7.0% for the amount of milk and from 0.1 to 13.8% for exterior indicators, which confirms their polygenic nature [66]. Wasike et al. [67] have made a similar conclusion based on the GWAS performed for milk goats in the USA.

The GWAS method was used to search for genes associated with the number of somatic cells (somatic cell count, SCC), selected as a sign of resistance to mastitis. Phenotypic data included SCC for 1941 Alpine and Saanen goats bred in France. In the Saanen breed, a significant association with SCC was shown by an SNPs identified on chromosome 19 in a region from 33 to 42 Mbp in length, which included candidate genes associated with a response to infections caused by

intramammary strains, the retinoic acid receptor α (*RARA*) gene and STAT transcription factor genes (*STAT3*, *STAT5A*, *STAT5B*). However, these associations were not found for the Alpine breed [68].

In Eastern Europe, 10 genes were identified in goats that affect resistance to mastitis and gastrointestinal infections. These were the genes for pentraxin 3 (*PTX3*), interleukin-6 (*IL6*), C-type 4 lectin domain family member 4 (*CLEC4E*), interleukin-8 (*IL8*), interleukin-1 receptor antagonist (*IL1RN*), interleukin-15alpha receptor subunit (*IL15RA*), a member of the tumor necrosis factor 13 (*TNFSF13*) superfamily, cytokine signaling suppressor 3 (*SOCS3*), tumor necrosis factor (*TNF*) and toll-like receptor 3 (*TLR3*) [69].

Another French GWAS study attempted to identify genes associated with extra lobes and udder teats in goats. The sample included 810 Saanen and 1185 Alpine goats, however, no significant associations between SNPs and these traits could be found [70].

Desire et al. [71] used GWAS to evaluate genomic breeding value estimator (GEBV) and identify SNPs associated with milk yield and body weight gain. Phenotypic data covered a period of one year for 320 individuals. The obtained GEBV accuracy value was low (0.28 for both indicators). Nevertheless, the authors believe that with an increase in the number of animals, the period of studies and the total number of observations, the accuracy of the genomic estimation will increase [71].

Martin et al. [72] sequenced the *DGAT1* gene and identified 29 polymorphisms, of which R251L and R396W not previously described were associated with reduced milk fat. The frequency of occurrence of the R396W mutation in Saanen and Alpine goats was 13.0% and 7.0%, respectively, the frequency of R251L for both breeds was 3.5% [72].

When using a one-step approach in combination with genomic best linear unbiased prediction (GBLUP), the accuracy of estimating the breeding value of Alpine and Saanen goats (825 individuals), which constitute the breeding core on farms in France, was increased from 22 to 37% by compared with the two-stage method and was higher than the traditional pedigree estimate [73]. Another work used several prediction estimates. The estimates were based on the best linear unbiased prediction (BLUP), single-step genomic best linear unbiased prediction (ssGBLUP), and three weighted analyses (weighted single-step genomic best linear unbiased prediction, WssGBLUP; single-step genomic best linear unbiased prediction with the maximum weight of SNPs included in the chromosomal region, $WssGBLUP_{Max}$; single-step genomic best linear unbiased prediction with the sum of the weights of the SNPs included in the chromosomal region, $WssGBLUP_{Sum}$) calculated for SNPs with regard to their effect on milk protein content. The accuracy of GEBV with ssGBLUP has improved by 5-7% compared to the traditional BLUP model. WssGBLUP more accurately identified SNPs associated with α_{s1}-casein content and proved to be more effective in predicting genomic selection values than unweighted ssGBLUP. In addition, the authors indicate that using WssGBLUP was somewhat easier to perform calculations, which speeded up genomic analysis [74].

In a Spanish study involving 50,649 records of milk production from 19,067 Florida goats, it was found that the ssGBLUP method improved the average accuracy of breeding value estimates by 1.06% compared to classical BLUP. The correlation between matrix A (pedigree) and matrix G (gene) was 0.826. The correlation between EBV (breeding value estimator) and GEBV (genomic breeding value estimator) was 0.989, but when comparing only EBV-genotyped animals, the correlation between these estimates decreased to 0.952, and the average accuracy increased by 5.86% [75].

In order to reduce the cost of genotyping in the control of origin, Talenti et al. [76], based on an analysis of 109 Alpine goats, proposed two low-density panels comprising 130 and 114 SNPs with random match probabilities of 1.51×10^{-57} and 2.94×10^{-34}, respectively. The results made it possible to determine family ties with absolute accuracy. Subsequently, an improved panel containing 195 SNPs was developed. It has been shown that at a comparable cost, the 195 SNP chip can replace microsatellite markers, but with much higher accuracy [77].

Goat wool color is a polygenic trait that is often determined by epistatic gene interactions [78]. These include genes for the melanocortin 1 receptor (*MC1R*) and its endogenous antagonist, the agouti signaling protein (*ASIP*). The *MC1R* gene plays a key role in the synthesis of melanin pigments and the control of the amount of eumelanin (black/brown) or pheomelanin (red/yellow). This has been demonstrated in several studies examining the effect of *MC1R* on color in cattle and sheep [79]. Similarly, mutations in the *MC1R* gene are associated with wool color in goats of the Girgentana, Maltese, Derivata di Siria, Murciano-Granadina, and Camosciata delle Alpi, and Saanen goats [80]. *ASIP* has an epistatic effect on the *MC1R* gene and reduces MC1R activity, which leads to increased pheomelanin synthesis. Yellow or pheomelanin pigmentation is due to the action of the dominant allele at the *ASIP* locus, while black/brown or eumelanin pigmentation is due to the action of the recessive allele [81]. In Saanen goats, the dominant allele A^{wt} (white/red) seems to be responsible for the white coat color [82]. Duplication of regions in the *ASIP* gene leads to the formation of white and black colors [83]. Another gene that affects the coat color of goats is the proto-oncogene receptor tyrosine kinase (*KIT*) gene, which is considered one of the key genes in color formation in many animal species [80, 84-86].

Wang et al. [87], based on sequencing genotyping performed on goats from eight populations, reported several genes under positive selection pressure. The *ASIP* gene was associated with coat color, the fibroblast growth factor 5 (*FGF5*) gene was associated with wool productivity, and the NADPH oxidase activator gene 1 (*NOXA1*) was associated with adaptation to altitude hypoxia [87]. Further editing of the *FGF5* gene in goat embryos led to an increase in the number of secondary hair follicles and fiber length, which confirms the positive association of the gene with cashmere productivity and the expediency of its inclusion in down goat breeding programs [88].

Guo et al. [89] performed genome-wide sequencing of 38 goats of three Chinese breeds, the Nanjiang Yellow, Jintang Black, and Tibetan cashmere and compared them with the genomes of 30 goats of five other breeds, as well as with the genomes of 21 bezoar goats from AdaptMap databases. As a result, a new SNP (c.-253G>A) associated with down productivity and adaptation to low temperatures in Tibetan cashmere goats was identified in the 5′-UTR region of the *FGF5* gene. A high frequency of occurrence of the *AGG* allele in the exon 12 of the desmoglein 3 gene (*DSG3*), which determines cell adhesion and is expressed mainly in the skin, has also been established [89]. Genome comparison of cashmere goats of different breeds have shown that loci under selection pressure are associated with color (*IRF4*, *EXOC2*, *RALY*, *EIF2S2*, *KITLG*), reproduction (*KHDRBS2*) and adaptation to altitude (*EPAS1*) [90]. The selection pressure for the endothelial PAS domain-containing protein 1 (*EPAS1*) gene was established by Song et al. [90] in exome sequencing of 330 Tibetan cashmere goats well adapted to mountainous environments [90].

We examined selective loci in a population of native Karachay goats ($n = 37$) by analyzing runs of homozygosity (ROH). In total, 17 ROH regions larger than 0.1 Mb were identified, which were found in the genome in more than 50% of

Karachay goats (including 6 ROH regions identified in more than 60% of animals) (Table 2). To confirm these data and select positional candidate genes, it is necessary to study a larger population of goats of the Karachay breed.

2. Runs of homozygosity (ROHs) in the genomes of more than 50% of Karachay goats (*Capra hircus*)

Chromosome	SNP number	Position		Length, Mb	Candidate genes
		start	end		
1	6	123,995,551	124,276,659	0.281	
3	7	91,992,725	92,358,697	0.366	
7	5	47,720,691	47,985,489	0.265	
7	10	50,213,129	50,678,375	0.465	
	4[a]	50,385,448	50,599,960	0.215	*HTR4*, *FBXO38*
11	13	14,570,133	15,147,019	0.577	
	7[a]	14,850,176	15,108,357	0.258	*BIRC6*, *TTC27*
11	12	37,444,185	37,989,059	0.545	
	10[a]	37,518,114	37,955,681	0.438	*CLHC1*, *RPS27A*, *MTIF2*, *CCDC88A*[b], *CFAP36*[b], *PPP4R3B*[b], *PNPT1*[b]
11	3	95,963,081	96,081,413	0.118	
12	7	24,713,474	25,070,617	0.357	
12	13	34,478,328	35,027,103	0.549	
	7[a]	34,509,187	34,826,053	0.317	
13	8	60,716,743	61,161,390	0.445	
	5[a]	60,913,235	61,123,452	0.210	*HCK*, *TM9SF4*, *PLAGL2*, *POFUT1*, *KIF3B*, *ASXL1*
14	11	74,881,431	75,466,670	0.585	
	7[a]	74,881,431	75,240,511	0.359	*MMP16*
21	8	54,788,196	55,179,468	0.391	
23	5	27,849,491	28,042,905	0.193	
25	5	3,491,087	3,753,620	0.263	
27	3	32,676,747	32,783,591	0.107	
27	3	32,905,720	33,015588	0.110	
28	7	15,314,246	15 639,373	0.325	

N o t e. [a] — ROH in the genomes of more than 60% animals; [b] — genes present in more than 70% animals ("Studies of the genomic diversity of goats of different breeds, searching for selection marks in the population of Karachay goats based on full genome SNP genotyping, biochemical blood analysis and phenotype". Moscow, 2020).

Thus, in the world's goat raising, there are many breeds and populations of goats intended for various us, the vast majority of which are aboriginal. Goat biodiversity has been fairly well studied using microsatellite DNA loci. To obtain new fundamental knowledge about the origin of goats, genetic drift, and genetic relationships between domestic goats and their wild ancestors, genome-wide analysis and genome scanning using DNA chips have been widely used recently. Data were obtained on the relationship of the goat genes *CSN1S1*, *CSN1S2*, *CSN2*, *BLG*, *RARA*, *STAT*, *PTX3*, *IL6*, *IL8*, *DGAT1* with milk productivity and milk quality. The association has been demonstrated of *MC1R*, *ASIP*, and *KIT* genes with the color of wool and down, *FGF5*, *EPAS1*, and *NOXA1* genes with wool productivity and adaptation to high-altitude hypoxia. At present, certain progress has been made in understanding the formation of goat biodiversity, the prospects of the genomic approach in the selection of wool and milk breeds. However, the loci associated with economically important traits (reproduction, down and wool productivity, wool color, the amount of milk and the content of milk protein and fat, somatic cell counts, etc.) and those associated with adaptiveness and resistance to diseases are still little studied. Therefore, efforts should be focused on these issues and searching for candidate genes based on genomic and omics technologies.

REFERENCES

1. Skapetas B., Bampidis V. Goat production in the world: present situation and trends. Livestock Research for Rural Development, 2016, 28(11): 200
2. Erokhin A.I., Karasev E.A., Erokhin S.A. Dinamika pogolov'ya koz i proizvodstva koz'ego moloka i myasa v mire i v Rossii. *Ovtsy, kozy, sherstyanoe delo*, 2020, 4: 22-25 (doi: 10.26897/2074-0840-

2020-4-22-25) (in Russ.).
3. Skidan I.N., Gulyaev A.E., Kaznacheev K.S. *Voprosy pitaniya*, 2015, 84(2): 81-95 (in Russ.).
4. Shuvarikov A.S., Kanina K.A., Robkova T.O., Yurova E.A. *Fermer. Povolzh'e,* 2019, 7(84): 92-93 (in Russ.).
5. Grigoryan L.N., Khatataev S.A., Sverchkova S.V. V sbornike: *Ezhegodnik po plemennoi rabote v ovtsevodstve i kozovodstve v khozyaistvakh Rossiiskoi Federatsii (2005 god)* [In: Yearbook on breeding sheep and goat on the farms of the Russian Federation (2005)]. Moscow, 2006: 312-313 (in Russ.).
6. Dunin I.M., Amerkhanov Kh.A., Safina G.F., Grigoryan L.N., Khatataev S.A., Khmelevskaya G.N. Kozovodstvo Rossii i ego plemennye resursy. *Ezhegodnik po plemennoi rabote v ovtsevodstve i kozovodstve v khozyaistvakh Rossiiskoi Federatsii (2019 god)* [Yearbook on breeding sheep and goat on the farms of the Russian Federation (2019)]. Moscow, 2020: 323-325 (in Russ.).
7. Novopashina S.I., Sannikov M.Yu., Khatataev S.A., Kuz'mina T.N., Khmelevskaya G.N., Stepanova N.G., Tikhomirov A.I., Marinchenko T.E. *Sostoyanie i perspektivnye napravleniya uluchsheniya geneticheskogo potentsiala melkogo rogatogo skota: nauchnyi i analiticheskii obzor* [State and promising ways for improving the genetic potential of small ruminants: scientific and analytical review]. Moscow, 2019 (in Russ.).
8. Deniskova T.E., Dotsev A.V., Fornara M.S., Sermyagin A.A., Reyer H., Wimmers K., Brem G., Zinov'eva N.A. The genomic architecture of the Russian population of saanen goats in comparison with worldwide Saanen gene pool from five countries. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2020, 55(2): 285-294 (doi: 10.15389/agrobiology.2020.2.285rus).
9. Koshkina O.A., Deniskova T.E., Zinov'eva N.A. *Agrarnaya nauka Evro-Severo-Vostoka*, 2020, 21(4): 355-368 (doi: 10.30766/2072-9081.2020.21.4.355-368) (in Russ.).
10. Mekuriaw G., Gizaw S., Dessie T., Mwai O., Djikeng A., Tesfaye K. A review on current knowledge of genetic diversity of domestic goats (*Capra hircus*) identified by microsatellite loci: how those efforts are strong to support the breeding programs? *Journal of Life Science and Biomedicine*, 2016, 6(2): 22-32.
11. Azhar P., Chakraborty D., Iqbal Z., Malik A., Ajaz qaudir, Asfar A., Bhat I.A. Microsatellite markers as a tool for characterization of small ruminants: a review. *International Journal of Current Microbiology and Applied Sciences,* 2018, 7(1): 1330-1342 (doi: 10.20546/ijcmas.2018.701.162).
12. Wright S. The genetical structure of populations. *Ann. Eugenics*, 1951, 15: 323-354.
13. Nei M. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences*, 1973, 70(12): 3321-3323 (doi: 10.1073/pnas.70.12.3321).
14. Nei M. Genetic distance between populations. *The American Naturalist*, 1972, 106(949): 283-392 (doi: 10.1086/282771).
15. Wang Y., Wang J., Zi X.-D., Huatai C.-R., Ouyang X., Liu L.-S. Genetic diversity of Tibetan goats of Plateau type using microsatellite markers. *Archives Animal Breeding*, 2011, 54(2): 188-197 (doi: 10.5194/aab-54-188-2011).
16. Di R., Farhad Vahidi S.M., Ma Y.H., He X.H., Zhao Q.J., Han J.L., Guan W.J., Chu M.X., Sun W., Pu Y.P. Microsatellite analysis revealed genetic diversity and population structure among Chinese cashmere goats. *Animal Genetics*, 2011, 42(4): 428-431 (doi: 10.1111/j.1365-2052.2010.02072.x).
17. Kharzinova V.R., Petrov S.N., Dotsev A.V., Bezborodova N.A., Zinov'eva N.A. *Ovtsy, kozy, sherstyanoe delo*, 2019, 3: 7-12 (in Russ.).
18. Selionova M.I., Aibazov M.M., Mamontova T.V., Petrov S.N., Kharzinova V.R., Dotsev A.V. Zinovieva N. A. Genetic differentiation of Russian goats and wild relatives based on microsatellite loci. *Journal of Animal Science*, 2020, 98(4): 19-20 (doi: 10.1093/jas/skaa278.037).
19. Beketov S.V., Piskunov A.K., Voronkova V.N., Petrov S.N., Kharzinova V.R., Dotsev A.V., Zinov'eva N.A., Selionova M.I., Stolpovskii Yu.A. *Genetika*, 2021, 57(7): 810-819 (doi: 10.31857/S0016675821070031) (in Russ.).
20. Wang G.Z., Chen S.S., Chao T.L., Ji Z.B., Hou L., Qin Z.J., Wang J.M. Analysis of genetic diversity of Chinese dairy goats via microsatellite markers. *Journal of Animal Science*, 2017, 95(5): 2304-2313 (doi: 10.2527/jas.2016.1029).
21. Araújo A.M., Guimarães S.E.F., Machado T.M.M., Lopes P.S., Pereira C.S., Silva F.L.R., Rodrigues M.T., Columbiano V.S., da Fonseca C.G. Genetic diversity between herds of Alpine and Saanen dairy goats and the naturalized Brazilian Moxotó breed. *Genetics and Molecular Biology*, 2006, 29(1): 67-74 (doi: 10.1590/S1415-47572006000100014).
22. Seilsuth S., Seo J.H., Kong H.S., Jeon G.J. Microsatellite analysis of the genetic diversity and population structure in dairy goats in Thailand. *Anim. Biosci.*, 2016, 29(3): 327-332 (doi: 10.5713/ajas.15.0270).
23. Mastrangelo S., Tolone M., Montalbano M., Tortorici L., Gerlando R., Sardina M.T., Portolano B. Population genetic structure and milk production traits in Girgentana goat breed. *Animal Production Science*, 2016, 57(3): 430-440 (doi: 10.1071/AN15431).
24. Sardina M., Tortorici L., Mastrangelo S., Di Gerlando R., Tolone M., Portolano B. Application of microsatellite markers as potential tools for traceability of Girgentana goat breed dairy products. *Food Research International*, 2015, 74: 115-122 (doi: 10.1016/j.foodres.2015.04.038).

25. Wei C., Lu J., Xu L., Liu G., Wang Z., Zhang L., Zhao F., Han X., Du L., Liu C. Genetic structure of Chinese indigenous goats and the special geographical structure in the Southwest China as a geographic barrier driving the fragmentation of a large population. *PLoS ONE*, 2014, 9(4): e94435 (doi: 10.1371/journal.pone.0094435).
26. Dixit S.P., Verma N.K., Aggarwal R.A.K., Vyas M.K., Rana J., Sharma A., Tyagi P., Arya P., Ulmek B.R. Genetic diversity and relationship among Indian goat breeds based on microsatellite markers. *Small Ruminant Research*, 2010, 91(2): 153-159 (doi: 10.1016/j.smallrumres.2010.02.015).
27. Nomura K., Ishii K., Dadi H., Takahashi Y., Minezawa M., Cho C.Y., Sutopo, Faruque M.O., Nyamsamba D., Amano T. Microsatellite DNA markers indicate three genetic lineages in East Asian indigenous goat populations. *Animal Genetics*, 2012, 43(6): 760-767 (doi: 10.1111/j.1365-2052.2012.02334.x).
28. Cañón J., García D., García-Atance M.A., Obexer-Ruff G., Lenstra J.A., Ajmone-Marsan P., Dunner S., The ECONOGENE Consortium. Geographical partitioning of goat diversity in Europe and the Middle East. *Animal Genetics*, 2006, 37(4): 327-334 (doi: 10.1111/j.1365-2052.2006.01461.x).
29. Weir B.S., Cockerham C.C. Estimating *F*-statistics for the analysis of population structure. *Evolution*, 1984, 38(6): 1358-1370 (doi: 10.1111/j.1558-5646.1984.tb05657.x).
30. Kalinowski S.T. Counting alleles with rarefaction: private alleles and hierarchical sampling designs. *Conservation Genetics*, 2004, 5(4): 539-543 (doi: 10.1023/B:COGE.0000041021.91777.1a).
31. Nicoloso L., Bomba L., Colli L., Negrini R., Milanesi M., Mazza R., Sechi T., Frattini S., Talenti A., Coizet B., Chessa S., Marletta D., D'Andrea M., Bordonaro S., Ptak G., Carta A., Pagnacco G., Valentini A., Pilla F., Ajmone-Marsan P., Crepaldi P., the Italian Goat Consortium. Genetic diversity of Italian goat breeds assessed with a medium-density SNP chip. *Genetics, Selection, Evolution*, 2015, 47(1): 62 (doi: 10.1186/s12711-015-0140-6).
32. Mdladla K., Dzomba E.F., Huson H.J., Muchadeyi F.C. Population genomic structure and linkage disequilibrium analysis of South African goat breeds using genome-wide SNP data. *Animal Genetics*, 2016, 47(4): 471-482 (doi: 10.1111/age.12442).
33. Brito L.F., Kijas J.W., Ventura R.V., Sargolzaei M., Porto-Neto L.R., Cõnovas A., Feng Z., Jafarikia M., Schenkel F.S. Genetic diversity and signatures of selection in various goat breeds revealed by genome-wide SNP markers. *BMC Genomics*, 2017, 18: 229 (doi: 10.1186/s12864-017-3610-0).
34. Zhang B., Chang L., Lan Y.X., Nadeem A., Guan F.L., Fu K.D., Li B., Yan X.C., Zhang B.H., Zhang Y.X., Huang Z.A., Chen H., Yu J., Li B.S. Genome-wide definition of selective sweeps reveals molecular evidence of trait-driven domestication among elite goat (*Capra* species) breeds for the production of dairy, cashmere, and meat. *GigaScience,* 2018, 7(12): giy105 (doi: 10.1093/gigascience/giy105).
35. Zonaed Siddiki A.M.A.M., Miah G., Islam M.S., Kumkum M., Rumi M.H., Baten A., Hossain M.A. Goat genomic resources: the search for genes associated with its economic traits. *International Journal of Genomics*, 2020, 2020(1): 5940205 (doi: 10.1155/2020/5940205).
36. Khaertdinov R.R., Gafiatullin F.I., Afanas'ev M.P. Features of milk protein content in main species of agricultural animals. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2011, 2: 81-85 (in Russ.).
37. Vorozhko I.V., Skidan I.N., Chernyak O.O., Gulyaev A.E. *Voprosy pitaniya*, 2016, 85 (5): 13-21 (in Russ.).
38. Barillet F. Genetic improvement for dairy production in sheep and goats. *Small Ruminant Research*, 2007, 70(1): 60-75 (doi: 10.1016/j.smallrumres.2007.01.004).
39. Dodds K.G., McEwan J.C., Davis G.H. Integration of molecular and quantitative information in sheep and goat industry breeding programmes. *Small Ruminant Research*, 2007, 70(1): 32-41 (doi: 10.1016/j.smallrumres.2007.01.010).
40. Marletta D., Criscione A., Bordonaro S., Guastella A.M., D'Urso G. Casein polymorphism in goat's milk. *Lait*, 2008, 87(6): 491-504 (doi: 10.1051/lait:2007034).
41. Devold T.G., Nordbø R., Langsrud T., Svenning C., Brovold M.J., Sørensen E.S., Christensen B., Ådnøy T., Vegarud G.E. Extreme frequencies of the α_{s1}-casein 'null' variant in milk from Norwegian dairy goats – implications for milk composition, micellar size and renneting properties. *Dairy Science and Technology*, 2010, 91(1): 39-51 (doi: 10.1051/DST/2010033).
42. Ollier S., Chauvet S., Martin P., Chilliard Y., Leroux C. Goat's α_{s1}-casein polymorphism affects gene expression profile of lactating mammary gland. *Animal*, 2008, 2(4): 566-573 (doi: 10.1017/S1751731108001584).
43. Jordana J., Amills M., Diaz E., Angulo C., Serradilla J.M., Sanchez A. Gene frequencies of caprine α_{s1}-casein polymorphism in Spanish goat breeds. *Small Ruminant Research*, 1996, 20(3): 215-221 (doi: 10.1016/0921-4488(95)00813-6).
44. Enne G., Feligini M., Greppi G.F., Iametti S., Pagani S. Gene frequencies of caprine α_{s1}-casein polymorphism in dairy goats, IDF Seminar «Milk Protein Polymorphism II». *Palmerston North*, 1997: 275-279.
45. Küpper J., Chessa S., Rignanese D., Caroli A., Erhardt G. Divergence at the casein haplotypes

in dairy and meat goat breeds. *Journal Dairy Research*, 2010, 77(1): 56-62 (doi: 10.1017/S0022029909990343).
46. Chilliard Y., Rouel J., Leroux C. Goat's alpha-s1 casein genotype influences its milk fatty acid composition and delta-9 desaturation ratios. *Animal Feed Science and Technology*, 2006, 131(3-4): 474-487 (doi: 10.1016/j.anifeedsci.2006.05.025).
47. Silanikove N., Leitner G., Merin U., Prosser C.G. Recent advances in exploiting goat's milk: quality, safety and production aspects. *Small Ruminant Research*, 2010, 89(2): 110-124 (doi: 10.1016/j.smallrumres.2009.12.033).
48. Wang K., Hailong Y., Xu H., Yang Q., Zhang S., Pan C., Chen H., Zhu H., Liu J., Qu L., Lan X. A novel indel within goat casein alpha S1 gene is significantly associated with litter size. *Gene*, 2018, 671: 161-169 (doi: 10.1016/j.gene.2018.05.119).
49. Shuvarikov A.S., Pastukh O.N., Zhukova E.V., Zhizhin N.A. *Izvestiya TSKHA*, 2019, 3: 130-148 (doi: 10.34677/0021-342X-2019-3-130-148) (in Russ.).
50. Fatikhov A.G., Khaertdinov R.A., Kamaldinov I.N. *Molochnokhozyaistvennyi vestnik*, 2017, 1(25): 64-69 (in Russ.).
51. Kravtsova O.A., Spiridonova S.V., Faizov T.Kh. *Sposob geneticheskogo otbora molochnykh koz. Patent RU 2620977. Zayavka № 2015140586 ot 24.09.2015. Opubl. 30.05.2017 g. Byul. № 16* [Method for genetic selection of dairy goats. Patent RU 2620977. Appl. № 2015140586 24.09.2015. Publ. 30.05.2017. Bull. № 16] (in Russ.).
52. Goncharenko G.M., Grishina N.B., Khoroshilova T.S., Romanchuk I.V., Kargachakova T.B., Podkorytov N.A. *Sibirskii vestnik sel'skokhozyaistvennoi nauki*, 2018, 48(4): 63-71 (doi: 10.26898/0370-8799-2018-4-9) (in Russ.).
53. Visscher P.M., Wray N.R., Zhang Q., Sklar P., McCarthy M.I., Brown M.A., Yang J. 10 Years of GWAS discovery: biology, function, and translation. *American Journal of Human Genetics*, 2017, 101(1): 5-22 (doi: 10.1016/j.ajhg.2017.06.005).
54. Meuwissen T., Hayes B., Goddard M. Genomic selection: a paradigm shift in animal breeding. *Animai Frontiers*, 2016, 6(1): 6-14 (doi: 10.2527/af.2016-0002).
55. Ibtisham F. Zhang L., Xiao M., An L., Ramzan M.B., Nawab A., Zhao Y., Li G., Xu Y. Genomic selection and its application in animal breeding. *Thai Journal of Veterinary Medicine*, 2017, 47(3): 301-310.
56. Stella A., Nicolazzi E.L., Tassell C., Rothschild M., Colli L., Rosen B., Sonstegard T., Crepaldi P., Tosser-Klopp G., Joost S., the AdaptMap Consortium. AdaptMap: exploring goat diversity and adaptation. *Genetics, Selection, Evolution*, 2018, 50: 61 (doi: 10.1186/s12711-018-0427-5).
57. Tosser-Klopp G., Bardou F., Bouchez O., Cabau C., Crooijmans R., Dong Y., Donnadieu-Tonon C., Eggen A., Heuven H.C.M., Jamli S., Jiken A.J., Klopp C., Lawley C.T., McEwan J., Martin P., Moreno C.R., Mulsant P., Nabihoudine I., Pailhoux E., Palhiere I., Rupp R., Sarry J., Sayre B.L., Tircazes A., Wang J., Wang W., Zhang W., the International Goat Genome Consortium. Design and characterization of a 52K SNP chip for goats. *PLoS ONE*, 2014, 9(1): e86227 (doi: 10.1371/journal.pone.0086227).
58. Rubin C.J., Megens H.-J., Barrio A.M., Maqboo K., Sayyab S., Schwochow D., Wang C., Carlborgd Ö., Jerna P., Jørgensene C.B., Archibald A.L., Fredholm M., Groenen M.A.M., Andersson L. Strong signatures of selection in the domestic pig genome. *Proceedings of the National Academy of Sciences*, 2012, 109(48): 19529-19536 (doi: 10.1073/pnas.1217149109).
59. Rubin C.-J., Zody M.C., Eriksson J., Meadows J.R.S., Sherwood E., Webster M.T., Jiang L., Ingman M., Sharpe T., Ka S., Hallböök F., Besnier F., Carlborg O., Bed'hom B., Tixier-Boichard M., Jensen P., Siegel P., Lindblad-Toh K., Andersson L. Whole-genome resequencing reveals loci under selection during chicken domestication. *Nature,* 2010, 464(7288): 587-591 (doi: 10.1038/nature08832).
60. Kemper K.E., Saxton S.J., Bolormaa S., Hayes B. J., Goddard M.E. Selection for complex traits leaves little or no classic signatures of selection. *BMC Genomics*, 2014, 15: 246 (doi: 10.1186/1471-2164-15-246).
61. Zhao F., McParland S., Kearney F., Du L., Berry D.P. Detection of selection signatures in dairy and beef cattle using high-density genomic information. *Genetics, Selection, Evolution*, 2015, 47: 49 (doi: 10.1186/s12711-015-0127-3).
62. Xu L., Bickhart D.M., Cole J.B., Schroeder S.G., Song J., Tassell C.P., Sonstegard T.S., Liu G.E. Genomic signatures reveal new evidences for selection of important traits in domestic cattle. *Molecular Biology and Evolution*, 2015, 32(3): 711-725 (doi: 10.1093/molbev/msu333).
63. Kijas J.W., Lenstra J.A., Hayes B., Boitard S., Neto L.P., Cristobal M.S., Servin B., McCulloch R., Whan V., Gietzen K., Paiva S., Barendse W., Ciani E., Raadsma H., McEwan J., Dalrymple B. other members of the International Sheep Genomics Consortium. 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).
64. 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*, 2015, 116(3): 255-264 (doi: 10.1038/hdy.2015.94).

65. Lachance J., Tishkoff S.A. SNP ascertainment bias in population genetic analyses: Why it is important, and how to correct it. *BioEssays*, 2013, 35(9): 780-786 (doi: 10.1002/bies.201300014).
66. Mucha S., Mrode R., Coffey M., Kizilaslan M., Desire S., Conington J. Genome-wide association study of conformation and milk yield in mixed-breed dairy goats. *Journal Dairy Science*, 2018, 101(3): 2213-2225 (doi: 10.3168/jds.2017-12919).
67. Wasike C.B., Rolf M., Silva N.C.D., Puchala R., Sahlu T., Goetsch A.L., Gipson T.A. 1683 Genome-wide association analysis of residual feed intake and milk yield in dairy goats. *Journal of Animal Science*, 2016, 94(5): 820 (doi: 10.2527/jam2016-1683).
68. Martin P., Palhière I., Maroteau C., Clément V., David I., Tosser Klopp G., Rupp R. Genome-wide association mapping for type and mammary health traits in French dairy goats identifies a pleiotropic region on chromosome 19 in the Saanen breed. *Journal Dairy Science*, 2018, 101(6): 5214-5226 (doi: 10.3168/jds.2017-13625).
69. Ilie D.E., Kusza S., Sauer M., Gavojdian D. Genetic characterization of indigenous goat breeds in Romania and Hungary with a special focus on genetic resistance to mastitis and gastrointestinal parasitism based on 40 SNPs. *PLoS ONE*, 2018, 13(5): e0197051 (doi: 10.1371/journal.pone.0197051).
70. Martin P., Palhière I., Tosser-Klopp G., Rupp R. Heritability and genome-wide association mapping for supernumerary teats in French Alpine and Saanen dairy goats. *Journal Dairy Science*, 2016, 99(11): 8891-8900 (doi: 10.3168/jds.2016-11210).
71. Desire S., Mucha S., Coffey M., Mrode R., Broadbent J., Conington J. Deriving genomic breeding values for feed intake and body weight in dairy goats. *Proceedings of the World Congress on Genetics Applied to Livestock Production*, 2016, 11: 818.
72. Martin P., Palhière I., Maroteau C., Bardou P., Canale-Tabet K., Sarry J., Woloszyn F., Bertrand-Michel J., Racke I., Besir H., Rupp R., Tosser-Klopp G. A genome scan for milk production traits in dairy goats reveals two new mutations in *DGAT1* reducing milk fat content. *Scientific Reports*, 2017, 7: 1872 (doi: 10.1038/s41598-017-02052-0).
73. Cérillier C., Larroque H., Robert-Granié C. Comparison of joint versus purebred genomic evaluation in the French multi-breed dairy goat population. *Genetics, Selection, Evolution*, 2014, 46: 67 (doi: 10.1186/s12711-014-0067-3).
74. Teissier M., Larroque H., Robert-Granié C. Weighted single-step genomic BLUP improves accuracy of genomic breeding values for protein content in French dairy goats: A quantitative trait influenced by a major gene. *Genetics, Selection, Evolution*, 2018, 50: 31 (doi: 10.1186/s12711-018-0400-3).
75. Molina A., Muñoz E., Díaz C., Menéndez-Buxadera A., Ramón M., Sánchez M., Carabaño M.J., Serradilla J.M. Goat genomic selection: impact of the integration of genomic information in the genetic evaluations of the Spanish Florida goats. *Small Ruminant Research*, 2018, 163: 72-75 (doi: 10.1016/j.smallrumres.2017.12.010).
76. Talenti A., Nicolazzi E.L., Chessa S., Frattini S., Moretti R., Coizet B., Nicoloso L., Colli L., Pagnacco G., Stella A., Ajmone-Marsan P., Ptak G., Crepaldi P. A method for single nucleotide polymorphism selection for parentage assessment in goats. *Journal Dairy Science*, 2016, 99(5): 3646-3653 (doi: 10.3168/jds.2015-10077).
77. Talenti A., Palhière I., Tortereau F., Pagnacco G., Stella A., Nicolazzi E.L., Crepaldi P., Tosser-Klopp G. AdaptMap Consortium. Functional SNP panel for parentage assessment and assignment in worldwide goat breeds. *Genetics, Selection, Evolution*, 2018, 50: 55 (doi: 10.1186/s12711-018-0423-9).
78. Sturm R.A., Teasdale R.D., Box N.F. Human pigmentation genes: identification, structure and consequences of polymorphic variation. *Gene*, 2001, 277(1-2): 49-62 (doi: 10.1016/S0378-1119(01)00694-1).
79. Switonski M., Mankowska M., Salamon S. Family of melanocortin receptor (*MCR*) genes in mammals — mutations, polymorphisms and phenotypic effects. *Journal Applied Genetics*, 2013, 54: 461-472 (doi: 10.1007/s13353-013-0163-z).
80. Fontanesi L., Beretti F., Riggio V., Dall'Olio S., Gonzőlez E.G., Finocchiaro R., Davoli R., Russo V., Portolano B. Missense and nonsense mutations in melanocortin 1 receptor (*MC1R*) gene of different goat breeds: association with red and black coat colour phenotypes but with unexpected evidences. *BMC Genetics*, 2009, 10: 47 (doi: 10.1186/1471-2156-10-47).
81. Adalsteinsson S., Sponenberg D.P., Alexieva S., Russel A.J.F. Inheritance of goat coat colors. *Journal of Heredity*, 1994, 85(4): 267-272 (doi: 10.1093/oxfordjournals.jhered.a111454).
82. Martin P.M., Palhière I., Ricard A., Tosser-Klopp G., Rupp R. Genome wide association study identifies new loci associated with undesired coat color phenotypes in Saanen goats. *PLoS ONE*, 2016, 11(3): e0152426 (doi: 10.1371/journal.pone.0152426).
83. Norris B.J., Whan V.A. A gene duplication affecting expression of the ovine *ASIP* gene is responsible for white and black sheep. *Genome Research*, 2008, 18(8): 1282-1293 (doi: 10.1101/gr.072090.107).
84. David V.A., Menotti-Raymond M., Wallace A.C., Roelke M., Kehler J., Leighty R., Eizirik E., Hannah S.S., Nelson G., Schäffer A.A., Connelly C.J., O'Brien S.J., Ryugo D.K. Endogenous retrovirus insertion in the KIT oncogene determines white and white spotting in domestic cats.

G3 Genes|Genomes|Genetics, 2014, 4(10): 1881-1891 (doi: 10.1534/g3.114.013425).
85. Dürig N., Jude R., Holl H., Brooks S.A., Lafayette C., Jagannathan V., Leeb T. Whole genome sequencing reveals a novel deletion variant in the *KIT* gene in horses with white spotted coat colour phenotypes. *Animal Genetics*, 2017, 48(4): 483-485 (doi: 10.1111/age.12556).
86. Holl H., Isaza R., Mohamoud Y., Ahmed A., Almathen F., Youcef C., Gaouar S.B.S., Antczak D.F., Brooks S.A. A frameshift mutation in KIT is associated with white spotting in the Arabian camel. *Genes*, 2017, 8(3): 102 (doi: 10.3390/genes8030102).
87. Wang X., Liu J., Zhou G., Guo J., Yan H., Niu Y., Li Y., Yuan C., Geng R., Lan X., An X., Tian X., Zhou H., Song J., Jiang Y., Chen Y. Whole-genome sequencing of eight goat populations for the detection of selection signatures underlying production and adaptive traits. *Scientific Reports*, 2016, 6: 38932 (doi: 10.1038/srep38932).
88. Wang X., Cai B., Zhou J., Zhu H., Niu Y., Ma B., Yu H., Lei A., Yan H., Shen Y., Shi L., Zhao X., Hua J., Huang X., Qu L., Chen Y. Disruption of *FGF5* in cashmere goats using CRISPR/Cas9 results in more secondary hair follicles and longer fibers. *PLoS ONE*, 2016, 11(10): e0164640 (doi: 10.1371/journal.pone.0164640).
89. Guo J., Tao H., Li P., Li L., Zhong T., Wang L., Ma J., Chen X., Song T., Zhang H. Whole-genome sequencing reveals selection signatures associated with important traits in six goats' breeds. *Scientific Reports*, 2018, 8: 10405 (doi: 10.1038/s41598-018-28719-w).
90. Song S., Yao N., Yang M., Liu X., Dong K., Zhao Q., Pu Y., He X., Guan W., Yang N., Ma Y., Jiang L. Exome sequencing reveals genetic differentiation due to high-altitude adaptation in the Tibetan cashmere goat (*Capra hircus*). *BMC Genomics*, 2016, 17: 122 (doi: 10.1186/s12864-016-2449-0).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1049-1062
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 636.32/.38:636.082:577.21

doi: 10.15389/agrobiology.2021.6.1049eng
doi: 10.15389/agrobiology.2021.6.1049rus

INTRAMUSCULAR FATTY ACID COMPOSITION IN SHEEP: PHENOTYPIC VARIABILITY, HERITABILITY, AND CANDIDATE GENES

(review)

S.N. KOVALCHUK ✉

Institute of Innovative Biotechnologies in Animal Husbandry — the Branch of Ernst Federal Research Center for Animal Husbandry, 12/4, ul. Kostyakova, Moscow, 127422 Russia, e-mail s.n.kovalchuk@mail.ru (✉ corresponding author)
ORCID:
Kovalchuk S.N. orcid.org/0000-0002-5029-0750
The author declares no conflict of interests
Acknowledgements:
Funded by the Ministry of Science and Higher Education of the Russian Federation (theme No. AAAA-A19-119051590015-1)
Received October 2, 2021

Abstract

Sheep husbandry contributes significantly to global food production. Improving the biochemical parameters of meat is one of the urgent goals of sheep breeding programs due to the changed customers' requirements for food quality, in particular its dietary properties. The fatty acid composition is one of the important indicators of meat quality. High concentrations of saturated fatty acids in the human diet are known to increase plasma cholesterol concentrations which increases the risk of developing diabetes, obesity, and cardiovascular disease (A.P. Simopoulos, 2001; F.B. Hu et al., 2001). Improving the dietary properties of sheep meat by breeding animals with the increased content of unsaturated fatty acids is one of the possible measures that could reduce the incidence of these diseases. In addition, intramuscular fatty acid composition affects flavor, aroma, juiciness, and tenderness of the meat and the digestibility of fat. These reasons determine the relevance of identifying genetic markers associated with intramuscular fatty acid composition in sheep and their use in sheep breeding programs. This review analyzes data on phenotypic variability, inheritance of the intramuscu-lar fatty acid composition in sheep, and candidate genes identified due to genome-wide association studies (GWAS) with DNA microarrays technology (R. Bumgarner 2013) and high-throughput RNA sequencing method (RNA-seq) applicable in studying genetic mechanisms that are involved in the formation of animal phenotypes at the gene expression level (A. Oshlack et al., 2010; K.O. Mutz et al., 2013; R. Stark et al., 2019). Research results demonstrate that the quantitative indicators of the intramuscular fatty acid composition in different breeds of sheep and the degree of heritability of this trait vary widely which indicates the possibility of changing the profiles of the fatty acid composition of mutton through the use of genetic methods in sheep breeding programs (E. Karamichou et al., 2006; H.D. Daetwyler et al., 2012; S.I. Mortimer et al., 2014; S. Bolormaa et al., 2016; G.A. Rovadoscki et al., 2017**).** Summarizing GWAS и RNA-seq results, the most significant candidate genes associated with the fatty acid composition of sheep meat are i) *acot11*, *baat*, *pnpla3*, *lclat1*, *isyna1*, *elovl6*, *agpat9*, *me1, acaca*, *dgat2*, *plcxd3*, *fads2*, *scd*, *cpt1a*, *pisd*, *lipg*, *b4galt6*, *acsm1*, *acsl1*, *aacs*, and *fasn* which encode the enzymes of fat and fatty acids metabolism; ii) the genes encoding fatty acid transporters FABP3, FABP4, FABP5, SLC27A6, APOL6, and COPB2; iii) *mlxipl*, *ppard*, *wnt11, foxo3*, *tnfaip8*, *npas2*, *fndc5*, *adipoq*, *adipor2*, *trhde*, *cidec*, *ccdc88c*, *tysnd1* and *sgk2* genes which encode the transcription factors and effector proteins, regulating energy and fat metabolism (X. Miao et al., 2015; S. Bolormaa et al., 2016; L. Sun et al., 2016; G.A. Rovadoscki et al., 2017; R. Arora et al., 2019). These data allow a deeper understanding of the genetic mechanisms underlying the phenotypic variability of intramuscular fatty acid composition in sheep, which is a necessary background for successful selection strategies in sheep husbandry.

Keywords: sheep, fatty acids, genetic markers, GWAS, RNA-seq, SNP

Sheep farming significantly contributes to world food production. Currently, gene pool of sheep bred in more than 150 countries comprises more than 2300 breeds. In the Russian Federation, 46 breeds of sheep are raised of which 15

are fine-wool sheep, the share of which in 2020 was 53.6% of the total livestock at agricultural enterprises, 14 are semi-fine-wool sheep (5.0%), 2 are semi-coarse-wool sheep (1.4%), 15 are coarse-haired sheep (34.3%) [1]. The general trend of modern world sheep breeding is the reduction in the number of woolly sheep. From 2000 to 2020 in Russia, the share of fine-wool sheep decreased by 26.9%, of semi-fine-wool sheep 2.6-fold, while the share of coarse-wool breeds bred for meat production increased 6.4-fold [1]. The main reason for the reduction in the need for sheep wool is the rapid growth in the production of synthetic fibers, the quality of which, in many respects, is close to natural at a much lower cost. Currently, the share of meat products in the gross income from the sale of all products obtained from sheep approximates to 90% [2, 3]. The intensification of sheep breeding and the growing demand for mutton in many countries is accompanied by the emergence of new, more productive sheep breeds. Selection is aimed at creating sheep breeds with a combined high wool and meat productivity [3].

One of the ways to improve sheep breeds is to improve the biochemical parameters of meat, which is due to the changed requirements for the quality of food products, in particular, for their dietary properties. The average content of saturated fatty acids in mutton is 1.464 g/100 g of meat, which is higher than in beef and pork (respectively 1.149 and 0.400 g/100 g meat) [4]. Lamb significantly exceeds beef and pork (more than 1.5-fold and 10-fold) in the content of polyunsaturated ω-3 and ω-6 fatty acids [4], which are not synthesized in the human body [5], but are involved in the synthesis of eicosanoids, cell signaling, regulation of enzyme and neurotransmitter activity, neuronal migration, and other vital processes [6, 7]. For an adult, the physiological need for ω-6 fatty acids is 8-10 g per day, for ω-3 fatty acids 0.8-1.6 g per day, with the optimal ratio of ω-6 to ω-3 fatty acids within 5:1-10:1 [8].

A high content of saturated fatty acids in the human diet is known to increase the blood cholesterol concentration and, as a result, increase the risk of cardiovascular diseases, diabetes and obesity [9, 10]. In addition, the fatty acid composition of meat affects its consumer properties, e.g., flavor, odor, juiciness, tenderness and digestibility. The more unsaturated fatty acids in the composition of the fat, the lower its pour point and the higher the digestibility. Therefore, it is important to identify and use in breeding genetic markers associated with the fatty acid composition of sheep meat.

The emergence in recent decades of high-throughput DNA sequencing (next generation sequencing, NGS) technologies [11, 12] and their widespread use has made it possible to establish the nucleotide sequences of the genomes of most agricultural animal species, including sheep [13, 14]. In turn, this contributed to the development of DNA chip technology [15] for genome-wide association analysis to identify candidate genes and genomic variations (single nucleotide polymorphisms, SNPs) associated with economically important traits in crops and domestic animals [16-18].

The later developed RNA sequencing technology (RNA-seq) makes it possible to study the genetic mechanisms of phenotype formation based on a comparative analysis of gene expression profiles. [19-22]. The integrated use of these approaches contributes to the understanding of the genetic mechanisms underlying the variability of economically useful traits of farm animals, which serves as the necessary scientific basis for the development of successful breeding programs in animal husbandry [23, 24].

This review analyzes and summarizes the results of studies of the phenotypic variability and heritability of fatty acid composition of sheep muscle tissue, as well as data on candidate genes identified using genome-wide association search and high-throughput RNA sequencing.

The heritability of the content of fatty acids in the muscle tissue of sheep. The quality of lamb, including its fatty acid composition, depends on the breed [25-28], sex and age of the animals [29-31], as well as on the diet [32-34]. Quantitative indicators of the fatty acid composition of sheep meat of different breeds vary widely and differ in breeds of both the same and different directions of productivity (Table 1).

1. Lamb fatty acid composition in various breeds of sheep (*Ovis aries*) (*M*±SEM)

Fatty acids	Breed					
	Edilbay [29]	Romanian [31]	Prekos [27]	Karachai [30]	Kubashev [28]	Tsigai [28]
	meat-fat	meat-wool				wool-met
Saturated:						
myristic, $C_{14:0}$	8.11±0.10	2,51±0,45	5,00±0,25	3,50±0,11	2,42±0,19	4,98±0,12
pentadecanoic, $C_{15:0}$		0,68±0,51		0,99±0,03	0,76±0,06	
palmitic, $C_{16:0}$	24.15±0.14	22,31±1,53	25,00±0,08	25,32±1,19	22,29±0,29	25,02±0,07
stearic, $C_{18:0}$	21.98±0.23	24,71±0,63	25,00±0,10	22,51±0,96	46,76±0,34	25,02±0,11
Monounsaturated:						
palmitoleic, $C_{16:1}$	1.38±0.11	2.54±0,13		2,54±0,08	4,33±0,20	
heptadecenoic, $C_{17:1}$	0.60±0.09	0,54±0,12			2,01±0,14	
oleic, $C_{18:1}$	32.8±0.22	41,09±1,68	39,00±0,18	39,44±1,16	15,8±0,24	38,98±0,23
Polyunsaturated:						
linoleic, $C_{18:2\omega6}$	5.32±0.14	2,54±1,09	4,00±0,15	2,24±0,09		3,99±0,09
linolenic, $C_{18:3\omega3}$	0.99±0.07	0,93±0,08	0,50±0,02	0,86±0,02	0,73±0,09	0,55±0,02
arachidonic, $C_{20:4\omega6}$	0.27±0.03		1,50±0,04	0,090±0,004		1,46±0,05

N o t e. Gaps mean no data.

2. Reported heritability (h^2) **of fatty acid content in muscle tissue of various breeds of sheep (*Ovis aries*)** (*M*±SEM)

Fatty acids	h^2			
	[36]	[37]	[38]	[39]
Saturated:				
myristic, $C_{14:0}$	0.19±0.14		0,15	0,44±0,045
palmitic, $C_{16:0}$	0.29±0.17		0,11	0,25±0,033
stearic, $C_{18:0}$	0.24±0.15		0,19	0,30±0,037
Monounsaturated:				
palmitoleic, $C_{16:1}$	0.31±0.18			0,30±0,035
oleic, $C_{18:1}$	0.27±0.17			0,28±0,035
Polyunsaturated:				
arachidonic, $C_{20:4\omega6}$	0.60±0.17	0,15±0,04	0,16	
linoleic, $C_{18:2\omega6}$	0.10±0.09	0,22±0,04	0,15	0,27±0,034
conjugated linoleic, CLA_{c9t11}	0.48±0.06			0,34±0,045
α-линоленовая кислота, $C_{18:\omega3}$	0.30±0.02			0,46±0,045
Total content of saturated fatty acids	0.90±0.16			0,32±0,039
Total content of monounsaturated fatty acids	0.73±0.18			0,31±0,038
Total content of polyunsaturated fatty acids	0.40±0.16			0,28±0,034
Total content of ω-3 fatty acids				0,37±0,045
Total content of ω-6 fatty acids				0,27±0,034
Polyunsaturated/saturated fatty acids				0,28±0,034
ω6/ω3 ж fatty acids				0,33±0,042

N o t e. Data on Texel, Border Leicester, Polled Dorset, Suffolk, East Friesian, Merino sheep [36], Merino sheep [37], Merino, Poll Dorset, Border Leicester, Suffolk, Texel, Corriedale, Coopworth sheep and crosses [38], Santa Inês [39] are submitted. Gaps mean no data.

For the first time, the degree of heritability of the content of fatty acids in the muscle tissue of sheep was estimated by Karamichou et al. [35] in 2006 based on a study of two lines of Scottish Blackface sheep that differed in the fatty acid composition of the *longissimus dorsi* muscle. It was shown that the total content of saturated and monounsaturated fatty acids are highly inherited traits (h^2 heritability coefficients of 0.90 and 0.73, respectively); the total content of polyunsaturated fatty acids is a moderately inherited trait ($h^2 = 0.40$) (Table 2).

Later, similar studies were carried out for more than a dozen breeds and crosses of sheep [36-39]. Daetwyler et al. [36] conducted a genomic assessment of the breeding value of sheep based on the analysis of the databases of the Cooperative

Research Center for Sheep Industry Innovation [40] and SheepGENOMICS [41], including 14039 breeds Texel, Border Leicester, Polled Dorset, Suffolk, East Friesian and Merino. It was revealed that the intramuscular fat content refers to moderately inherited traits ($h^2 = 0.49$), while this indicator for eicosapentaenoic and docosapentaenoic polyunsaturated fatty acids was significantly lower and amounted to 0.26 and 0.24, respectively [36], which agrees with the data of Mortimer et al. [37]. Bolormaa et al. [38] studied 10613 Merino, Poll Dorset, Border Leicester, Suffolk, Texel, Corriedale, Coopworth and cross breed sheep and found low heritability coefficients ($h^2 = 0.15$-0.19) for both polyunsaturated arachidonic, linoleic, and for saturated $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ fatty acids (see Table 2). The results of a study of 216 sheep of the Santa Inês breed revealed a moderate heritability of all fatty acids studied by the authors, among which the highest values of the coefficient of heritability were in α-linolenic and myristic acids [39].

Thus, the heritability coefficients of the content of fatty acids in the muscle tissue of sheep varied over a wide range, which indicates a significant genetic variability of the estimated traits in different breeds and, therefore, the possibility of changing the profiles of the fatty acid composition of meat in sheep through the use of genetic methods in breeding.

Quantitative trait loci and candidate genes associated with fatty acid content. More than 20 QTLs (quantitative trait loci) associated with fatty acid content in sheep muscle tissue have been annotated in the SheepQTLdb database [42, 43].

For the first time, loci of quantitative traits of the fatty acid composition of sheep meat were identified by Karamichou et al. [35] in 2006. A total of 21 QTLs were found on chromosomes 1, 2, 3, 5, 14, 18, 2 and 21, most of which were associated with the content of certain fatty acids, and not with their total number [35]. Rovadoscki et al. [39] performed genome-wide association studies based on the genotyping of 216 Santa Inês sheep using the OvineSNP50 BeadChip DNA chip (Illumina, Inc., USA), as a result of which 27 QTLs were detected on chromosomes 1, 2, 3, 5, 8, 12, 14, 15, 16, 17 and 18 and 23 potential candidate genes were found, including *dgat2*, *trhde*, *tph2*, *me1*, *parp14*, and *mrps30* associated with fatty acid content in sheep muscle tissue (Table 3). Thus, QTLs of the total content of saturated fatty acids were found on chromosomes 3, 14, and 15 and included the *tph2*, *trhde*, *dgat2*, *wnt11*, and *npas2* genes. The *tph2* gene encodes the enzyme tryptophan hydroxylase 2 (TPH2), which is associated with the serotonergic system and is involved in various physiological processes, including lipid metabolism in adipose tissue [44, 45]. The enzyme pyroglutamyl peptidase II (TRHDE, product of the *trhde* gene) inactivates thyrotropin-releasing hormone, which regulates energy metabolism [46]. The association of the *trhde* gene with the content of visceral fat in Merino sheep was previously shown [47]. The neuronal PAS domain-containing protein 2 (NPAS2) plays an important role in the PPAR signaling pathway that regulates lipid metabolism with the participation of the PPARα receptor (peroxisome proliferator-activated receptor α), which controls fatty acid beta-oxidation [48, 49]. The enzyme diacylglycerol O-acyltransferase 2 (DGAT2) plays a key role in triglyceride biosynthesis [50, 51]. The *wnt11* gene is associated with the Wnt signaling pathway which has an inhibitory effect on adipogenesis [52-55].

Four QTLs were found on chromosomes 1, 3, and 15, associated with the total amount of monounsaturated fatty acids (see Table 3) and containing the *copb2* and *dgat2* genes. The COPB2 protein (coatomer subunit beta 2) plays an important role in the metabolic pathways associated with the intracellular transport of cholesterol and sphingolipids from the endoplasmic reticulum to the Golgi apparatus [56]. The QTL for oleic acid ($C_{18:1}$) is located on chromosome 15, overlaps

with the QTL for stearic acid ($C_{18:0}$) and includes the *dgat2* gene. For α-linolenic ($C1_{8:3\omega3}$), linoleic ($C_{18:2\omega6}$), conjugated linoleic (CLA_{c9t11}) polyunsaturated fatty acids, as well as total polyunsaturated fatty acids, 11 QTLs and 12 candidate genes were found, including *me1*, *tnfaip8*, *plcxd3*, *ccdc88c* and *cacna1c* located on eight chromosomes (see Table 3). The ME1 enzyme (malic enzyme 1) is associated with the tricarboxylic acid cycle, in which NADPH and acetyl-CoA necessary for the biosynthesis of fatty acids are synthesized [57]. The TNFAIP8 protein (tumor necrosis factor TNF-alpha-induced protein 8) is involved in maintaining immune homeostasis and regulating the expression of genes encoding lipid metabolism enzymes [58]. PLCXD3 (phosphatidylinositol-specific phospholipase C, X domain containing 3) refers to phospholipases that break down phospholipids into fatty acids and other lipophilic molecules [39)]. The *cdc88c* gene product regulates the Wnt signaling pathway that affects lipid metabolism and adipogenesis [53]. The CACNA1C protein (voltage-dependent l-type calcium channel subunit alpha-1 C), like long-chain fatty acids, is involved in the functioning of calcium channels [59, 60].

3. Candidate genes associated with fatty acid content in muscle tissue of sheep (*Ovis aries*)

Gene and the encoded protein	Chromosome	Method	Function	References
adipoq (adiponectin)	1	RNA-seq	Regulation of energy homeostasis	[66]
adipor2 (adiponectin receptor 2)	1	RNA-seq	Regulation of energy homeostasis	[66]
acot11 (acyl-CoA-thioesterase 11b)	1	RNA-seq	Lipid metabolism enzyme	[66]
copb2 (coatomer subunit beta 2)	1	GWAS	Intracellular fat transport	[39]
baat (bile acid-coenzyme A: amino acid N-acyltransferase)	2	RNA-seq	Lipid metabolism enzyme	[84]
cyp27a1 (sterol 26-hydroxylase)	2	GWAS	Breakdown of cholesterol	[38]
fabp5 (fatty acid binding protein 5)	2	RNA-seq	Transport of long-chain fatty acids, compensation for loss of FABP4 in adipocytes	[80]
fndc5 (6fibronectin type III domain-containing protein 5)	2	RNA-seq	Regulation of adipose tissue metabolism	[66]
fabp3 (fatty acid binding protein 3)	2	RNA-seq	Regulation of intramuscular fat content, adipogenesis	[66]
trhde (pyroglutamyl-peptidase II)	3	GWAS	Regulation of energy metabolism	[39]
apol6 (apolipoprotein L6)	3	GWAS	Lipid transport	[38]
cacna1c (voltage-dependent l-type calcium channel subunit alpha-1 C)	3	GWAS	Transmembrane transport of calcium ions	[39]
npas2 (neuronal PAS-containing domain protein 2)	3	GWAS	Regulation of fat metabolism with PPAR	[39]
tph2 (tryptophan hydroxylase 2)	3	GWAS	Biosynthesis of serotonin	[39]
pnpla3 (adiponutrin)	3	GWAS	Release of fatty acids and glycerol via hydrolysis of triglycerides	[38]
lclat1 (lysocardiolipin acyltransferase 1)	3	RNA-seq	Lipid metabolism enzyme catalyzing the acylation of polyglycerophospholipids	[84]
tnfaip8 (tumor necrosis factor (TNF)-alpha-induced protein 8)	5	GWAS	Regulation of the expression of enzymes involved in the metabolism of lipids and fatty acids	[39]
slc27a6 (solute carrier family 27 member 6)	5	RNA-seq	Fatty acid transport	[84]
isyna1 (inositol-3-phosphate synthase 1)	5	GWAS	Biosynthesis of phospholipids	[38]
snora70 (small nucleolar RNA, H/ACA box 70)	6	GWAS	RNA processing	[38]
elovl6 (elongation of very long chain fatty acids protein 6)	6	GWAS	Fatty acid elongation	[38, 80]
agpat9 (1-acylglycerol-3-phosphate O-acyltransferase 9)	6	GWAS	Biosynthesis of triglycerides	[38]
foxo3 (forkhead box protein O3)	8	GWAS	Transcription factor regulating glucose metabolism, cell cycle and apoptosis	[39]
me1 (malic enzyme 1)	8	GWAS	Biosynthesis of fatty acids	[39]
fabp4 (fatty acid binding protein 4)	9	RNA-seq	Delivery of fatty acids to mitochondria	[66]
dgkh (diacylglycerol kinase eta)	10	RNA-seq	Regulation of intracellular concentrations of diacylglycerol and phosphatidic acid	[84]

Continued Table 3

acaca (acetyl-coA carboxylase 1)	11	GWAS	Biosynthesis of fatty acids	[38]
fasn (fatty acid synthase)	11	GWAS	De novo fatty acid biosynthesis, fat deposition and fatty acid anabolism	[38]
synrg (synergin gamma)	11	GWAS	Participation in the transport of proteins through the Golgi apparatus	[38]
sgk2 (serum/glucocorticoid regulated kinase 2)	13	GWAS	Participation in the intracellular signaling pathway PI3K/AKT/mTOR, regulating glucose metabolism, cell proliferation and apoptosis	[38]
gys1 (glycogen synthase, muscle)	14-я	GWAS	Intramuscular glycogen synthesis	[38]
dgat2 (diacylglycerol O-acyltransferase 2)	15	GWAS	Biosynthesis of triglycerides	[39]
wnt11 (Wnt family member 11)	15	GWAS	Regulation of adipogenesis	[39]
plcxd3 (phosphatidylinositol-specific phospholipase C, X domain containing 3)	16	GWAS	Breakdown of phospholipids into fatty acids and other lipophilic molecules	[39]
cdh12 (кадгерин 12; cadherin 12)	16	GWAS	Intercellular adhesion protein	[39]
aacs (acetoacetyl-CoA synthetase)	17	RNA-seq	Enzyme for the biosynthesis of cholesterol and fatty acids	[66]
pisd (phosphatidylserine decarboxylase proenzyme, mitochondrial)	17	RNA-seq	Phospholipid biosynthetic enzyme	[84]
fbln5 (fibulin-5)	18	GWAS	Participation in the formation of elastic fibers	[39]
ccdc88c (coiled-coil domain-containing protein 88C)	18	GWAS	Downregulation of the Wnt signaling pathway involved in lipid metabolism	[39]
cidec (CIDE-N domain-containing protein)	19	RNA-seq	Deposition of fats in adipocytes, regulation of adipocyte apoptosis	[66]
ppard (peroxisome proliferator-activated receptor delta)	20	RNA-seq	Transcription factor regulating lipid metabolism	[66]
fads2 (fatty acid desaturase 2)	21	GWAS	Biosynthesis of unsaturated fatty acids	[38]
scd (stearoyl-CoA desaturase)	22	GWAS	Biosynthesis of unsaturated fatty acids	[38]
cpt1a (carnitine-palmitoyltransferase 1)	21	RNA-seq	Breakdown of long chain fatty acids	[84]
lipg (lipase endothelial)	23	RNA-seq	Fat metabolism	[84]
b4galt6 (beta 1,4-galactosyltransferase 6)	23	RNA-seq	Sphingolipid metabolism	[84]
mlxipl (MLX interacting protein like)	24	GWAS	Transcription factor that activates promoters of triglyceride synthesis genes	[38]
acsm1 (acyl-coenzyme A synthetase ACSM1, mitochondrial)	24	RNA-seq	Biosynthesis of fatty acids	[66]
tysnd1 (trypsin like peroxisomal matrix peptidase 1)	25	RNA-seq	Participation in the processing of proteins involved in beta-oxidation of fatty acids	[66]
acsl1 (long-chain-fatty-acid-CoA ligase 1)	26	GWAS	Fatty acid metabolism	[38]

Bolormaa et al. [38] used GWAS technology to study 10613 sheep and identified several potential candidate genes involved in the biosynthesis of fatty acids and triglycerides, the most significant of which are *fasn*, *mlxipl*, *elovl6*, *acaca*, *synrg*, *acsl1*, *isyna1*, *sgk2*, *fads2*, and *agpat9* genes. The *fasn*, *acaca*, *elovl6*, and *fads2* genes encode enzymes that are directly involved in fatty acid biosynthesis [62]. The *agpat9* gene encodes 1-acylglycerol-3-phosphate-O-acyltransferase 9 (AGPAT9), a key enzyme in triglyceride biosynthesis that catalyzes the conversion of glycerol-3-phosphate to lysophosphatidic acid during the synthesis of triglycerides [63]. The MLXIPL (MLX interacting protein like) protein activates promoters of triglyceride synthesis genes [64]. The enzymes long-chain-fatty-acid-CoA ligase 1 (ACSL1) and inositol-3-phosphate synthase 1 (ISYNA1) are involved in lipid biosynthesis and fatty acid degradation [65].

Arora et al. [66] performed a comparative analysis of the transcriptome profiles of *musculus longissimus thoracis* of Bandur sheep and local Indian sheep based on the RNA-seq method. Expression levels of the *adipoq*, *adipor2*, *fabp3*,

fabp4, *aacs*, *acsm1*, *acot11*, *cidec*, *fndc5*, *ppard*, and *tysnd1* genes associated with fatty acid metabolism were higher in Bandur sheep, distinguished by meat tenderness, high fat and oleic acid content compared to local sheep populations. The *fabp3*, *fabp4*, and *adipoq* genes encode proteins that play an important role in the regulation of lipid and glucose homeostasis in adipocytes [67, 68]. FABP3 and FABP4 belong to the family of fatty acid-binding proteins (FABPs). FABP3 is involved in the metabolism of long-chain fatty acids, transporting them to the mitochondria for oxidation, and also regulates adipogenesis [69]. FABP4 is one of the predominant proteins in the soluble fraction of adipose tissue, the function of which is to regulate lipolysis in adipocytes by activating hormone-sensitive lipase (HSL), leading to an increase in intracellular fatty acids [70, 71]. Recent studies have shown a negative correlation between *fabp4* gene transcription and the ratio of polyunsaturated to saturated fatty acids in *musculus longissimus dorsi* of Chinese Tan sheep [72]. The protein ADIPOQ (adiponectin) and its receptor ADIPOR2 (adiponectin receptor 2) are involved in maintaining energy homeostasis by regulating glucose levels and fatty acid oxidation [73, 74]. Previously, 9 sheep haplotypes for the *adipoq* gene were identified [75], and haplotypes B1 and A3 were shown to be associated with an increase in lean meat yield in New Zealand Romney sheep [76]. Peptidase TYSND1 (trypsin like peroxisomal matrix peptidase 1) is involved in the processing of proteins involved in beta-oxidation of fatty acids [77]. The *fabp4*, *adipoq*, and *fabp5* genes associated with fat deposition also turned out to be the most transcribed in the tail adipose tissue of fat-tailed sheep [78, 79].

Sun et al. [80] performed a comparative analysis of the transcriptome profiles of the *longissimus dorsi* muscle in two Chinese sheep breeds and identified 960 genes with different expression levels, including *elovl6* and *fabp5*, directly associated with the synthesis and transport of fatty acids [81, 82]. The *elovl6* gene encoding elongation of very long chain fatty acids protein 6 (ELOVL6) was also proposed by Bolormaa et al. [38] as a candidate gene associated with fatty acid content in sheep muscle tissue. It should be noted that polymorphism in the promoter region of the *elovl6* gene in pigs is associated with the content of palmitic and palmitoleic acids in muscles and fat [83].

Miao et al. [84] using transcriptome analysis of *musculus longissimus dorsi* of Dorset and Small Tail Han sheep, identified differentially expressed genes *cpt1a*, *baat*, and *slc27a6* associated with fatty acid biosynthesis and metabolism [85-87]. Expression of the *cpt1a* and *slc27a6* genes was also high in the tail adipose tissue of sheep [88].

There are data on the relationship of allelic variants of the calpastatin gene with the fatty acid composition of lipids in the muscle tissue of lambs. In sheep, two allelic variants of the *cast* gene, *N* and *M* were identified [89], and it was shown that the carriers of the *NN* genotype had more myristic (C*14:0)*, palmitic ($C_{16:0}$), stearic ($C_{18:0}$), arachidic ($C_{20:0}$), palmetic ($C_{16:1}$) and arachidonic ($C_{20:4}$) fatty acids than in lambs of the *MM* genotype [90]. SNPs C24T, G62A, G65T, and T69 were also found in the intron 5, c.197A > T and c.282G > T in the exon 6 of the *cast* gene; six corresponding genotypes are B, C, D, F, I, and J. Animals of genotype I have been shown to have a lower palmitic acid content and a ratio of ω6 to ω3 fatty acids and a higher content of palmitoleic acid compared to carriers of other genotypes [91].

Thus, at present, genome-wide association studies and sequencing of transcriptomes of sheep muscle tissue identified QTLs and a number of candidate genes associated with the content of fatty acids in muscle tissue. Of these, the genes *acot11*, *baat*, *pnpla3*, *lclat1*, *isyna1*, *elovl6*, *agpat9*, *me1*, *acaca*, *dgat2*, *plcxd3*, *fads2*, *scd*, *cpt1a*, *pisd*, *lipg*, *b4galt6*, *acsm1*, *acsl1*, *aacs* and *fasn* encode enzymes of fat and fatty acids metabolism. The genes *fabp3*, *fabp4*, *fabp5*, *slc27a6*, *apol6*

and *copb2* encode transporter proteins of fatty acids and fats. The genes *mlxipl*, *ppard*, *wnt11*, *foxo3*, *tnfaip8*, *npas2*, *fndc5*, *adipoq*, *adipor2*, *trhde*, *cidec*, *ccdc88c*, *tysnd1* and *sgk2* encode transcription factors and effector proteins that regulate energy and fat metabolism. Further research is needed to validate the identified candidate genes and their allelic variants as genetic markers of fatty acid content in sheep muscle tissue for use in breeding to improve lamb quality.

REFERENCES

1. *Ezhegodnik po plemennoi rabote v ovtsevodstve i kozovodstve v khozyaistvakh Rossiiskoi Federatsii (2020 god)* /Pod redaktsiei T.A. Moroz [Yearbook on pedigree breeding in sheep and goat on the farms of the Russian Federation (2020). T.A. Moroz (ed.)]. Moscow, 2021 (in Russ.).
2. Erokhin A.I., Karasev E.A., Yuldashbaev Yu.A. *Zootekhniya*, 2014, 12: 12-13 (in Russ.).
3. Erokhin A.I., Karasev E.A., Erokhin S.A., Sycheva I.N. *Ovtsy, kozy, sherstyanoe delo*, 2021, 2: 20-22 (doi: 10.26897/2074-0840-2021-2-20-22) (in Russ.).
4. Williams P. Nutritional composition of red meat. *Nutrition & Dietetics*, 2007, 64(s4): S113-S119 (doi: 10.1111/j.1747-0080.2007.00197.x).
5. Ikem A., Shanks B., Caldwell J., Garth J., Ahuja S. Estimating the daily intake of essential and nonessential elements from lamb m. *longissimus thoracis et lumborum* consumed by the population in Missouri (United States). *Journal of Food Composition and Analysis*, 2015, 40: 126-135 (doi: 10.1016/j.jfca.2014.12.022).
6. Yehuda S., Rabinovitz S., Carasso R.L., Mostofsky D.I. The role of polyunsaturated fatty acids in restoring the aging neuronal membrane. *Neurobiology of Aging*, 2002, 23(5): 843-853 (doi: 10.1016/s0197-4580(02)00074-x).
7. Bazinet R.P., Layé S. Polyunsaturated fatty acids and their metabolites in brain function and disease. *Nature Reviews Neuroscience*, 2014, 15: 771-785 (doi: 10.1038/nrn3820).
8. Poznyakovskii V.M. *Gigienicheskie osnovy pitaniya, kachestvo i bezopasnost' pishchevykh produktov* [Food hygiene, food quality and safety]. Novosibirsk, 2005 (in Russ.).
9. Simopoulos A.P. n-3 fatty acids and human health: defining strategies for public policy. *Lipids*, 2001, 36(S1): S83-S89 (doi: 10.1007/s11745-001-0687-7).
10. Hu F.B., Manson J.E., Willett W.C. Types of dietary fat and risk of coronary heart disease: a critical review. *Journal of the American College of Nutrition*, 2001, 20(1): 5-19 (doi: 10.1080/07315724.2001.10719008).
11. Barba M., Czosnek H., Hadidi A. Historical perspective, development and applications of next-generation sequencing in plant virology. *Viruses*, 2014, 6(1): 106-136 (doi: 10.3390/v6010106).
12. Heather J.M., Chain B. The sequence of sequencers: the history of sequencing DNA. *Genomics*, 2016, 107(1): 1-8 (doi: 10.1016/j.ygeno.2015.11.003).
13. Jiang Y., Xie M., Chen W., Talbot R., Maddox J.F., Faraut T., Wu C., Muzny D.M., Li Y., Zhang W., Stanton J.A., Brauning R., Barris W.C., Hourlier T., Aken B.L., Searle S.M.J., Adelson D.L., Bian C., Cam G.R., Chen Y., Cheng S., DeSilva U., Dixen K., Dong Y., Fan G., Franklin I.R., Fu S., Guan R., Highland M.A., Holder M.E., Huang G., Ingham A.B., Jhangiani S.N., Kalra D., Kovar C.L., Lee S.L., Liu W., Liu X., Lu C., Lv T., Mathew T., McWilliam S., Menzies M., Pan S., Robelin D., Servin B., Townley D., Wang W., Wei B., White S.N., Yang X., Ye C., Yue Y., Zeng P., Zhou Q., Hansen J.B., Kristensen K., Gibbs R.A., Flicek P., Warkup C.C., Jones H.E., Oddy V.H., Nicholas F.W., McEwan J.C., Kijas J., Wang J., Worley K.C., Archibald A.L., Cockett N., Xu X., Wang W., Dalrymple B.P. The sheep genome illuminates biology of the rumen and lipid metabolism. *Science*, 2014, 344(6188): 1168-1173 (doi: 10.1126/science.1252806).
14. Li X., Yang J., Shen M., Xie X.L., Liu G.J., Xu Y.X., Lv F.H., Yang H., Yang Y.L., Liu C.B., Zhou P., Wan P.C., Zhang Y.S., Gao L., Yang J.Q., Pi W.H., Ren Y.L., Shen Z.Q., Wang F., Deng J., Xu S.S., Salehian-Dehkordi H., Hehua E., Esmailizadeh A., Dehghani-Qanatqestani M., Štěpánek O., Weimann C., Erhardt G., Amane A., Mwacharo J.M., Han J.L., Hanotte O., Lenstra J.A., Kantanen J., Coltman D.W., Kijas J.W., Bruford M.W., Periasamy K., Wang X.H., Li M.H. Whole-genome resequencing of wild and domestic sheep identifies genes associated with morphological and agronomic traits. *Nature Communications*, 2020, 11(1): 2815 (doi: 10.1038/s41467-020-16485-1).
15. Bumgarner R. Overview of DNA microarrays: types, applications, and their future. *Current Protocols in Molecular Biology*, 2013, 101: 22.1.1-22.1.11 (doi: 10.1002/0471142727.mb2201s101).
16. Dekkers J.C. Application of genomics tools to animal breeding. *Current Genomics*, 2012, 13(3): 207-212 (doi: 10.2174/138920212800543057).
17. Koopaee H.K., Koshkoiyeh A.E. SNPs genotyping technologies and their applications in farm animals breeding programs. *Brazilian Archives of Biology and Technology*, 2014, 57(1): 87-95 (doi: 10.1590/S1516-89132014000100013).
18. 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., other members of the International Sheep Genomics Consortium. 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).
19. Mardis E.R. The impact of next-generation sequencing technology on genetics. *Trends in Genetics*, 2008, 24(3): 133-141 (doi: 10.1016/j.tig.2007.12.007).
20. Mutz K.O., Heilkenbrinker A., Lönne M., Walter J.G., Stahl F. Transcriptome analysis using next-generation sequencing. *Current Opinion in Biotechnology*, 2013, 24(1): 22-30 (doi: 10.1016/j.copbio.2012.09.004).
21. Oshlack A., Robinson M.D., Young M.D. From RNA-seq reads to differential expression results. *Genome Biology*, 2010, 11(12): 220 (doi: 10.1186/gb-2010-11-12-220).
22. Stark R., Grzelak M., Hadfield J. RNA sequencing: the teenage years. *Nature Reviews Genetics*, 2019, 20(11): 631-656 (doi: 10.1038/s41576-019-0150-2).
23. VanRaden P.M. Efficient methods to compute genomic predictions. *Journal of Dairy Science*, 2008, 91(11): 4414-4423 (doi: 10.3168/jds.2007-0980).
24. Goddard M.E., Hayes B.J. Genomic selection. *Journal of Animal Breeding and Genetics*, 2007, 124(6): 323-330 (doi: 10.1111/j.1439-0388.2007.00702.x).
25. Alekseeva A., Magomadov T., Yuldashbaev Yu. *Glavnyi zootekhnik*, 2018, 7: 32-37 (in Russ.).
26. Glagoev A.Ch. *Metody povysheniya produktivnosti i effektivnosti ispol'zovaniya porodnykh resursov v ovtsevodstve. Doktorskaya dissertatsiya* [Methods for increasing productivity and efficiency of sheep breed resources in breeding. DSc Thesis]. Michurinsk—Naukograd, 2019 (in Russ.).
27. Glagoev A.Ch., Negreeva A.N., Shchugoreva T.E. *Tekhnologii pishchevoi i pererabatyvayushchei promyshlennosti APK — produkty zdorovogo pitaniya*, 2021, 1: 137-144 (in Russ.).
28. Erokhin A.I., Karasev E.A., Yuldashbaev Yu.A., Magomadov T.A., Medvedev M.V. *Izvestiya Timiryazevskoi sel'skokhozyaistvennoi akademii*, 2012, 2: 126-135 (in Russ.).
29. Muratova V.V. *Myasnaya produktivnost' i otsenka kachestva myasa molodnyaka ovets edil'baevskoi porody raznykh vesovykh kategorii. Kandidatskaya dissertatsiya* [Meat productivity and meat quality assessment of young Edilbaev sheep of different weight categories. PhD Thesis]. Saratov, 2020 (in Russ.).
30. Kipkeev M.Kh., Sel'kin I.I. *Sel'skokhozyaistvennyi zhurnal*, 2004, 1-1: 23-28 (in Russ.).
31. Mugaev M.A., Khatataev S.A., Grigoryan L.N. Parameters of meat quality in young sheeps of the romanovskaya breed in connection with lambing season. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2011, 6: 110-115 (in Russ.).
32. Liu K., Ge S., Luo H., Yue D., Yan L. Effects of dietary vitamin E on muscle vitamin E and fatty acid content in Aohan fine-wool sheep. *Journal of Animal Science and Biotechnology*, 2013, 4(1): 21 (doi: 10.1186/2049-1891-4-21).
33. Knapik J., Ropka-Molik K., Pieszka M. Genetic and nutritional factors determining the production and quality of sheep meat — a review. *Annals of Animal Science*, 2017, 17(1): 23-40 (doi: 10.1515/aoas-2016-0036).
34. Chai J., Diao Q., Zhao J., Wang H., Deng K., Qi M., Nie M., Zhang N. Effects of rearing system on meat quality, fatty acid and amino acid profiles of Hu lambs. *Animal Science Journal*, 2018, 89(8): 1178-1186 (doi: 10.1111/asj.13013).
35. Karamichou E., Richardson R.I., Nute G.R., Gibson K.P., Bishop S.C. Genetic analyses and quantitative trait loci detection, using a partial genome scan, for intramuscular fatty acid composition in Scottish Blackface sheep. *Journal of Animal Science*, 2006, 84(12): 3228-3238 (doi: 10.2527/jas.2006-204).
36. Daetwyler H.D., Swan A.A., van der Werf J.H., Hayes B.J. Accuracy of pedigree and genomic predictions of carcass and novel meat quality traits in multi-breed sheep data assessed by cross-validation. *Genetics Selection Evolution*, 2012, 44: 33 (doi: 10.1186/1297-9686-44-33).
37. Mortimer S.I., van der Werf J.H.J., Jacob R.H., Hopkins D.L., Pannier L., Pearce K.L., Gardner G.E., Warner R.D., Geesink G.H., Hocking Edwards J.E., Ponnampalam E.N., Ball A.J., Gilmour A.R., Pethick D.W. Genetic parameters for meat quality traits of Australian lamb meat. *Meat Science*, 2014, 96(2): 1016-1024 (doi: 10.1016/j.meatsci.2013.09.007).
38. Bolormaa S., Hayes B.J., van der Werf J.H., Pethick D., Goddard M.E., Daetwyler H.D. Detailed phenotyping identifies genes with pleiotropic effects on body composition. *BMC Genomics*, 2016, 17: 224 (doi: 10.1186/s12864-016-2538-0).
39. Rovadoscki G.A., Pertile S.F.N., Alvarenga A.B., Cesar A.S.M., Pértille F., Petrini J., Franzo V., Soares W.V.B., Morota G., Spangler M.L., Pinto L.F.B., Carvalho G.G.P., Lanna D.P.D., Coutinho L.L., Mourão G.B. Estimates of genomic heritability and genome-wide association study for fatty acids profile in Santa Inкs sheep. *BMC Genomics*, 2018, 19(1): 375 (doi: 10.1186/s12864-018-4777-8).
40. van der Werf J.H.J., Kinghorn B.P., Banks R.G. Design and role of an information nucleus in sheep breeding programs. *Animal Production Science*, 2010, 50(12): 998-1003 (doi: 10.1071/AN10151).
41. White J.D, Allingham P.G., Gorman C.M., Emery D.L., Hynd P., Owens J., Bell A., Siddell J., Harper G., Hayes B.J., Daetwyler H.D., Usmar J., Goddard M.E., Henshall J.M., Dominik S.,

Brewer H., van der Werf J.H.J., Nicholas F.W., Warner R., Hofmyer C., Longhurst T., Fisher T., Swan P., Forage R., Oddy V.H. Design and phenotyping procedures for recording wool, skin, parasite resistance, growth, carcass yield and quality traits of the SheepGENOMICS mapping flock. *Animal Production Science*, 2012, 52(3): 157-171 (doi: 10.1071/AN11085).
42. Hu Z.L., Park C.A., Reecy J.M. Developmental progress and current status of the Animal QTLdb. *Nucleic Acids Research*, 2016, 44(D1): D827-D833 (doi: 10.1093/nar/gkv1233).
43. Hu Z.L., Park C.A., Reecy J.M. Building a livestock genetic and genomic information knowledgebase through integrative developments of Animal QTLdb and CorrDB. *Nucleic Acids Research*, 2019, 47(D1): D701-D710 (doi: 10.1093/nar/gky1084).
44. Sumara G., Sumara O., Kim J.K., Karsenty G. Gut-derived serotonin is a multifunctional determinant to fasting adaptation. *Cell Metabolism*, 2012, 16(5): 588-600 (doi: 10.1016/j.cmet.2012.09.014).
45. Laporta J., Hernandez L.L. Serotonin receptor expression is dynamic in the liver during the transition period in Holstein dairy cows. *Domestic Animal Endocrinology*, 2015, 51: 65-73 (doi: 10.1016/j.domaniend.2014.11.005).
46. Aliesky H.A., Pichurin P.N., Chen C.R., Williams R.W., Rapoport B., McLachlan S.M. Probing the genetic basis for thyrotropin receptor antibodies and hyperthyroidism in immunized CXB recombinant inbred mice. *Endocrinology*, 2006, 147(6): 2789-2800 (doi: 10.1210/en.2006-0160).
47. Cavanagh C.R., Jonas E., Hobbs M., Thomson P.C., Tammen I., Raadsma H.W. Mapping quantitative trait loci (QTL) in sheep. III. QTL for carcass composition traits derived from CT scans and aligned with a meta-assembly for sheep and cattle carcass QTL. *Genetics Selection Evolution*, 2010, 42(1): 36 (doi: 10.1186/1297-9686-42-36).
48. Hwang D. Fatty acids and immune responses-a new perspective in searching for clues to mechanism. *Annual Review of Nutrition*, 2000, 20: 431-456 (doi: 10.1146/annurev.nutr.20.1.431).
49. Muoio D.M., MacLean P.S., Lang D.B., Li S, Houmard J.A., Way J.M., Winegar D.A., Corton J.C., Dohm G.L., Kraus W.E. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. Evidence for compensatory regulation by PPAR delta. *Journal of Biological Chemistry*, 2002, 277(29): 26089-26097 (doi: 10.1074/jbc.M203997200).
50. Yen C.-L.E., Stone S.J., Koliwad S., Harris C., Farese R.V. Jr. Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *Journal of Lipid Research*, 2008, 49(11): 2283-2301 (doi: 10.1194/jlr.R800018-JLR200).
51. Drackley J.K. Lipid metabolism. In: *Farm animal metabolism and nutrition*. J.P.F. D'Mello (ed.). CABI, New York, 2000.
52. Ross S.E., Hemati N., Longo K.A., Bennett C.N., Lucas P.C., Erickson R.L., MacDougald O.A. Inhibition of adipogenesis by Wnt signaling. *Science*, 2000, 289(5481): 950-953 (doi: 10.1126/science.289.5481.950).
53. Takada I., Kouzmenko A.P., Kato S. Wnt and PPARgamma signaling in osteoblastogenesis and adipogenesis. *Nature Reviews Rheumatology*, 2009, 5(8): 442-447 (doi: 10.1038/nrrheum.2009.137).
54. Galic S., Oakhill J.S., Steinberg G.R. Adipose tissue as an endocrine organ. *Molecular and Cellular Endocrinology*, 2010, 316(2): 129-139 (doi: 10.1016/j.mce.2009.08.018).
55. Cristancho A.G., Lazar M.A. Forming functional fat: a growing understanding of adipocyte differentiation. *Nature Reviews Molecular Cell Biology*, 2011, 12: 722-734 (doi: 10.1038/nrm3198).
56. Waters M., Serafini T., Rothman J. 'Coatomer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature*, 1991, 349: 248-251 (doi: 10.1038/349248a0).
57. Kuhajda F.P., Jenner K., Wood F.D., Hennigar R.A., Jacobs L.B., Dick J.D., Pasternack G.R. Fatty acid synthesis: a potential selective target for antineoplastic therapy. *Proceedings of the National Academy of Sciences*, 1994, 91(14): 6379-6383 (doi: 10.1073/pnas.91.14.6379).
58. Niture S., Gyamfi M.A., Lin M., Chimeh U., Dong X., Zheng W., Moore J., Kumar D. TNFAIP8 regulates autophagy, cell steatosis, and promotes hepatocellular carcinoma cell proliferation. *Cell Death and Disease*, 2020, 11(3): 178 (doi: 10.1038/s41419-020-2369-4).
59. Huang J.M., Xian H., Bacaner M. Long-chain fatty acids activate calcium channels in ventricular myocytes. *Proceedings of the National Academy of Sciences*, 1992, 89(14): 6452-6456 (doi: 10.1073/pnas.89.14.6452).
60. Xiao Y.F., Gomez A.M., Morgan J.P., Lederer W.J., Leaf A. Suppression of voltage-gated L-type Ca^{2+} currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes. *Proceedings of the National Academy of Sciences*, 1997, 94(8): 4182-4187 (doi: 10.1073/pnas.94.8.4182).
61. Murphy E.F., Jewell C., Hooiveld G.J., Muller M., Cashman K.D. Conjugated linoleic acid enhances transepithelial calcium transport in human intestinal-like Caco-2 cells: an insight into molecular changes. *Prostaglandins, Leukotrienes & Essential Fatty Acids,* 2006, 74(5): 295-301 (doi: 10.1016/j.plefa.2006.03.003).
62. Leonard A.E., Pereira S.L., Sprecher H., Huang Y.S. Elongation of long-chain fatty acids. *Progress in Lipid Research*, 2004, 43(1): 36-54 (doi: 10.1016/s0163-7827(03)00040-7).

63. Shindou H., Shimizu T. Acyl-CoA:lysophospholipid acyltransferases. *Journal of Biological Chemistry*, 2009, 284(1): 1-5 (doi: 10.1074/jbc.R800046200).
64. Kooner J.S., Chambers J.C., Aguilar-Salinas C.A., Hinds D.A., Hyde C.L., Warnes G.R., Gymez Pérez F.J., Frazer K.A., Elliott P., Scott J., Milos P.M., Cox D.R., Thompson J.F. Genome-wide scan identifies variation in MLXIPL associated with plasma triglycerides. *Nature Genetics*, 2008, 40: 149-151 (doi: 10.1038/ng.2007.61).
65. Füllekrug J., Ehehalt R., Poppelreuther M. Outlook: membrane junctions enable the metabolic trapping of fatty acids by intracellular acyl-CoA synthetases. *Frontiers in Physiology*, 2012, 3: 401 (doi: 10.3389/fphys.2012.00401).
66. Arora R., Kumar N.S., Sudarshan S., Fairoze M.N., Kaur M., Sharma A., Girdhar Y.M.S.R., Devatkal S.K., Ahlawat S., Vijh R.K., Manjunatha S.S. Transcriptome profiling of longissimus thoracis muscles identifies highly connected differentially expressed genes in meat type sheep of India. *PLoS ONE,* 2019, 14(6): e0217461 (doi: 10.1371/journal.pone.0217461).
67. Fischer H., Gustafsson T., Sundberg C.J., Norrbom J., Ekman M., Johansson O., Jansson E. Fatty acid binding protein 4 in human skeletal muscle. *Biochemical and Biophysical Research Communications*, 2006, 346(1): 125-130 (doi: 10.1016/j.bbrc.2006.05.083).
68. Stern J.H., Rutkowski J.M., Scherer P.E. Adiponectin, leptin, and fatty acids in the maintenance of metabolic homeostasis through adipose tissue crosstalk. *Cell Metabolism*, 2016, 23(5): 770-784 (doi: 10.1016/j.cmet.2016.04.011).
69. Li B., Zerby H.N., Lee K. Heart fatty acid binding protein is upregulated during porcine adipocyte development. *Journal of Animal Science*, 2007, 85(7): 1651-1659 (doi: 10.2527/jas.2006-755).
70. Coe N.R., Simpson M.A., Bernlohr D.A. Targeted disruption of the adipocyte lipid-binding protein (aP2 protein) gene impairs fat cell lipolysis and increases cellular fatty acid levels. *Journal of Lipid Research*, 1999, 40(5): 967-972 (doi: 10.1016/S0022-2275(20)32133-7).
71. Furuhashi M., Saitoh S., Shimamoto K., Miura T. Fatty acid-binding protein 4 (FABP4): pathophysiological insights and potent clinical biomarker of metabolic and cardiovascular diseases. *Clinical Medicine Insights*: Cardiology, 2015, 8(Suppl. 3): 23-33 (doi: 10.4137/CMC.S17067).
72. Xu X., Chen W., Yu S., Fan S., Ma W. Candidate genes expression affect intramuscular fat content and fatty acid composition in Tan sheep. *Genetics and Molecular Research*, 2020, 19(4): GMR18550 (doi: 10.4238/gmr18550).
73. Wolf G. Adiponectin: a regulator of energy homeostasis. *Nutrition Review*, 2003, 61(8): 290-292 (doi: 10.1301/nr.2003.aug.290-292).
74. Kadowaki T., Yamauchi T. Adiponectin and adiponectin receptors. *Endocrine Reviews*, 2005, 26(3): 439-451 (doi: 10.1210/er.2005-0005).
75. An Q.M., Zhou H.T., Hu J., Luo Y.Z., Hickford J.G. Haplotypes and sequence variation in the ovine adiponectin gene (ADIPOQ). *Genes*, 2015, 6(4): 1230-1241 (doi: 10.3390/genes6041230).
76. An Q., Zhou H., Hu J., Luo Y., Hickford J.G.H. Haplotypes of the ovine adiponectin gene and their association with growth and carcass traits in New Zealand Romney lambs. *Genes*, 2017, 8(6): 160 (doi: 10.3390/genes8060160).
77. Okumoto K., Kametani Y., Fujiki Y. Two proteases, trypsin domain-containing 1 (Tysnd1) and peroxisomal lon protease (PsLon), cooperatively regulate fatty acid β-oxidation in peroxisomal matrix. *Journal of Biological Chemistry*, 2011, 286(52): 44367-44379 (doi: 10.1074/jbc.M111.285197).
78. 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).
79. Deniskova T.E., Kunz E., Medugorac I., Dotsev A.V., Brem G., Zinovieva N.A. A study of genetic mechanisms underlying the fat tail phenotype in sheep: methodological approaches and identified candidate genes (review). *Agricultural Biology*, 2019, 54(6): 1065-1079 (doi: 10.15389/agrobiology.2019.6.1065eng).
80. Sun L., Bai M., Xiang L., Zhang G., Ma W., Jiang H. Comparative transcriptome profiling of longissimus muscle tissues from Qianhua Mutton Merino and Small Tail Han sheep. *Scientific Reports*, 2016, 6: 33586 (doi: 10.1038/srep33586).
81. Zimmerman A.W., Veerkamp J.H. New insights into the structure and function of fatty acid-binding proteins. *Cellular and Molecular Life Sciences*, 2002, 59(7): 1096-1116 (doi: 10.1007/s00018-002-8490-y).
82. Senga S., Kobayashi N., Kawaguchi K., Ando A., Fujii H. Fatty acid-binding protein 5 (FABP5) promotes lipolysis of lipid droplets, de novo fatty acid (FA) synthesis and activation of nuclear factor-kappa B (NF-κB) signaling in cancer cells. *Biochimica et Biophysica Acta (BBA) — Molecular and Cell Biology of Lipids*, 2018, 1863(9): 1057-1067 (doi: 10.1016/j.bbalip.2018.06.010).
83. Corominas J., Ramayo-Caldas Y., Puig-Oliveras A., Pérez-Montarelo D., Noguera J.L., Folch J.M., Ballester M. Polymorphism in the ELOVL6 gene is associated with a major QTL effect on fatty acid composition in pigs. *PLoS ONE*, 2013, 8(1): e53687 (doi: 10.1371/journal.pone.0053687).
84. Miao X., Luo Q., Qin X. Genome-wide analysis reveals the differential regulations of mRNAs

and miRNAs in Dorset and Small Tail Han sheep muscles. *Gene,* 2015, 562(2): 188-196 (doi: 10.1016/j.gene.2015.02.070).
85. Pucci S., Zonetti M., Fisco T., Polidoro C., Bocchinfuso G., Palleschi A., Novelli G., Spagnoli L.G., Mazzarelli P. Carnitine palmitoyl transferase-1A (CPT1A): a new tumor specific target in human breast cancer. *Oncotarget*, 2016, 7: 19982-19996 (doi: 10.18632/oncotarget.6964).
86. O'Byrne J., Hunt M.C., Rai D.K., Saeki M., Alexson S.E. The human bile acid-CoA:amino acid N-acyltransferase functions in the conjugation of fatty acids to glycine. *Journal of Biological Chemistry*, 2003, 278(36): 34237-34244 (doi: 10.1074/jbc.M300987200).
87. Anderson C.M., Stahl A. SLC27 fatty acid transport proteins. *Molecular Aspects of Medicine*, 2013, 34(2-3): 516-528 (doi: 10.1016/j.mam.2012.07.010).
88. 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. *Scientific Reports*, 2017, 7(1): 14491 (doi: 10.1038/s41598-017-14967-9).
89. Palmer B.R., Roberts N., Hickford J.G., Bickerstaffe R. Rapid communication: PCR-RFLP for MspI and NcoI in the ovine calpastatin gene. *Journal of Animal Science*, 1998, 76(5): 1499-1500 (doi: 10.2527/1998.7651499x).
90. Chizhova L.N., Karpova E.D., Surzhikova E.S., Zabelina M.V. *Ovtsy, kozy, sherstyanoe delo*, 2021, 2: 12-15 (doi: 10.26897/2074-0840-2021-2-12-15) (in Russ.).
91. Aali M., Moradi-Shahrbabak H., Moradi-Shahrbabak M., Sadeghi M., Yousefi R. Association of the calpastatin genotypes, haplotypes, and SNPs with meat quality and fatty acid composition in two Iranian fat- and thin-tailed sheep breeds. *Small Ruminant Research*, 2017, 149: 40-51 (doi: 10.1016/j.smallrumres.2016.12.026).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1063-1078
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 636.2:619:618.177-089.888.11

doi: 10.15389/agrobiology.2021.6.1063eng
doi: 10.15389/agrobiology.2021.6.1063rus

EMBRYO SURVIVAL TO ACCELERATE GENETIC PROGRESS IN DAIRY HERDS

(review)

O.A. SKACHKOVA ✉, A.V. BRIGIDA

Institute of Innovative Biotechnology in Animal Husbandry — Branch of the Ernst Federal Research Center for Animal Husbandry, 12/4, ul. Kostyakova, Moscow, 127422 Russia, e-mail oaskachkova@mail.ru (✉ corresponding author), brigida_86@mail.ru
ORCID:
Skachkova O.A. orcid.org/0000-0003-4960-0712 Brigida A.V orcid.org/0000-0002-0139-8087

The authors declare no conflict of interests
Acknowledgements:
The work was done within the framework of the State assignment No. 121052600344-8.
Received July 14, 2021

Abstract

Continuity of genetic progress and the use of advanced technologies in the breeding of highly productive livestock are the distinctive features of modern dairy cattle breeding (G.R. Wiggans et al., 2017; B.V. Sanches et al., 2019). An example of Holstein cows of North American selection indicates the achievement of genetic changes (more than 56,0 %) in animals over 50 years (1963-2013), when milk yield doubled from 6619 kg to 12662 kg (A. Garcia-Ruiz et al., 2016). Along with this, genetic improvements aimed at higher milk yields have decreased the reproductive capacity and impaired health of cows (J. Kropp et al., 2014; L. Hyun-Joo et al., 2015, B. Fessenden et al., 2020) that is a global problem (E.S. Ribeiro et al., 2012; K.J. Perkel et al., 2015). High-yielding cows are 30-50 % susceptible to mastitis, metritis, lameness and other diseases (I. Cruz et al., 2021), and the average calving rate is about 40-50 % with 90-95 % fertilization (M.G. Diskin et al., 1980; P. Humblot, 2001). The embryonic period of cows which is up to 42-45 days of gestation (J. Peippo et al., 2011) is characterized by high (up to 40 %) embryonic mortality (D.C. Wathes, 1992; K.J. Perkel et al., 2015; P. Rani et al., 2018), the multifactorial etiology of which has not yet been elucidated. Loss of genetic potential (unborn bull sires, replacement heifers, mothers of bull sires, and embryo donor cows) slows down selection process in dairy herds (M. Ptaszynska, 2009). This review focuses on the genetic predisposition of the embryo to survival as one of the important factors determining the onset and development of pregnancy of dairy cows. Blastocysts retain the ability to survive in stressful conditions of in vivo or in vitro production after cryopreservation-thawing (J.L.M. Vasconcelos et al., 2011; C. Galli, 2017; H. Erdem et al., 2020) and bisection (microsurgical division of the embryo in half for two demi-embryos) (Y. Hashiyada, 2017). The information on embryo survivability becomes more genetically founded as candidate genes associated with high embryo competence to development are found (M.C. Summers and J.D. Biggers, 2003; A. El-Sayed et al., 2006). Molecular genetic technologies make it possible to study the entire set of genes that endow the blastocyst with the ability to develop sustainably (A.M. Zolini et al., 2020), as well as epigenetic changes of gene expression patterns before and after embryo implantation (A. Gad et al., 2012; P. Humblot, 2018). It will help to develop methods for marker-assessed diagnostics of embryonic disorders, to regulate embryonic genes expression, to elevate the pregnancy rate in cows possessing economically valuable traits and, finally, to accelerate genetic progress in dairy cattle populations.

Keywords: genomic selection, transcriptomes, high-yielding cows, embryonic mortality, genetic progress, molecular genetic markers

Holstein cattle are common in herds in many parts of the world [1]. However, the increase in milk productivity caused numerous health problems in cows (in 30.0-50.0% of cases of all registered diseases, these are mastitis, metritis, lameness, milk fever, ketosis) [2], and also led to a decrease in reproductive ability [3-7], which was the result of one-sided selection and genetic improvements and has a negative impact on modern dairy cattle breeding [8-10].

The average calving rate in high-yielding cows is about 40-50% with a fertilization of 90-95% [11, 12]. Frequent events include high (up to 40.0%) embryonic mortality [13-15] in the period from fertilization to 42-45 days of pregnancy [16]. Fetal death between 40 and 80 days of pregnancy occurs in 2.0-6.0% of cases, in the remaining period in 4.0% of cases [17]. In addition, industrial housing conditions lead to injury, stress, hyperthermia, pyrexia [18] and, consequently, to a longer period between calving, infertility, a significant percentage of early culling [19]. This slows down the rate of genetic progress. i.e., the continuous improvement in productivity rates provided by the continuity of the breeding process, the effectiveness of which depends on the rapid reproduction of animals with economically significant genotypes.

The etiology of the high embryonic mortality that is recorded in herds is still not understood [20]. Among the factors influencing embryonic death in pregnant high-yielding cows are the oviduct environment which regulates the development of the embryo up to the blastocyst stage [21-23], and the uterine environment before embryo implantation [24, 25]. In addition, attention is focused on the natural mechanism of adaptation of the blastocyst to environmental conditions, due to the genetic predisposition of the embryo to survival [17, 26, 27] which is determined by hereditary factors [28], i.e., the genetic information transmitted to the embryo from the egg [29] and from the sperm [30, 31].

Molecular studies of the relationship between gene expression and early embryonic development or its delay can provide insight into the genetic and epigenetic mechanisms that ensure the viability of the embryo.

The purpose of this review is to summarize data on the ability of bovine embryos to survive when obtained in vivo or in vitro, after cryopreservation and thawing, after microsurgical division, and during transplantation and pregnancy.

Embryo transplantation technology (ETT), widely practiced in the breeding of high-producing animals, makes it possible to obtain a large number of embryos from genetically valuable donor cows fertilized by the semen of outstanding sires in a short period [32, 33]. Among the methods that form the basis of TTE, MOET (multiple ovulation and embryo transfer method) through which embryos are obtained in vivo [34] and IVP (in vitro production method) designed to obtain embryos in vitro [35] are important.

The transfer of in vivo or in vitro derived embryos to less valuable recipient heifers allows faster reproduction of more offspring than natural reproduction [36, 37]. According to the International Embryo Transfer Society (IETS), more than 20 million dairy and beef cattle embryos were received worldwide between 2000 and 2019, and in 2019 in 39 countries, which accounted for approximately half of the world livestock (Russia, USA, Canada, Brazil, France, Italy, etc.), and 1,419,336 commercial embryos suitable for transplantation have been produced [38]. With TTE, a significant part of embryos in vivo degenerates and dies before reaching the blastocyst stage. On days 6-7, in superovulated dairy donor cows with a productivity of 85-95%, approximately 50% of viable embryos were retrieved [39].

Embryo survival with the MOET method. The essence of the MOET method is that in a genetically valuable cow (an embryo donor), the growth and maturation of many egg-producing follicles (superovulation induction) is artificially activated by the administration of follicle-stimulating hormone (FSH) preparations. On day 7 after insemination of a donor cow, in vivo embryos are removed from its reproductive organs and transplanted (freshly obtained or frozen-thawed) to less valuable recipients [40]. The technological process and the means used in this manipulation imply stresses and traumatization of both the resulting embryos and donor cows. Donor cows get into a stressful situation already in

preparation for the superovulation induction procedure, when the animal is caught and fixed. The FSH preparation is used in strict accordance with the scheme (8-10-fold injection every 12 hours for 4-5 days). In response to multifactorial influences, physiological and metabolic processes in the body change. The number of in vivo embryos produced by donor cows varies over a wide range [41-43]. According to international practice, 30% of donors have no ovarian response to exogenous gonadotropins [44], 30% of donors show an extremely low ovarian response with a number of ovulations of 1-3, which corresponds to the natural process of ovulation. And only in one third of donors there is a superovulatory response with the number of ovulations from 5 to 12 [45-47].

The oviduct of the female cattle serves as a place for the fertilization of the egg and the location of the embryo during the first 4 days. At the 16-cell stage of development (early morula), the embryo moves into the uterine cavity, where it develops to the morula stage and on day 7 to the blastocyst stage (pre-implantation stage of development) [48, 49]. On days 8-9, the blastocyst cavity significantly increases in size, its zona pellucida stretches, becomes thin, and breaks (hatching process), removing the embryo from the zona pellucida [39, 50]. Further, the blastocyst attaches to the endometrium of the uterus, the low receptivity of which causes failures in the implantation of the embryo, including during IVF programs. The processes that determine the readiness of the endometrium to accept an embryo in cattle are not well understood [51)], while in humans, the genes of the HOX family (Homeobox) and the proteins encoded by them, for example, HOX10 and HOX11, are known, which are involved in the regulation of implantation and serve as key regulators of receptivity processes. endometrium [52, 53].

In MOET programs, 7-day-old embryos are removed from the reproductive organs of a donor cow in a non-surgical way using specialized equipment. The technical removal of an embryo from its natural environment increases stress [54]. In addition, unavoidable losses of embryos occur during retrieval, ranging from 60-80 to 20-30% of the counted number of corpora lutea [55].

After retrieval, the embryos are placed in an artificial environment and labeled based on the International Embryo Transfer Society (IETS) guidelines [56, 57]. The stages of embryo development are determined (stage codes from 1 to 8) and their quality is assessed for suitability for transplantation (quality codes from 1 to 4). Embryos with a quality code of 1 (excellent or good), which are at the stages of development from compact morula (stage code 4) to blastocyst (stage codes 5 or 6), provide the highest pregnancy rates, including after cryopreservation. Embryos with quality codes 2 (satisfactory) and 3 (poor) after cryopreservation show low pregnancy rates in recipients, so they are used for transplantation only in a fresh form. In embryo collections, in addition to embryos suitable for transplantation, as a rule, there are oocytes (unfertilized eggs), unicellular or degenerated embryos that are not viable (quality code 4) and must be disposed of. According to many years of world practice, an average of 58.0% of embryos suitable for transplantation are detected in embryo collections, and the rest are degenerated embryos (11.0%) and unfertilized eggs (31.0%) [58]. After one session of MOET, on average, 6.2 in vivo embryos suitable for transplantation are obtained from one donor cow [59], and over 40 embryos in vivo for 1 year when using this method every 45 days [60]. Cases have been registered when up to 50 in vivo embryos suitable for transplantation were obtained from one donor cow during one session of superovulation stimulation [45]. The ability of freshly obtained in vivo embryos to survive when transplanted to recipients is evidenced by the pregnancy rate of 45.0-55.0%, after transplantation of frozen-thawed embryos, this figure is 30.0-45.0% [61-65]. Therefore, a significant part of the embryos

repeatedly subjected to technological stresses demonstrates the ability to survive which is confirmed by the birth of calves.

Embryo survival in the IVP system. The IVP method is even more aggressive than MOET, but both methods serve as an important tool in cattle breeding to increase the number of offspring from animals of high genetic value, which maximizes the reproductive capacity of cows over a shorter period of time [66].

In the production of IVP embryos, eggs are obtained in vivo or post mortem (after the slaughter of the animal). A transvaginal aspiration method is used, which is commonly known as the OPU (ovum pick-up) method. i.e., the collection of immature oocytes from the ovaries of donor cows under ultrasound control [33, 67]. The essence of IVP is that the resulting oocytes are cultivated in the laboratory under in vitro conditions for maturation (in vitro maturation, IVM), artificially matured oocytes are subjected to in vitro fertilization (IVF), after which the fertilized oocytes (zygotes) are cultured in a growth medium (in vitro culture, IVC) to develop the embryo to the blastocyst stage [68]. Oocytes are able to resume meiosis during IVM, split after fertilization (IVF), develop to the blastocyst stage in IVC, and induce pregnancy leading to the birth of healthy offspring, which is generally interpreted as developmental competence of oocytes [69].

In addition to hormonal stimulation, follicular wave phase, follicle diameter, feeding conditions, and donor age, the developmental competence of oocytes is affected by the in vitro culture process [70]. The transfer of oocytes from one culture medium to another, as well as the composition of the medium and culture conditions during IVC, can cause physico-chemical (temperature, osmolality and pH), oxidative (pro-oxidant and antioxidant balance) and energy (use and accumulation of nutrients, synthesis) in the embryo. ATP) stresses leading to misregulation of homeostasis at an early stage of development [71].

With the use of molecular technologies, it became possible to study various indicators of embryo development at all stages of IVP. It has been shown that the development of embryos under certain culture conditions leads not only to a change in the expression of genes associated with metabolism and growth, but also to a change in the concept and development of the fetus after transfer to recipients [72]. Embryo stress responses during in vitro culture correlate with transcriptomic changes associated with energy metabolism, signaling pathways, and extracellular matrix remodeling [71, 72]. It is assumed that the transcriptomic changes that occur during the blastulation period are the result of the adaptation of the embryo to environmental factors, and such adaptation, under suboptimal cultivation conditions, can cause epigenetic changes leading to metabolic imbalance that negatively affects the process of implantation, development of the embryo, and its health in the postnatal period [71, 73].

Embryos derived from in vitro matured oocytes are less viable than embryos derived from naturally ovulating oocytes [74-78]. As practice shows, 90% of oocytes extracted from the follicles of a donor cow are capable of meiosis and maturation, 80% of fertilized oocytes (zygotes) develop to the 2-cell stage, but only 30-40% of them can develop to the blastocyst stage [39, 79-81]. On one donor cow, the IVP method is used every 15 days, receiving more than 72 in vitro embryos within one year, when three in vitro embryos are produced on average in one technological cycle [60]. After transfer of embryos in vitro, the pregnancy rate in recipients is 10-40% lower compared to embryos in vivo, in addition, 60.0% of pregnancies are terminated during the first 6 weeks, and live calves are born in 27% of cases [82]. Compared to in vivo embryos, in vitro embryos are characterized by lower cryotolerance during cryopreservation [83-85], and the engraftment rates of vitrified embryos in vitro, recorded by Sanches et al. [86], on day 30 after

transplantation, were 35.89±3.87% (84/234) vs. 51.35±1.87% (133/259) after transplantation of freshly obtained embryos. The observed (albeit small) percentage of calves that developed from oocytes subjected to numerous manipulations outside their natural environment indicates the presence of an adaptation mechanism, the understanding of which will become possible with the accumulation of experimental data.

Microsurgical division of the embryo in half and the survival of demi-embryos. Cattle are singletons that give birth to one calf per year. With natural reproduction, the appearance of twin calves (mono- and dizygotic twins) occurs extremely rarely, in 3-5% of cases in dairy cattle and in no more than 1% of cases in beef cattle, the percentage of birth of monozygotic twins is even lower [87]. In dairy cattle, the probability of having monozygotic twins occurs in no more than 0.001% of calvings [88].

The developed method of microsurgical division of the embryo in vivo in half (bisection) [89-92] offered a simple way to increase 2-fold the number of in vivo embryos. During bisection, the embryo (at the morula or blastocyst stage) is placed on a laboratory watch glass or in a Petri dish with an artificial nutrient medium. After fixation, the embryo is divided under a microscope into two halves [93, 94], which should be of the same size, and blastomeres and trophoblast cells should be evenly distributed [95]. The bisection method is based on the unique property of totipotency that mammalian gametes (egg and sperm) acquire immediately after fertilization: the zygote begins to split, forming blastomeres, while each blastomere is able to generate a full-fledged organism, but this ability is lost in the course of embryo development with the onset of cell differentiation [96]. After microsurgical division of the embryo, each of the halves within several hours (from 1 to 3 hours) in a nutrient medium at room temperature restores the spherical shape typical of the embryo (demi-embryo) [89, 97]. Immediately after recovery, demi-embryos can be transferred to recipients.

Embryonic death in demi-embryos is recorded much more often in comparison with intact (intact) embryos [98], but after the demi-embryo engraftment, it develops similarly to the intact one [95]. According to Hashiyada [99], the pregnancy rate in recipients after transplantation of demi-embryos in vivo is 36.4-53.2%, according to Lopatarova et al. [100] 48.8-56.5%. The absence of pregnancy after demi-embryo transfer is mainly due to damage and loss of blastomeres during the bisection procedure, as well as with insufficiently effective methods of culturing halves of a divided embryo [101]. Despite the damage caused to the embryo during bisection, for several decades, thousands of twin calves without signs of developmental anomalies were obtained from demi-embryos around the world [102]. However, the bisection method has not been widely used in practice, since it is difficult to perform such manipulations in a farm environment [33].

The development of molecular technologies has expanded the scope of the bisection method, which makes it possible to conduct scientific research on monozygotic genetically homologous demi-embryos [99]. Thus, the expression patterns of genes associated with the genetically determined ability of the embryo to survive in the mother-embryo system were studied. In the studies of Zolini et al. [17, 27], to identify marker genes that correlate with embryo survival, one part of a demi-embryo was transplanted into the recipient, and other part was used for RNA-seq analysis. The bisection method is also used in breeding farms in testing sires for offspring. This reduces the interval between generations, which allows the use of such bulls at a younger age [99].

The genetically determined ability of the embryo to survive. Gene polymorphism has been recognized as the most effective mechanism that ensures both the homeostasis of the organism and the dynamic constancy of

the population [103]. The regulation of gene activity and activation of regulatory genes play an important role [104]. Due to gene polymorphism, the embryo is programmed to be resistant to damage, and its genotype has an individual potential for variability depending on environmental conditions [103, 105].

Before the advent of modern molecular genetic technologies, the study of genes involved in early embryonic development was difficult, but whole genome studies are now possible using advanced microarrays that allow profiling of gene expression based on quantitative measurements [106]. Zolini et al. [17, 27] studied gene activity in transferred bovine embryo survivors and non-survivors. In embryos obtained in vivo, among the genes differentially expressed in viable and nonviable embryos, the most transcribed cluster was associated with membrane proteins, especially those involved in the development and functioning of the nervous system, in particular in the formation of the olfactory function [17]. Interestingly, in the survivors derived from in vivo embryo transplantation, there were the genes for oxidative phosphorylation the activity of which was suppressed [17]. In case of engraftment of embryos obtained in vitro [27], many differentially expressed genes involved in survival were associated with cellular responses to stress. The authors suggested that this is a consequence of disturbances caused by embryo culture. It also turned out that the set of genes associated with the survival of embryos, and the biological functions associated with these genes, are significantly different in embryos obtained in vivo and in vitro.

In bioptates of 7-day-old bovine blastocysts, Salehi et al. [106] revealed 6765 genes associated with numerous biological processes, such as regulation of the metaphase-anaphase transition of the cell cycle, regulation of chromosome segregation, mitochondrial translation, ubiquitination associated with the K48 protein, and mitotic nuclear fission. El-Sayed et al. [72], who studied gene expression in in vitro blastocyst bioptates transplanted into recipients, showed that the regulation of gene activity was different in the absence of pregnancy and in the case of calf birth. For a number of genes, such as *TNF* (pro-inflammatory cytokine), *EEF1A1* (enzymatic delivery of aminoacyl-tRNA to the ribosome), *PTTG1* (oncogene), *AKR1B1* (glucose metabolism), and *CD9* (implantation inhibitor gene), increased expression was found, which correlated with the inability to induce pregnancy. The implantation-associated genes (*COX2* and *CDX2*), genes for carbohydrate metabolism (*ALOX15*), growth factor (*BMP15*), signal transduction (*PLAU*), and placental development (*PLAC8*) were involved in calf birth. In bovine blastocysts cultured in vitro, Suwik et al. [78], when profiling transcripts of the *IGF1R*, *IGF2R*, *OCT4*, *SOX2*, and *PLAC8* genes, showed a change in their expression depending on the stage of blastocyst development and quality. In a transcriptomic analysis of in vitro blastocysts obtained from oocytes exposed to elevated concentrations of non-esterified fatty acids (NEFA), Van Hoeck et al. [107] found physiological changes in developing embryos and a decrease in their survival compared to controls.

At present, the entire set of genes and transcriptomes associated with the characteristics of the development and survival of the bovine embryo is not fully understood. It is expected that the study of transcriptome abnormalities will lead to methodological progress in assessing embryonic competence [71, 108, 109] which is understood as its development from the zygote stage (single-cell embryo) to the blastocyst (multi-cell embryo, pre-implantation stage), capable of causing pregnancy, culminating in the birth of a calf [109].

Epigenetic aspect of embryo survival. In living organisms, epigenetic regulation of gene activity is widespread, which is not associated with a change in the primary structure of DNA, but modifies the functioning of the genome depending on internal and external factors [110]. It has been shown that

epigenetic regulation is carried out through chemical modification of the DNA structure (DNA methylation, histone modifications, non-coding RNAs) or chromatin [111]. DNA methylation in blastocysts is a reversible and dynamic epigenetic mechanism involved in the remodeling of the chromatin structure, including in critical regulatory regions of the genome, and thereby affecting gene expression [112]. To date, the complex relationship between epigenetic modifications, chromatin state, and transcriptional activity in bovine embryos has not been sufficiently studied [111]. A comparative analysis of the degree of modification of certain parts of the genome in normal and pathological conditions can reveal epigenetic predictors associated with disturbances in the regulation of gene expression, which are associated with the survival of the embryo.

While still in the oviduct, the embryo undergoes epigenetic changes that affect its subsequent development, implantation and postnatal phenotype [113, 114] which is important for ensuring the correct set of genes transcribed during embryonic genome activation (zygotic genome activation, ZGA) [115)]. After fertilization, the first zygotic divisions occur in the mode of transcriptomic silence, which persists until the activation of the embryonic genome is completed. In this regard, early embryos in vitro show increased sensitivity to culture-related stress compared to later stages of pre-implantation development [71]. A study by Dobbs et al. [116] showed dynamic changes in DNA methylation in bovine embryos, that is, a decrease in methylation from the 2-cell to 6-8-cell stage during ZGA followed by an increase during further development to the blastocyst stage. This indicates that embryonic cells after ZGA acquire transcriptomic variability, providing sensitivity to external environmental conditions [117, 118].

With the expansion of experimental data, epigenetic studies of gene expression patterns in response to changes in environmental conditions before and after embryo implantation will become a source of important information on the regulation of embryonic development in MS [119, 120].

The role of MOET, IVP and embryo bisection in molecular genetic studies of embryonic development. Molecular technologies make it possible to obtain a large amount of genomic information on many biological processes in the animal body. Significant progress has been made through genomic selection (particularly in dairy farming) [80, 121].

Prior to the introduction of TTE in livestock farming practices in the 1980s, genetic progress in dairy herds was slow due to the long breeding cycle and one calf per cow [122]. The advent of MOET and IVP accelerated it by shortening the generation interval and using the best females. The combination of these methods with genomic selection for milk production traits further reduced the generation gap and increased the genetic effect due to the high selection accuracy [34]. An example of Holstein cows of North American breeding indicates the achievement of genetic changes (more than 56.0%) in the body of animals over 50 years (1963-2013), when the annual milk yield doubled from 6619 kg to 12662 kg [1]. Also, thanks to the use of TTE in combination with genomic selection for 7 years (from 2008 to 2015), in the USA, when producing genetically valuable cows and sires, the interval between generations was sharply reduced from about 7 years to < 2.5 years, and when obtaining bull-producing cows from 4 to 2.5 years [1]. There is evidence that more than 90% of Scandinavian dairy cows in Denmark, Sweden and Finland in 2018 were born from bulls that were only 3.1 years old [123]. Therefore, despite the fact that genomic selection has been used for a relatively short time, the results achieved confirm its positive impact on the efficiency of dairy cattle breeding [1].

Currently, research is ongoing on a set of genes and transcriptomic data associated with the embryonic development of cattle. It is expected that the

identification of molecular genetic markers specific for the development of a certain pathological process in early embryos will contribute to the development of methods for assessing pathogenic factors leading to early embryonic death. The CattleQTLdb database (https://www.animalgenome.org/cgi-bin/QTLdb/BT/index) integrates ever-growing volumes of data on quantitative trait loci (QTL) obtained in different countries of the world during the study of the bovine genome , as well as providing tools to study the genetic mechanisms that control traits of interest in this farm animal species [124]. In CattleQTLdb, you can quickly find relevant genotype-phenotype information for trait analysis [125], including those associated with various aspects of fertility and successful pregnancy.

The results of experiments on the study of transcriptomes of bovine embryos (from oocytes to late blastocysts), including those using next-generation sequencing (NGS) technologies, are summarized at http://emb-bioinfo.fsaa.ulaval.ca/ IMAGE/. However, the assessment of the relationship between the transcriptome profile of the preimplantation blastocyst and the onset of pregnancy in cows is still difficult, since there are no unified algorithms and approaches to interpreting data from different sources.

The decisive factors affecting the reliability of genomic estimates and predictions are the increase in the number of individuals in the reference population, which determines the relationship between phenotypes and markers, as well as the increase in the size of the reference population and the accuracy of the phenotypes of interest [127]. The MOET and IVP methods are becoming important for maintaining the genetic potential [120], making it possible to obtain tens of hundreds of embryos from genetically valuable animals in a short period of time [128]. In addition, in vitro embryos serve as a model object for molecular studies of biological functions from the unicellular stage to the blastocyst [37] and the study of oocyte maturation, fertilization, early development and implantation [78].

The use of bisection is common in the study of transcriptomes in embryos in connection with the onset of pregnancy [17, 27].

Thus, the methods of multiple ovulation, in vitro maturation, microsurgical division of embryos in half (bisections) and transplantation are quite well developed and are applicable to obtain viable embryos from genetically valuable donor cows, which, after transplantation to recipients and engraftment, can develop up to the birth of offspring. Combined with genomic research, these methods form the basis of modern reproductive biotechnologies used to accelerate genetic progress in herds. Significant factors determining the onset and development of pregnancy include the genetically determined ability of the embryo to survive in different environmental conditions, so the search for candidate genes associated with embryonic development is an important area of research aimed at increasing pregnancy rate in high-yielding cows. Modulation of the expression of embryonic genes may become a promising direction in reproduction. To implement this approach, genetic and epigenetic markers are needed to detect both violations of embryonic development and the high competence of the embryo.

REFERENCES

1. García-Ruiz A., Cole J.B., VanRaden P.M., Wiggans G. R., Ruiz-López F.J., Van Tassell C.P. Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. *Proceedings of the National Academy of Sciences*, 2016, 113(28): 3995-4004 (doi: 10.1073/pnas.1519061113).
2. Cruz I., Pereira I., Ruprechtera G., Barca J., Meikle A., Larriestra A. Clinical disease incidence during early lactation, risk factors and association with fertility and culling in grazing dairy cows in Uruguay. *Preventive Veterinary Medicine*, 2021, 191: 105359 (doi: 10.1016/j.prevetmed.2021.105359).

3. Royal M., Mann G.E., Flint A.P. Strategies for reversing the trend towards subfertility in dairy cattle. *The Veterinary Journal*, 2000, 160(1): 53-60 (doi: 10.1053/tvjl.1999.0450).
4. Lucy M.C. Reproductive loss in high-producing dairy cattle: where will it end? *Journal of Dairy Science*, 2001, 84(6): 1277-1293 (doi: 10.3168/jds.S0022-0302(01)70158-0).
5. Kropp J., Pecagaricano F., Salih S.M., Khatib H. Invited review: Genetic contributions underlying the development of preimplantation bovine embryos. *Journal of Dairy Science*, 2014, 97(3): 1187-1201 (doi: 10.3168/jds.2013-7244).
6. Hyun-Joo L., Ho-Beak Y., Harim I., Jihoo P., Yong-il C., Yeon-Seop J., Kwang-Seok K., Seok-Ki I. Survey on the incidence of reproductive disorders in dairy cattle. *Journal of Embryo Transfer*, 2015, 30(1): 59-64 (doi: 10.12750/JET.2015.30.1.59).
7. Fessenden B., Weigel D.J., Osterstock J., Galligan D.T., Di Croce F. Validation of genomic predictions for a lifetime merit selection index for the US dairy industry. *Journal of Dairy Science*, 2020, 103(11): 10414-10428 (doi: 10.3168/jds.2020-18502).
8. Randel R.D., Welsh T.H. Jr. Joint alpharma-beef species symposium: interactions of feed efficiency with beef heifer reproduction development. *Journal of Animal Science*, 2013, 91(3): 1323-1328 (doi: 10.2527/jas.2012-5679).
9. Ribeiro E.S., Galvão K.N., Thatcher W.W., Santos J.E.P. Economic aspects of applying reproductive technologies to dairy herds. *Animal Reproduction*, 2012, 9(3): 370-387.
10. Perkel K.J., Tscherner A., Merrill C., Lamarre J., Madan P. The ART of selecting the best embryo: a review of early embryonic mortality and bovine tmbryo viability assessment methods. *Molecular Reproduction Development*, 2015, 82(11): 822-838 (doi: 10.1002/mrd.22525).
11. Diskin M.G., Sreenan J.M. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. *Journal Reproduction Fertility*, 1980, 59: 463-468 (doi: 10.1530/jrf.0.0590463).
12. Humblot P. Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. *Theriogenology*, 2001, 56(9): 1417-1433 (doi: 10.1016/s0093-691x(01)00644-6).
13. Wathes D.C. Embryonic mortality and the uterine environment. *Journal of Endocrinology*, 1992, 134(3): 321-325 (doi: 10.1677/joe.0.1340321).
14. Reese S.T., Pereira M.C., Vasconcelos J.L.M., Smith M.F., Geary T.V., Peres R.F.G., Perry G.A., Pohler K.G. Markers of pregnancy: how early can we detect pregnancies in cattle using pregnancy-associated (PAGs) and microRNAs? *Animal Reproduction*, 2016, 13(3): 200-208 (doi: 10.21451/1984-3143-AR878).
15. Rani P., Dutt R., Singh G., Chandolia R.R. Embryonic mortality in cattle — a review. *International Journal of Current Microbiology and Applied Sciences*, 2018, 7(7): 1501-1516 (doi: 10.20546/ijcmas.2018.707.177).
16. Peippo J., Machaty Z., Peter A. Terminologies for the pre-attachment bovine embryo. *Theriogenology*, 2011, 76(8): 1373-1379 (doi: 10.1016/j.theriogenology.2011.06.018).
17. Zolini A.M., Block J., Rabaglino M.B., Rincon G., Hoelker M., Bromfield J.J., Salilew-Wondim D., Hansen P.J. Genes associated with survival of female bovine blastocysts produced in vivo. *Cell and Tissue Research*, 2020, 382: 665-678 (doi: 10.1007/s00441-020-03257-y).
18. Bovine reproduction. In: *Compendium of animal reproduction*. M. Ptaszynska (ed.). Intervet International BV, 2009.
19. Khatib H., Huang, W., Wang X., Tran, A H., Bindrim A.B., Schutzkus V., Monson R.L., Yandell B.S. Single gene and gene interaction effects on fertilization and embryonic survival rates in cattle. *Journal of Dairy Science*, 2009, 92(5): 2238-2247 (doi: 10.3168/jds.2008-1767).
20. Santos J.E.P., Thatcher W.W., Chebel R.C., Cerri R.L.A., Galvão K.N. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. *Animal Reproduction Science*, 2004, 82-83: 513-535 (doi: 10.1016/j.anireprosci.2004.04.015).
21. Watson A.J., Westhusin M.E., Winger Q.A. IGF paracrine and autocrine interactions between conceptus and oviduct. *Journal of Reproduction and Fertility*, 1999, 54: 303-315.
22. Avilés M., Coy P., Rizos D. The oviduct: a key organ for the success of early reproductive events. *Animal Fronttiers*, 2015, 5(1): 25-31 (doi: 10.2527/af.2015-0005).
23. Rizos D., Maillo V., Lonergan P. Role of the oviduct and oviduct-derived products in ruminant embryo development. *Animal Reproducrion*, 2016, 13(3): 160-167 (doi: 10.21451/1984-3143-AR863).
24. Forde N., Spencer T.E., Bazer F.W., Song G., Roche J.F., Lonergan P. Effect of pregnancy and progesterone concentration on expression of genes encoding for transporters or secreted proteins in the bovine endometrium. *Physiological Genomics*, 2010, 41(1): 53-62 (doi: 10.1152/physiolgenomics.00162.2009).
25. Talukder A.K., Marey M.A., Shirasuna K., Kusama K., Shimada M., Imakawa K., Miyamoto A. Roadmap to pregnancy in the first 7 days post-insemination in the cow: Immune crosstalk in the corpus luteum, oviduct, and uterus. *Theriogenology*, 2020, 150: 313-320 (doi: 10.1016/j.theriogenology.2020.01.071).
26. Kues W.A., Sudheer S., Herrmann D., Carnwath J. W., Havlicek V., Besenfelder U., Lehrach H., Adjaye J., Niemann H. Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development in vivo. *Proceedings of the National*

Academy of Sciences, 2008, 105(50): 19768-19773 (doi: 10.1073/pnas.0805616105).
27. Zolini A.M., Block J., Rabaglino M.B., Tríbulo P., Hoelker M., Rincon G., Bromfield J.J., Hansen P.J. Molecular fingerprint of female bovine embryos produced in vitro with high competence to establish and maintain pregnancy. *Biology of Reproduction*, 2020, 102(2): 292-305 (doi: 10.1093/biolre/ioz190).
28. Ledoux D., Ponsart C., Grimard B., Gatien J., Deloche M.C., Fritz S., Lefebvre R., Humblot P. Sire effect on early and late embryonic death in French Holstein cattle. *Animal*, 2015, 9(5): 766-774 (doi: 10.1017/S1751731114003140).
29. Hansen P.J., Block J., Loureiro B., Bonilla L., Hendricks K.E.M. Effects of gamete source and culture conditions on the competence of in vitro-produced embryos for post-transfer survival in cattle. *Reproduction, Fertility and Development*, 2010, 22(1): 59-66 (doi: 10.1071/RD09212).
30. Trasler J.M., Hales B.F., Robaire B. Paternal cyclophosphamide treatment of rats causes fetal loss and malformations without affecting male fertility. *Nature*, 1985, 316(6024): 144-146 (doi: 10.1038/316144a0).
31. Kumaresan A., Gupta M.D., Datta T.K., Morrell J.M. Sperm DNA integrity and male fertility in farm animals: a review. *Frontiers in Veterinary Science*, 2020, 7: 321 (doi: 10.3389/fvets.2020.00321).
32. Church R.B., Shea B.F. The role of embryo transfer in cattle improvement programs. *Canadian Journal of Animal Science*, 1977, 57(1): 33 (doi: 10.4141/cjas77-005).
33. Hasler J.F. Forty years of embryo transfer in cattle: a review focusing on the journal Theriogenology, the growth of the industry in North America, and personal reminisces. *Theriogenology*, 2014, 81(1): 152-169 (doi: 10.1016/j.theriogenology.2013.09.010).
34. Thomasen J.R., Willam A., Egger-Danner C., Sørensen A.C. Reproductive technologies combine well with genomic selection in dairy breeding programs. *Journal of Dairy Science*, 2016, 99(2): 1331-1340 (doi: 10.3168/jds.2015-9437).
35. Camargo L.S.A., Viana J.H.M., Sá W.F., Ferreira A.M., Ramos A.A., Vale Filho V.R. Factors influencing in vitro embryo production. *Animal Reproduction*, 2006, 3(1): 19-28.
36. Patel D., Haque N., Patel G., Chaudhari A., Madhavatar M., Bhalakiya N. Jamnesha N., Patel P. Implication of embryo transfer technology in livestock productivity. *International Journal of Current Microbiology and Applied Sciences*, 2018, 7: 1498-1510.
37. Sirard M.-A. 40 years of bovine IVF in the new genomic selection context. *Reproduction*, 2018, 156(1): R1-R7 (doi: 10.1530/REP-18-0008).
38. Viana J. 2019 Statistics of embryo production and transfer in domestic farm animals. *Embryo Technology Newsletter*, 2020, 38(4): 7-26.
39. Lonergan P., Fair T., Forde N., Rizos D. Embryo development in dairy cattle. *Theriogenology*, 2016, 86(1): 270-277 (doi: 10.1016/j.theriogenology.2016.04.040).
40. Petroman I., Pacala N., Petroman C. Utilization of gestagen hormones and pituitary FSH extracts in inducing the superovulation at embryo donor cows. *Journal of Food Agriculture & Environment*, 2009, 7(2): 193-195.
41. Desaulniers D.M., Lussier J.G., Gaff A.K., Bousquet D., Guilbault L.A. Follicular development and reproductive endocrinology during and after superovulation in heifers and mature cows displaying contrasting superovulatory responses. *Theriogenology*, 1995, 44(4): 479-497 (doi: 10.1016/0093-691X(95)00220-3).
42. Bó G.A., Mapletoft R.J. Historical perspectives and recent research on superovulation in cattle. *Theriogenology*, 2014, 81(1): 38-48 (doi: 10.1016/j.theriogenology.2013.09.020).
43. Naranjo-Chacón F., Montiel-Palacios F., Canseco-Sedano R., Ahuja-Aguirre C. Embryo production in middle-aged and mature *Bos taurus* × *Bos indicus* cows induced to multiple ovulation in a tropical environment. *Tropical Animal Health and Production*, 2019, 51: 2641-2644 (doi: 10.1007/s11250-019-01975-2).
44. Wohlres-Viana S., Arashiro E.K.N., Minare T.P., Fernandes C.A.C., Grazia J.G.V., Siqueira L.G.B., Machado M.A., Viana J.H.M. Differential expression of *LHCGR* and its isoforms is associated to the variability in superovulation responses of Gir cattle. *Theriogenology*, 2019, 26: 68-74 (doi: 10.1016/j.theriogenology.2018.12.004).
45. Bekele T., Mekuriaw E., Walelegn B. Bovine embryo transfer and its application: arwiew. *Journal of Health, Medicine and Nursing*, 2016, 26: 48-60.
46. Brigida A., Skachkova O., Bykova O., Sorokin V. Comparative evaluation of the efficiency of poliovulation induction in donor cows using "FSH-super" drug with various injection schemes. *Atlantis Press*, 2019, 167: 491-497 (doi: 10.2991/ispc-19.2019.110).
47. Cirit Ü., Özmen M.F., Küçükaslan İ., Köse M., Kutsal H.G., Çinar E.M. Effect of the interval from follicle aspiration to initiation of lengthened FSH treatment on follicular superstimulatory and superovulatory responses and embryo production in lactating Simmental cows. *Theriogenology*, 2019, 128: 218-224 (doi: 10.1016/j.theriogenology.2019.02.008).
48. Hackett A.J., Durnford R., Mapletoft R.J., Marcus G.J. Location and status of embryos in the gential tract of superovulated cows 4 to 6 days after insemination. *Theriogenology*, 1993, 40(6): 1147-1153 (doi: 10.1016/0093-691X(93)90285-D).
49. Seidel G.E., Elsen R.E. Embryo transfer in dairy cattle. Hoards Dairyman, 1989.

50. Spencer T.E., Forde N., Dorniak P., Hansen T.R., Romero J.J., Lonergan P. Conceptus derived prostaglandins regulate gene expression in the endometrium prior to pregnancy recognition in ruminants. *Reproduction*, 2013, 146(4): 377-387 (doi: 10.1530/REP-13-0165).
51. Binelli M., Scolari S.C., Pugliesi G., Hoeck V., Gonella-Diaza A.M., Andrade S.C.S., Gasparin G.R., Coutinho L.L. The transcriptome signature of the receptive bovine uterus determined at early gestation. *PLoS ONE*, 2015, 10(4): e0122874 (doi: 10.1371/journal.pone.0122874).
52. Hsieh-Li H.M., Witte D.P., Weinstein M., Branford W., Li H., Small K., Potter S.S. Hoxa 11 structure, extensive antisense transcription, and function in male and female fertility. *Development*, 1995, 121(5): 1373-1385.
53. Kwon H.E., Taylor H.S. The role of *HOX* genes in human implantation. *Annals of the New York Academy of Sciences*, 2004, 1034 (1): 1-18 (doi: 10.1196/annals.1335.001).
54. Spell A.R., Beal W.E., Corah L.R., Lamb G.C. Evaluating recipient and embryo factors that affect pregnancy rates of embryo transfer in beef cattle. *Theriogenology*, 2001, 56(2): 287-297 (doi: 10.1016/s0093-691x(01)00563-5).
55. Sartori R., Suárez-Fernández C.A., Monson R.L., Guenther J.N., Rosa G.J.M., Wiltbank M.C. Improvement in recovery of embryos/ova using a shallow uterine horn flushing technique in superovulated Holstein heifers. *Theriogenology*, 2003, 60(7): 1319-1330 (doi: 10.1016/s0093-691x(03)00147-x).
56. Bó G.A., Mapletoft R.J. Evaluation and classification of bovine embryos. *Animal Reproduction*, 2013, 10(3): 344-348.
57. Mapletoft R.J., Bo G.A. Bovine embryo transfer. In: *Reviews in veterinary medicine*. I. Revah (ed.). International Veterinary Information Service, 2016.
58. Castro Neto A.S., Sanches B.V., Binelli M., Seneda M.M., Perri S.H., Garcia J.F. Improvement in embryo recovery using double uterine flushing author links open overlay panel. *Theriogenology*, 2005, 63(5): 1249-1255 (doi: 10.1016/j.theriogenology.2004.03.022).
59. Quinton H. *Commercial embryo transfer activity in Europe 2020*. Association of embryo technology in Europe, 2020. Available: https://www.aete.eu/publications/statistics/. No date.
60. Baruselli P.S., Souza A.H., Sá Filho M.F., Marques M.O., Sousa Sales J.N. Genetic market in cattle (Bull, AI, FTAI, MOET and IVP): financial payback based on reproductive efficiency in beef and dairy herds in Brazil. *Animal Reproduction*, 2018, 15(3): 247-255 (doi: 10.21451/1984-3143-AR2018-0091).
61. Smith A.K., Grimmer S.P. Pregnancy rates for Grade 2 embryos following administration of synthetic GnRH at the time of transfer in embryo-recipient cattle. *Theriogenology*, 2002, 57(8): 2083-2091 (doi: 10.1016/s0093-691x(02)00704-5).
62. Dochi O., Yamamoto Y., Saga H., Yoshiba N., Kano N., Maeda J., Miyata K., Yamauchi A., Tominaga K., Oda Y., Nakashima T., Inohae S. Direct transfer of bovine embryos frozen-thawed in the presence of propylene glycol or ethylene glycol under on-farm conditions in an integrated embryo transfer program. *Theriogenology*, 1998, 49(5): 1051-1058 (doi: 10.1016/s0093-691x(98)00053-3).
63. Vasconcelos J.L.M., Jardina D.T.G., Sá Filho O.G., Aragon F.L., Veras M.B. Comparison of progesterone-based protocols with gonadotropin-releasing hormone or estradiol benzoate for timed artificial insemination or embryo transfer in lactating dairy cows. *Theriogenelogy*, 2011, 75(6): 1153-1160 (doi: 10.1016/j.theriogenology.2010.11.027).
64. Galli C. Achievements and unmet promises of assisted reproduction technologies in large animals: a personal perspective. *Animal Reproduction*, 2017, 14(3): 614-621 (doi: 10.21451/1984-3143-AR1005).
65. Erdem H., Karasahin T., Alkan H., Dursun S., Satilmis F., Guler M. Effect of embryo quality and developmental stages on pregnancy rate during fresh embryo transfer in beef heifers. *Tropical Animal Health and Production*, 2020, 52: 2541-2547 (doi: 10.1007/s11250-020-02287-6).
66. Guemra S., Santo E., Zanin R., Monzani P.S., Sovernigo T.C., Ohashi O.M., Leal C.L.V., 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).
67. Humblot P., Bourhis D.L., Fritz S., Colleau J.J., Gonzalez C., Joly C.G., Malafosse A., Heyman Y., Amigues Y., Tissier M., Ponsart C. Reproductive technologies and genomic selection in cattle. *Veterinary Medicine International*, 2010: 192787 (doi: 10.4061/2010/192787).
68. Varga E., Kiss R., Papp A.B. In vitro maturation of porcine, bovine and equine oocytes. Literature review. *Magyar Allatorvosok Lapja*, 2008, 130(9): 542-549.
69. Sirard M.-A., Richard F., Blondin P., Robert C. Contribution of the oocyte to embryo quality. *Theriogenology*, 2006, 65(1): 126-136 (doi: 10.1016/j.theriogenology.2005.09.020).
70. Soto-Heras S., Paramio M.T. Impact of oxidative stress on oocyte competence for in vitro embryo production programs. *Research in Veterinary Science*, 2020, 132: 342-350 (doi: 10.1016/j.rvsc.2020.07.013).
71. Cagnone G., Sirard M.-A. The embryonic stress response to in vitro culture: insight from genomic

analysis. *Reproduction*, 2016, 152(6): 247-261 (doi: 10.1530/REP-16-0391).
72. El-Sayed A., Hoelker M., Rings, F., Salilew D., Jennen D., Tholen E., Sirard M.A., Schellander K., Tesfaye D. Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiological Genomics*, 2006, 28(1): 84-96 (doi: 10.1152/physiolgenomics.00111.2006).
73. Summers M.C., Biggers J.D. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. *Human Reproduction Update*, 2003, 9(6): 557-582 (doi: 10.1093/humupd/dmg039).
74. Farin P.W., Farin C.E. Transfer of bovine embryos produced in vivo or in vitro: Survival and fetal development. *Biology of Reproduction*, 1995, 52(3): 676-682 (doi: 10.1095/biolreprod52.3.676).
75. Blondin P., Coenen K., Guilbault L.A., Sirard M.-A. In vitro production of bovine embryos: Developmental competence is acquired before maturation. *Theriogenology*, 1997, 47(5): 1061-1075 (doi: 10.1016/S0093-691X(97)00063-0).
76. Lonergan P., Fair T. In vitro-produced bovine embryos: dealing with the warts. *Theriogenology*, 2008, 69(1): 17-22 (doi: 10.1016/j.theriogenology.2007.09.007).
77. Lonergan P., Forde N. Maternal-embryo interaction leading up to the initiation of implantation of pregnancy in cattle. *Animal*, 2014, 8(1): 64-69 (doi: 10.1017/S1751731114000470).
78. Suwik K., Boruszewska D., Sinderewicz E., Kowalczyk-Zieba I., Staszkiewicz-Chodor J., Woclawek-Potocka I. Expression profile of developmental competence gene markers in comparison with prostaglandin $F_{2\alpha}$synthesis and action in the early- and late-cleaved pre-implantation bovine embryos. *Reproduction in Domestic Animals*, 2021, 56(3): 437-447 (doi: 10.1111/rda.13880).
79. Rizos D., Clemente M., Bermejo-Alvarez P., Fuente J., Lonergan P., Gutiérrez-Adán A. Consequences of in vitro culture conditions on embryo development and quality. *Reproduction in Domestic Animals*, 2008, 43(s4): 44-50 (doi: 10.1111/j.1439-0531.2008.01230.x).
80. Sanches B.V., Zangirolamo A.F., Seneda M.M. Intensive use of IVF by large-scale dairy programs. *Animal Reproduction*, 2019, 16(3): 394-401 (doi: 10.21451/1984-3143-AR2019-0058).
81. Aguila L., Treulen F., Therrien J., Felmer R., Valdivia M., Smith L.C. Oocyte selection for in vitro embryo production in bovine species: noninvasive approaches for new challenges of oocyte competence. *Animals*, 2020, 10(12): 2196 (doi: 10.3390/ani10122196).
82. Ealy A.D., Wooldridge L.K., McCoski S.R. Post-transfer consequences of in vitro-produced embryos in cattle. *Journal of Animal Science*, 2019, 97(6): 2555-2568 (doi: 10.1093/jas/skz116).
83. Pollard J.W., Leibo S.P. Chilling sensitivity of mammalian embryos. *Theriogenology*, 1994, 41(1): 101-106 (doi: 10.1016/S0093-691X(05)80054-8).
84. Rizos D., Ward F., Duffy P., Boland M.P., Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Molecular Reproduction Development*, 2002, 61(2): 234-248 (doi: 10.1002/mrd.1153).
85. Canon-Beltran K., Giraldo-Giraldo J., Cajas Y.N., Beltrán-Breña P., Hidalgo C.O., Vásquez N., Leal C.L.V., Gutiérrez-Adán A., González E.M., Rizos D. Inhibiting diacylglycerol acyltransferase-1 reduces lipid biosynthesis in bovine blastocysts produced in vitro. *Theriogenology*, 2020, 158: 267-276 (doi: 10.1016/j.theriogenology.2020.09.014).
86. Sanches B.V., Lunardelli P.A., Tannura J.H., Cardoso B.L., Pereira M.H.C., Gaitkoski G., Basso A.C., Arnold D.R., Seneda M.M. A new direct transfer protocol for cryopreserved IVF embryos. *Theriogenology*, 2016, 85(6): 1147-1151 (doi: 10.1016/j.theriogenology.2015.11.029).
87. Wakchaure R., Ganguly S. Twinning in cattle: a review. *ARC Journal of Gynecology and Obstetrics*, 2016, 1(4): 1-3 (doi: 10.20431/2456-0561.0104001).
88. Winchester C.F. Monozygotic twin beef cattle in nutrition research. *Science*, 1952, 116(3002): 3.
89. Ozil J.P. Production of identical twins by bisection of blastocysts in the cow. *Journal of Reproduction and Fertility,* 1983, 69(2): 463-468 (doi: 10.1530/jrf.0.0690463).
90. Williams T.J., Elsden R.P., Seidel G.E. Jr. Pregnancy rates with bisected bovine embryos. *Theriogenology*, 1984, 22(5): 521-531 (doi: 10.1016/0093-691x(84)90051-7).
91. Warfield S.J., Seidel G.E. Jr., Elsden R.P. Transfer of bovine demi-embryos with and without the zone pellucid. *Journal of Animal Science*, 1987, 65(3): 756-761 (doi: 10.2527/jas1987.653756x).
92. Matsumoto K., Miyake M., Utumi K., Iritani A. Bisection of rat, goat and cattle blastocysts by metal blade. *The Japanese Journal of Animal Reproduction*, 1987, 33(1): 1-5 (doi: 10.1262/jrd1977.33.1).
93. Ozil J.P., Heyman Y., Renard J.P. Production of monozygotic twins by micromanipulation and cervical transfer in the cow. *Vet Rec.*, 1982, 110(6): 126-127 (doi: 10.1136/vr.110.6.126).
94. Skrzyszowska, M., Smorąg, Z., Kątska, L. Demi-embryo production from hatching of zona-drilled bovine and rabbit blastocysts. *Theriogenology*, 1997, 48(4): 551-557 (doi: 10.1016/s0093-691x(97)00272-0).
95. Silva J.C.E., Diniz P., Costa L.L. Luteotrophic effect, growth and survival of whole versus half embryos and, their relationship with plasma progesterone concentrations of recipient dairy heifers. *Animal Reproduction Science*, 2008, 104(1): 18-27 (doi: 10.1016/j.anireprosci.2007.01.004).

96. Iturbide A., Torres-Padilla M.-E. A cell in hand is worth two in the embryo: recent advances in 2-cell like cell reprogramming. *Current Opinion in Genetics & Development*, 2020, 64: 26-30 (doi: 10.1016/j.gde.2020.05.038).
97. Bredbacka P., Jaakma U., Muursepp I. Production of calves following nonsurgical transfer of fresh and refrigerated bovine demi-embryos. *Agricultural and Food Science in Finland*, 1996, 5(5): 521-527 (doi: 10.23986/afsci.72764).
98. Saito S., Niemann H. In vitro and in vivo survival of bovine demi-embryos following simplified bisection and transfer of one or two halves per recipient. *Journal of Reproduction Development*, 1993, 39(3): 251-258 (doi: 10.1262/jrd.39.251).
99. Hashiyada Y. The contribution of efficient production of monozygotic twins to beef cattle breeding. *Journal of Reproduction and Development*, 2017, 63(6): 527-538 (doi: 10.1262/jrd.2017-096).
100. Lopatarova M., Cech S., Krontorad P., Holy L., Hlavicov J., Dolezel R. Sex determination in bisected bovine embryos and conception rate after the transfer of female demi-embryos. *Veterinarni medicina*, 2008, 53(11): 595-603 (doi: 10.17221/1864-VETMED).
101. Skrzyszowska M., Smorag Z. Cell loss in bisected mouse, sheep and cow embryos. *Theriogenology*, 1989, 32: 115-122 (doi: 10.1016/0093-691x(89)90527-x).
102. Casser E., Israel S., Boiani M. Multiplying embryos: experimental monozygotic polyembryony in mammals and its uses. *The International Journal Developmental Biology*, 2019, 63: 143-155 (doi: 10.1387/ijdb.190016mb).
103. Gladchuk I.Z., Doshchechkyn V.V. Subfertility: philosophy and methodology of the problem. Part II. *Reproductive Endocrinology*, 2018, 42: 8-15 (doi: 10.18370/2309-4117.2018.42.8-15).
104. Sontag L.B., Lorincz M.C., Georg Luebeck E. Dynamics, stability and inheritance of somatic DNA methylation imprints. *Journal of Theoretical Biology*, 2006, 242(4): 890-899 (doi: 10.1016/j.jtbi.2006.05.012).
105. Weber W. Populations and genetic polymorphisms. *Molecular Diagnosis*, 1999, 4(4): 299-307 (doi: 10.1016/S1084-8592(99)80006-X).
106. Salehi R., Tsoi S.C.M., Colazo M.G., Ambrose D.J., Robert C., Dyck M.K. Transcriptome profiling of in-vivo produced bovine pre-implantation embryos using two-color microarray platform. *Developmental Biology*, 2017, 119: e53754 (doi: 10.3791/53754).
107. Van Hoeck V., Rizos D., Gutierrez-Adan A., Pintelon I., Jorssen E., Dufort I., Sirard M.A., Verlaet A., Hermans N., Bols P.E.J., Leroy J.L.M.R. Interaction between differential gene expression profile and phenotype in bovine blastocysts originating from oocytes exposed to elevated non-esterified fatty acid concentrations. *Reproduction, Fertility and Development*, 2015, 27(2): 372-384 (doi: 10.1071/RD13263).
108. Lee K.-F., Chow J.F.C., Xu J.S., Chan S.T.H., Ip S.M., Yeung W.S.B., Notes A. A comparative study of gene expression in murine embryos developed in vivo, cultured in vitro, and cocultured with human oviductal cells using messenger ribonucleic acid differential display. *Biology of Reproduction*, 2001, 64(3): 910-917 (doi: 10.1095/biolreprod64.3.910).
109. Jones G.M., Cram D.S., Song B., Kokkali G., Pantos K., Trounson A.O. Novel strategy with potential to identify developmentally competent IVF blastocysts. *Human Reproduction*, 2008, 23(8): 1748-1759 (doi: 10.1093/humrep/den123).
110. Allis C.D., Jenuwein T. The molecular hallmarks of epigenetic control. *Nature Reviews Genetics*, 2016, 17: 487-500 (doi: 10.1038/nrg.2016.59).
111. Wu C., Sirard M.-A. Parental effects on epigenetic programming in gametes and embryos of dairy cows. *Frontiers in Genetics*, 2020, 11: 557846 (doi: 10.3389/fgene.2020.557846).
112. Laskowski D., Humblot P., Sirard M.A., Sjunnesson Y., Jhamat N., Båge R., Andersson G. DNA methylation pattern of bovine blastocysts associated with hyperinsulinemia in vitro. *Molecular Reproduction and Development*, 2018, 85(7): 599-611 (doi: 10.1002/mrd.22995).
113. Wrenzycki C., Herrmann D., Lucas-Hahn A., Korsawe K., Lemme E., Niemann H. Messenger RNA expression patterns in bovine embryos derived from in vitro procedures and their implications for development. *Reproduction, Fertility and Development*, 2005, 17(2): 23-35 (doi: 10.1071/rd04109).
114. Bressan F.F., De Bem T.H.C., Perecin F., Lopes F.L., Ambrosio C.E., Meirelles F.V., Miglino M.A. Unearthing the roles of imprinted genes in the placenta. *Placenta*, 2009; 30(10): 823-834 (doi: 10.1016/j.placenta.2009.07.007).
115. Baroux C., Autran D., Gillmor C.S., Grimanelli D., Grossniklaus U. The maternal to zygotic transition in animals and plants. *Cold Spring Harbor Symposia Quantitative Biology*, 2008, 73: 89-100 (doi: 10.1101/sqb.2008.73.053).
116. Dobbs K.B., Rodriguez M., Sudano M.J., Ortega M.S., Hansen P.J. Dynamics of DNA methylation during early development of the preimplantation bovine embryo. *PLoS ONE*, 2013, 8: 66230 (doi: 10.1371/journal.pone.0066230).
117. McKiernan S.H., Bavister B.D. Fertilization and early embryology: Timing of development is a critical parameter for predicting successful embryogenesis. *Human Reproduction*, 1994, 9(11): 2123-2129 (doi: 10.1093/oxfordjournals.humrep.a138403).
118. Soom A., Ysebaert M.T., Kruif A. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine

embryos. *Molecular Reproduction and Development*, 1997, 47(1): 47-56 (doi: 10.1002/(SICI)1098-2795(199705)47:1<47::AID-MRD7>3.0.CO;2-Q).
119. Gad A., Hoelker M., Besenfelder U., Havlicek V., Cinar U., Rings F., Held E., Dufort I., Sirard M.-A., Schellander K., Tesfaye D. Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. *Biology of Reproduction*, 2012, 87(4): 1-13 (doi: 10.1095/biolreprod.112.099697).
120. Humblot P. From clinics to (cow) mics: a reproductive journey. *Anim. Reprod.*, 2018, 15(3): 278-291 (doi: 10.21451/1984-3143-AR2018-0076).
121. Wiggans G.R., Cole J.B., Hubbard S.M., Sonstegard T.S. Genomic selection in dairy cattle: the USDA experience. *Annual Review of Animal Biosciences*, 2017, 5: 309-327 (doi: 10.1146/annurev-animal-021815-111422).
122. McDaniel B.T., Cassell B.G. Effects of embryo transfer on genetic change in dairy cattle. *Journal of Dairy Science*, 1981, 64(12): 2484-2492 (doi: 10.3168/jds.S0022-0302(81)82873-1).
123. Mäntysaari E.A., Koivula M., Strandén I. Symposium review: Single-step genomic evaluations in dairy cattle. *Journal of Dairy Science*, 2020, 103(6): 5314-5326 (doi: 10.3168/jds.2019-17754).
124. Hayes B.J., Bowman P.J., Chamberlain A.J., Goddard M.E. Invited review: Genomic selection in dairy cattle: progress and challenges. *Journal of Dairy Science*, 2009, 92(2): 433-443 (doi: 10.3168/jds.2008-1646).
125. Hu Z.-L., Park C.A., Reecy J.M. Building a livestock genetic and genomic information knowledgebase through integrative developments of Animal QTLdb and CorrDB. *Nucleic Acids Research*, 2019, 47(D1): D701-D710 (doi: 10.1093/nar/gky1084).
126. Robert C., Nieminen J., Dufort I., Gagnй D., Grant J.R., Cagnone G., Plourde D., Nivet A.L., Fournier É., Paquet É., Blazejczyk M., Rigault P., Juge N., Sirard M.A. Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Molecular Reproduction & Development*, 2011, 78: 651-664 (doi: 10.1002/mrd.21364).
127. Lund M.S., de Roos A.P., de Vries A.G., Druet T., Ducrocg V., Fritz S., Guillaume F., Guidbrandtsen B., Liu Z., Reents R., Schrooten C., Seefriehd F., Su G. A common reference population from four European Holstein populations increases reliability of genomic predictions. *Genetics Selelection Evolution*, 2011, 43(43) (doi: 10.1186/1297-9686-43-43).
128. Chesnais J.P., Cooper T.A., Wiggans G.R., Sargolzaei M., Pryce J.E., Miglior F. Using genomics to enhance selection of novel traits in North American dairy cattle. *Journal of Dairy Science*, 2016, 99(3): 2413-2427 (doi: 10.3168/jds.2015-9970).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1079-1098
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 636.2:578.2:577.212.3

doi: 10.15389/agrobiology.2021.6.1079eng
doi: 10.15389/agrobiology.2021.6.1079rus

CELLULAR AND EXTRACELLULAR LEVELS OF RETROVIRUS—HOST INTERACTIONS ON THE EXAMPLE OF THE BOVINE LEUKOSE VIRUS. 2. CRITICAL STAGES — MULTIPLICITY AND VERSATILITY

(review)

V.I. GLAZKO[1, 2], G.Yu. KOSOVSKY[2], L.M. FEDOROVA[2], T.T. GLAZKO[1, 2] ✉

[1]*Timiryazev Russian State Agrarian University—Moscow Agrarian Academy*, 49, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail vigvalery@gmail.com, tglazko@rambler.ru (✉ corresponding author);
[2]*Afanas'ev Research Institute of Fur-Bearing Animal Breeding and Rabbit Breeding*, 6, ul. Trudovaya, pos. Rodniki, Ramenskii Region, Moscow Province, 140143 Russia, e-mail gkosovsky@mail.ru, felami@mail.ru
ORCID:
Glazko V.I. orcid.org/0000-0002-8566-8717
Kosovsky G.Yu. orcid.org/0000-0003-3808-3086
Fedorova L.M. orcid.org/0000-0002-1514-3050
Glazko T.T. orcid.org/0000-0002-3879-6935
The authors declare no conflict of interests
Received September 7, 2021

Abstract

The wide spread of viral infections and the ease of overcoming the species-specific barriers require the identification of critical stages in the virus interaction with multicellular organisms of mammals and the analysis of key molecular genetic systems involved. To date, a large amount of data has already been accumulated on the diversity and complexity of such systems, as well as the involvement in them the wide range of metabolic pathways. In this regard, attempts to identify some common elements that are implemented in different infectious processes are of particular relevance. This paper is such attempt made on the example of the analysis of the main events of cattle infection by bovine leukemia virus (BLV). Systems involved in the entry of BLV genetic material into the cytoplasm of host cells, the suppression of innate and adaptive immunity, as well as interactions between the genomes of the BLV provirus and the host genome are the identified critical stages. The direct participants in the reception of viral proteins are parts of some host tansmembrane systems (G.Yu. Kosovsky et al., 2017; V.I. Glazko et al., 2018; L. Bai et al., 2019; H. Sato et al., 2020). During virus reproduction in host cells, host enzymes modify virus envelope proteins by (A. De Brogniez et al., 2016; W. Assi et al., 2020). Importantly, modifications of SARS-CoV-2 spike proteins, as well as BLV envelope proteins, have a significant impact on their pathogenicity (M. Hoffmann et al., 2020). Pathogenicity and depressing effect of both BLV and SARS-CoV-2 on innate and adaptive immunity is realized in part through the activation of T regulatory cells and an increase in the expression of the growth transforming factor TGF-β (L.Y. Chang et al., 2015; G.Yu. Kosovsky et al., 2017; W. Chen et al., 2020). Intracellular mechanisms of protection against retrotranspositions, recombinations between viruses and host retrotransposons, the formation of new elements of host regulatory networks such as microRNAs, and the integration of proviral DNA into the host genome are closely related and controlled by interfering RNA (RNAi) systems with the key gene *dicer1* (P.V. Maillard et al., 2019; E.Z. Poirier et al., 2021; G.Y. Kosovsky et al., 2020). These systems can provide a certain «resistance» of the host genome both to the integration of exogenous genetic material and to transpositions of own mobile genetic elements. Apparently, it is the polygenicity of the control of these critical stages of viral infection that leads to difficulties in predicting their development and developing methods for their prevention.

Keywords: bovine leukemia virus, SARS-CoV-2, HIV-1, transmembrane systems, innate and adaptive immunity, interfering RNA systems, transpositions, mobile genetic elements

In recent years, data have been accumulating that consistently destroy simplified ideas about the interaction of retroviruses with host cells. Traditionally, each event of this interaction is considered separately, which does not allow assessing the polyvariance of its implementation from a holistic point of view. Moreover, interactions of retroviruses with host cell populations are mostly studied in vitro which often leads to contradictory results and complicates the development of methods for predicting the pathogenesis and spread of the infection.

There are several key stages in the interactions of a virus with a multicellular host organism. The first thing a virus encounters when it enters the body is the need to bind viral envelope proteins to receptor proteins on host cell membranes. As a rule, several domains of viral proteins and a number of host cell receptors most of which are associated with transmembrane transport systems participate in the binging. The second stage is the interaction of the virus with the host's immune defense, namely innate and adaptive immunity, and the third key stage is the integration of proviral DNA into the genome of the host cells.

In this review, we consider the polyvariance of molecular genetic systems involved in these events on an example of bovine leukemia virus (BLV, *Retroviridae*, *Deltaretrovirus*) as one of the most studied retroviruses.

BLV belongs to the *Retroviridae* family along with human T-leukemia viruse type 1 and 2 (HTLV-1 and HTLV-2). BLV infection in about 70% of cows is asymptomatic (aleukemic stage). In 25-30% of animals, persistent lymphocytosis develops; B-cell lymphoma occurs in 1-5% of animals after a 4-5-year latent period [1)].

Vaccination against BLV is still ineffective, so by far the most common way to improve the health of the dairy herd is to prevent infected animals from breeding. This approach is costly and, in addition, leads to a decrease in the productive potential of the herd, since a relatively increased susceptibility to BLV infection is often associated with high milk production. The combination of two circumstances (the low incidence of leukemia in infected animals and the loss of a part of the highly productive gene pool in the course of herd health improvement) actualizes the issues of predicting individual risks of oncogenesis and infectious danger of BLV carriers [2]. In this regard, of particular importance is the study of the molecular mechanisms of the processes occurring at each of the three stages of BLV-induced pathogenesis listed above.

The complexity of the interaction of the envelope proteins of the virus with the proteins of the plasma membrane of the host cells. Previously, we considered the basics and implications of the binding of the BLV envelope protein, encoded by the *env* gene, to the cellular receptor of the adapter-related protein complex-3 (AP-3) which is involved in the transport of proteins into lysosomes [3]. We found that in BLV-infected cows, the expression of the gene encoding the AP3D1 receptor is higher, but it does not correlate with an increase in the number of lymphocytes, which is usually considered as a preleukemic condition [4]. Another target for the reception of BLV *env* gene products which promotes the fusion of infected and infection-free cells is the transmembrane transporter of cationic amino acids SLC7A1/CAT1 [5, 6].

The *env* genes of BLV and human T-cell leukemia virus type I (HTLV-1) are 36% identical in amino acid sequences [7]. Entry of these retroviruses into target cells is initiated by interaction between Env and host cell receptors. Glucose transporter 1 (GLUT1) [8], neuropilin 1 (NRP-1) [9] and heparan sulfate proteoglycan (HSPG) [10] have been identified as cellular receptors for HTLV-1 attachment and cell infection.

The structure of GLUT1 has 12 hydrophobic transmembrane domains, six extracellular loops, and seven intracellular domains [11]. Like GLUT1, cationic amino acid transporter 1 (CAT1)/SLC7A1 has 14 membrane domains and has been identified in mouse cells as a membrane receptor for ecotropic murine leukemia viruses (eMuLV) [12]. CAT1 is a 622 amino acid protein with pronounced hydrophobic characteristics and is involved in sodium-independent transport of arginine, lysine, and histidine [13, 14]. Two different motifs in the third extracellular loop of CAT1 bind to the N-terminus of the *env* gene product subunit (SU) which is a determinant for eMuLV infection [15, 16]. Human CAT1 cells do not

confer susceptibility to human immunodeficiency virus infection. However, expression of mouse CAT1 in human cells can lead to acquired susceptibility [17]. Like human cells, hamster cells are completely resistant to eMuLV infection [18], and in many other animals, CAT1 proteins are also not involved in eMuLV infection, indicating that CAT1 may be species-specific for eMuLV infection. Importantly, both AP-3 and CAT1 expression occurs in various mammalian tissues. Therefore, retroviruses have no preferential target cells. It is obvious that other host proteins can also ensure the success of virus contacts with differentiated host cell populations.

According to Matsuura et al. [19], proteins carrying an immunoreceptor tyrosine-based activation motif (ITAM) which is present in the cytoplasmic tails of several protein components of antigen receptors on T- and B-cells, may be a key element for BLV reception together with the Fc receptor of immunoglobulin E. This motif is designated as Yxx(L/I)-x6-8-Yxx(L/I) where x corresponds to a variable amino acid residue.

Proteins of several viruses (e.g., BLV which causes B-cell lymphomas or leukemia in cattle, Epstein-Barr virus which causes Burkitt B-cell lymphomas in humans, and human herpesvirus 8 which causes sarcomas and primary effusion B-cell lymphomas in humans) contain ITAMs. The targets of these viruses are, in particular, B-lymphocytes, as well as non-hematopoietic cells, such as epithelial and endothelial cells. The BLV envelope glycoprotein (Env) contains two overlapping copies of the sequence (YXXL/I)2 (ITAM) in the C-terminal domain of the transmembrane (TM) protein. The Env BLV protein is synthesized as the Pr72 precursor peptide which is glycosylated in the rough endoplasmic reticulum and Golgi apparatus. Pr72 is cleaved by the cellular protease into two mature proteins, the gp51 surface subunit and the gp30 subunit with transmembrane localization. Due to disulfide bonds, gp51 and gp30 proteins form a stable complex and are included in the emerging viral particles. The gp51 protein binds to the cationic amino acid transporter 1 (CAT1)/SLC7A1 which acts as a cellular receptor for BLV and is responsible, as mentioned above [17], for its broad host specificity. The gp30 protein contains three different domains. These are an extracellular domain that interacts with gp51 and contains at the N-terminus a region of about 12 hydrophobic amino acids, the so-called fusion peptide [20], a transmembrane domain that anchors the gp51-gp30 complex in the plasma membrane of infected cells and in the virion [21], and a 58 amino acid cytoplasmic tail containing three YXXL sequences which were originally identified as two sets of ITAMs [22].

The three YXXL sequences in the cytoplasmic tail of gp30 BLV also correspond to the YXXφ tyrosine-based motif, where X is a variable residue and φ is an amino acid with a hydrophobic side chain (23). The YXXφ motif functions as an endocytic sorting motif and binds directly to the μ2 subunit of adapter protein-2 (AP2) [24]. The AP2 complex plays an essential role in initiating clathrin-mediated endocytosis [25]. The Env protein of most retroviruses (e.g., human immunodeficiency virus HIV, simian immunodeficiency virus SIV, and HTLV-1) contains only one YXXφ motif [26-28]. For HIV, the YSPL sequence contained in the Env protein is important for viral endocytosis and is required for virus replication and infectivity [29]. In vivo, YXXL gp30 sequences mediated high proviral loads in sheep experimentally infected with BLV [30]. It was found that a mutation in the second YXXL sequence, which leads to the replacement of tyrosine at position 498 by alanine, significantly reduces viral infectivity by reducing both the frequency of virus entry into the cell and the incorporation of the viral envelope protein into virions [23]. Thus, two of the three YXXL sequences in gp30 seem to play a crucial role in the development of a viral infection, namely, in binding to

cell membrane proteins, in particular, to T- and B-lymphocytes.

Posttranslational modification of viral proteins, such as glycosylation and methylation of arginine, can significantly contribute to their reception by host cells. For example, gp51 contains eight asparagine (N) residues; they presumably serve as sites for N-glycosylation [31], which can significantly affect viral replication, antigen conformation, the ability to form syncytium in vitro [32, 33], and infectivity in vivo [32]. Glycosylation of Env occurs both when virions attach to cell membranes and when cells merge with cells to form syncytium [34-36]. Env-associated glycans can protect surface viral proteins from neutralizing antibodies [36, 37]. A family of protein-specific arginine-N-methyltransferases (PRMT) catalyzes arginine methylation [38]. PRMT5 is a type II arginine methyl transferase. Arginine methylation plays a critical role in the biology of several viruses, in particular of hepatitis delta virus, hepatitis B virus, human immunodeficiency virus 1, Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus, and also, as followed from the data presented [38], of BLV. The authors of this study report that high expression of PRMT5 occurred in BLV-infected cattle only at high but not low proviral loads [38]. As it turned out, this is also true for artificial BLV infection (from the earliest stages of BLV infection to the stage of lymphoma).

Note that the multicomponent nature of molecular systems ensuring interaction with the host cell is not unique to BLV. Due to the pandemic, SARS-CoV-2 (*Coronaviridae*, *Alphacoronavirus*) seems to be the virus in which the molecular genetic systems involved in such interactions are the most studied. In contacts of SARS-CoV-2 with mammalian cells, as in the case of BLV, it is possible to isolate host proteins that are directly involved in the binding of the SARS-CoV-2 envelope protein (spike), as well as a number of systems that are indirectly involved in this. SARS-CoV-2 is an enveloped virus with single-strand positive viral RNA (ssRNA). Its entry into human cells is initiated through the binding of a spike protein (S protein) of the viral envelope to the angiotensin-converting enzyme 2 (ACE2) receptor on host cells. The type 2 transmembrane serine protease (TMPRSS2) and the endosomal cysteine proteases cathepsin B and L (CatB/L) cleave the S protein is into S1 and S2 fragmens. TMPRSS2 is believed to be of paramount importance for the entry of SARS-CoV-2 into host cells. ACE2 and TMPRSS2 are expressed in different cell types, including not only capillary endothelial cells, but also pneumocytes, macrophages, and other cells [39]. The C-terminal domain of the S1 subunit is responsible for the binding of SARS-CoV-2 to ACE2, and the S2 subunit undergoes conformational changes that lead to the fusion of the virus envelope with the cell membrane and the penetration of the virus content into the target cell. In the cytoplasm, the RNA of the virus is released, and the viral RNA polymerase necessary for virus replication is synthesized. The innate immune response is the host's first line of defense against SARS-CoV-2 infection. Toll-like receptors recognize viral RNA, i.e., double-stranded (dsRNA) (TLR3 receptor) and single-stranded (ssRNA) (TLR7 and TLR8), and serve as triggers for innate immune responses, including the expression of type I interferon genes and a number of cytokines [40]. In addition to the direct reception of the virus by host cell proteins, other metabolic modifications initiated by cellular enzymes occur. For example, glycosylation or methylation of argenins at the S proteolysis site of the spike protein, resulting in its cleavage into S1 and S2 subunits, can play a certain role at this stage [39].

If we generalize these data for taxonomically unrelated DNA- and RNA-containing viruses from groups with different types of replication, we can draw an obvious conclusion. At the first contact of the virus with the cell and at the stage

of virion maturation in the cytoplasm, there are two complex events which can significantly affect the subsequent spread of the pathogen. These are the interaction of the envelope protein virus with several host cell plasma membrane proteins and post-translational modifications performed by host enzymes during the synthesis of viral proteins.

Virus pathogenicity and adaptive immunity. The next key step in the interaction of a virus with a multicellular host organism is the activation of an adaptive response. It begins with antigen presentation in which class II gene products of the major histocompatibility complex (MHC) play a decisive role. The major histocompatibility complex is controlled by a highly polymorphic set of genes responsible for peptide antigen presentation and immune response, thereof it is associated with disease susceptibility. BoLA is the major histocompatibility gene complex in cattle. In particular, BoLA-DRB3 is a highly polymorphic class II BoLA locus with 365 alleles registered in the immunopolymorphism database (IPD database) of the MHC (https://www.ebi.ac.uk/ipd/mhc/group/BoLA/). Its polymorphism is associated with many infectious diseases in cattle [41-43]. Associations of BoLA-DRB3 polymorphisms with BLV proviral load (PVL) and associated symptoms are well documented [44-46].

An association between some BoLA-DRB3 allelic variants and BLV resistance was first described over 30 years ago [47, 48]. BoLA-DRB3 polymorphisms have been shown to influence the regulation of PVL by BLV during experimental infection in cattle [49, 50]. However, in recent years, it has become clear that in Holstein cows, PVL BLV and the development of lymphoma may be associated with different allelic variants of BoLA-DRB3 [51].

BVL-induced lymphoma develops as a result of the interaction of elements of the viral genome and products of the host genome in addition to BoLA-DRB3. For example, the integration of the provirus BLV in the region of the host genes involved in oncogenesis affects their expression [52-55]. In a number of studies, it is noted that quite often the integration of the BLV provirus is detected in the areas of localization of retroviruses [56-60].

The interaction of viral genes with host genes is of particular importance. For example, products of a number of host lymphocytic genes (transcription factors, cell cycle regulators, protein kinases, phosphatases), which affect apoptosis, proliferation, promote cell immortalization and ultimately lead to oncogenesis, transactivate the viral Tax protein, an activator of transcription of proviral DNA integrated into the host genome [53, 58, 61-63]. In turn, activation of BLV proviral DNA is to some extent due to the fact that Tax reduces the methylation activity of the BLV promoter region [64].

Tax mediates the activation of gene expression via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway [61]. Tax reduces the stability of various inhibitors of NF-kB in the cytoplasm (such as IkBa and oxidoreductase containing the WW domain) and induces nuclear translocation of NF-kB [65]. Tax interacts with the RelA subunit of the NF-kB complex [65, 66]. It is known that Tax induces an increase in the expression of many host genes, in particular tumor necrosis factor alpha TNFα [67, 68]. Our studies revealed that in BLV-infected cows, regardless of their origin and farms where they were kept, one of the most common changes, along with differences in platelet counts [69], is a decreased expression of NK-lysine, one of the main proteins in cytotoxic granules of T-killers and NK cells, which indicates inhibition of innate immunity factors [4]. Summarizing these research data, we proposed a scheme explaining the inhibition of not only innate immunity, but also antibody genesis. In brief, the essence of this scheme is as follows. Tax induces an increase in the expression of

TNFα which activates Treg cells, the producers of transforming growth factor beta TGF-β [70]. The TGF-β inhibits the proliferation and activity of T-killers and NK-cells, the producers of NK-lysines and increases the number and activity of platelets, in particular platelet antiapoptotic activity that was also described by other researchers [71-73].

The data accumulated to date generally do not contradict our earlier hypothesis about the mechanism of the host's innate and adaptive immunity suppression by the BLV through activation of TNF, being one of the leading immune response regulators, by BLV proteins [4]. It should be noted that, in our opinion, the key factor of the pathogenicity (aggressiveness) of the virus is the ability of the virus proteins to suppress various links of the host's immunity.

Based on accumulating evidence, expression of microRNAs (miRNAs) and long non-coding RNA sequences may be another source of influence of BLV viral genome elements on host immune responses [74-77].

Retroviral miRNAs actively influence various metabolic pathways of the host not only through the suppression of translation of the host mRNA, but also through interactions with its miRNA profile [78] or by interfering with the processes by which microRNAs are involved in the regulation of cell division and innate immunity functions [79, 80]. In recent years, microRNAs have attracted increasing attention as evidence is accumulating that these small RNA molecules (18-23 nucleotides), being one of the leading components in the epigenome formation, significantly contribute to the regulation of gene expression profiles [81].

To date, the studies performed reveals a spectrum of genes and gene networks the regulation of which in modern highly productive cattle breeds fundamentally differ from that of ancient ancestral forms in targets for miRNA of more than 1600 structural genes. These genes are involved in various metabolic pathways, including those associated with immunity [82]. The expression profiles of miRNAs involved in the regulation of transcription of structural genes the products of which are active in various metabolic pathways, and in particular in the key functions of the immune system at different stages of cow lactation, have been revealed [83, 84].

In recent years, special attention has been paid to the study of the organization, expression, and targets of BLV miRNAs due to the known similarity of this retrovirus with human T-cell leukemia virus types I and II (HTLV-1 and HTLV-2) [52, 85-87]. Sequencing of a collection of small RNAs obtained from B-lymphomas of BLV-infected sheep made it possible to isolate 10 regions of 20-23 nucleotide sequences of five BLV microRNAs that were transcribed from proviral DNA between the *env* gene and exon 2 R3 with coordinates of BLV proviral DNA positions 6398-6906 [88]. It was found that BLV microRNA transcripts in lymphoma cells account for approximately 40% of all microRNAs in these cells, and that transcription occurs with the participation of RNA polymerase III. Revealed suppression of the whole genome proviral DNA expression is due to epigenetic modification of 5′LTR which prevents transcription while no suppression occurs at the region of localization of microRNA genes [86]. The authors believe that activation of BLV miRNA transcription in pre-leukemic and leukemic cell clones is due to the host immune system-mediated selection against cell clones expressing BLV proteins. Moreover, it turned out that one of the BLV miRNAs, the BLV-miR-B4 identical in the nucleotide sequence of the "grain" region (nucleotides 2-7) to miR-29 of the bovine genome, exceeds miR-29 which is a member of the family miR-17-92 (oncomir-1) in terms of expression [79, 89]. Overexpression of miR-29 is found in BLV-infected tumor cells, as well as in human and mouse B-lymphomas [90]. The available data also indicate that BLV

miRNA plays a significant role in BLV-induced oncogenesis, and its antagonist is an antisense transcript from the 3'-end of BLV proviral DNA [76]. Note that the relationship between the expression of BLV proviral DNA, BLV microRNA, and leukocytosis has not been sufficiently studied. In addition, study of BLV-infected cell cultures suggests some antagonism between expression of full-length BLV proviral DNA and microRNA due to selection against BLV-expressing cell clones performed by the host's immune system.

An additional factor of the aggressiveness of retroviruses is their mutagenic activity against various host genes that control the processes of cell division, apoptosis, and cell differentiation. Thus, mutations in the host *p53* gene and polymorphisms of tumor necrosis factor α (TNFα) are directly associated with the development of lymphoma [91-93]. It also turned out that the expression levels of DNA repair proteins MSH2 (DNA Mismatch Repair Protein Msh2) and EXO1 (Exonuclease 1) are associated with the development of BLV-induced lymphomas. This suggests that one of the mechanisms causing the onset of the disease is the accumulation of mutations in a number of host genes [94]. In addition, the expression of the arginine-N-methyltransferase (PRMT5) gene involved in virus reception by host cells positively correlates with a high BLV proviral load and the development of the lymphoma stage. It was shown that changes in BLV pathogenicity is associated with downregulation of PRMT5 expression [95].

Thus, as at the BLV reception stage, a set of BLV genomic elements actively influence a large number of links in the innate and adaptive immunity of the host and inhibit them. It can be expected that proteins such as Tax serve as the key effector of suppression; in addition, microRNAs encoded by BLV have a significant effect on lymphogenesis in BLV-infected cows [77, 96]. Apparently, this can explain the many years of unsuccessful vaccination of cattle with BLV proteins.

The complexity of variability in the integration of BLV proviral DNA into the host genome. Integration of proviral DNA into the host genome is the critical point of the multiple ways to control the penetration of the BLV genetic material into host cells and the interaction of the virus with factors of the innate and adaptive immunity of the host. This process is also complex and is ensured by different molecular genetic systems [3]. There also are two fundamental points essential for the appearance of aggressive cell clones which are precursors of lymphomas. These are the extent of integration itself and the predisposition to mutagenesis of proviral DNA integrated into the genome.

A number of works [52-53, 55, 77, 97] consider massive integration of BLV into the genomes of B-lymphocytes followed by elimination of most cell clones and the frequency of mutational events in the key BLV genes for oncogenesis which occur in these clones. Some of publications mention that quite often the BLV proviral DNA integrates in the sites of retrotransposon localization.

Whole genome sequencing of the bovine genome revealed that the frequency of occurrence of the trinucleotide microsatellite (AGC)n in the bovine genome is 90, being 142 times higher than in the human and dog genomes. It also turned out that 39% of microsatellite loci with the AGC core in the bovine genome are associated with the evolutionarily young and Bov-A2 SINE retrotransposon species-specific for cattle [98]. Bov-A2 in its origin is closely related in origin to the long dispersed nuclear element (LINE) BovB [99]. BovB is an autonomous retrotransposon for which horizontal transfer is known in a number of species [100].

We have previously obtained evidence of a close relationship between microsatellites and retrotransposons [101]. We then compared the frequency of

occurrence of genomic DNA fragments of different lengths flanked by inverted repeats of microsatellites (AGC)6G, (GAG)6C, and (AG)9C in Black-and-White Holstain cows differing in BLV infection, as detected by the presence of in BLV proviral DNA genomes, and milk production. It turned out that the polymorphism of such fragments in the (AGC)6G and (GAG)6C spectra coincides in animals infected with BLV and distinguishes them from infection-free individuals regardless of their milk production. On other hand, the (AG)9C spectra differentiate cows by milk production but not by the presence of proviral BLV DNA in the genomes [102, 103]. Sequencing DNA fragments flanked by the (AGC)6G inverted repeat turned out that in animals infected with BLV there is an "overrepresentation" of homology regions with retrotransposons and their recombination products compared to cows free from the BLV infection [104]. Analysis of the associations of sequences localized between the inverted repeat (GAG)6C in the genomes of Black-and-White Holsteini cattle with structural genes showed that in most cases such sequences are associated with the genes of the immune and cell signaling systems or with their 5'-flanks in the intergenic space. In cows infected with bovine leukemia virus, in contrast to infection-free cows, such a sequence was found in the NK-lysine gene [105]. In addition, the NonLTR/SINE/SINE2 superfamily were detected in genomic DNA fragments flanked by an inverted repeat of the Helitron transposon at a frequency that was 5 times higher in the BLV-infected animals than in infection-free cows. In general, in the sequenced fragments, the density of transposable genetic elements (in particular, retrotransposons) in BLV-infected animals was higher than in infection-free ones [106-108].

Based on these data, we put forward a hypothesis that there are certain intracellular mechanisms the weakening of which promotes the BLV proviral DNA integration into the host genome while in the animals resistant to the infection it does not occur [104]. To designate the phenomenon of increased resistance to retrotranspositions and integration of DNA copies of retroviral sequences into the genome we suggest the term "genomic resistance to retrotranspositions".

Interestingly, the genomic resistance to retrotranspositions that we noted is confirmed by data on the consequences of the coronavirus infection COVID-19 caused by SARS-CoV-2, a virus with a different type of genome replication than in BLV. This may testify in favor of the universality of the mechanism of genomic resistance to retrotranspositions. Thus, infection of human lung and intestinal cells with SARS-CoV-2 induced increased expression of retrotransposons [109, 110]. It is assumed that the activation of retrotransposons leads to an increase in the amount of reverse transcriptase they encode, and the resulting higher intracellular level of this enzyme further increases the risk of retrotranspositions [109, 110]. Thus, chimeric transcripts (recombination products) of a retrotransposon and SARS-CoV-2 RNA have been found, which suggests the potential insertion of proviral fragments into the human genome [109, 110]. Note that the leading and terminating parts of the SARS-CoV-2 genome often form chimeric RNA. Therefore, SARS-CoV-2 can enter human cells and interact with retrotransposons in the host genome, causing more severe symptoms in patients with underlying diseases [109, 110].

One of the mechanisms of intracellular defense against retroviruses is the activity of the endogenous nuclease Dicer [111, 112]. The internal cellular antiviral mechanisms are part of the innate immune response and include RNA interference (RNAi) and the interferon system (IFN). The two systems operate quite differently, although both can be induced by viral long double-stranded RNA (dsRNA) or high base-pair single-stranded RNA (ssRNA). The source of dsRNA can be the virus itself (in the case of a virus with a dsRNA genome) or two complementary RNA strands formed as viral RNA replication intermediates

or convergent viral DNA transcripts. Paired regions of ssRNA, also commonly referred to as dsRNA, are localized at high density in hairpins in viral genomes or in viral transcripts. Both types of dsRNA (viral genomic double-stranded RNA or hairpin structures in viruses with single-stranded genomic RNAs) are absent in appreciable amounts in uninfected cells and act as signs of viral infection, inducing innate antiviral immune responses. Long dsRNA is cleaved by type III endoribonuclease Dicer into small interfering RNAs (siRNAs), the RNA duplexes 21-24 nucleotides long. One strand of each siRNA duplex binds to the Argonaute (Ago) protein which, together with available proteins, forms an RNA-induced silencing complex (RISC) and mediates endonucleolytic cleavage (slicing) of complementary target RNAs. Ago is involved in an RNAi- and miRNA-mediated process that leads to mRNA degradation and/or inhibits translation [111].

Available data indicate that the interferon and RNAi systems are antagonistic as different components of antiviral defense in mammals [112]. Our own studies have shown that cows with high leukocytosis tend to have lower expression of the *ifn-α* gene compared to animals free from BLV infection and those with moderate leukocytosis [4]. Note that the first group exhibits a statistically significant increase ($p < 0.5$) in the expression of the Dicer1 (*dc1*) and *ago2* genes compared to the second of these three groups [113]. Interestingly, we also found statistically significant ($p < 0.5$) positive correlations between the expression of these two genes in cows free of BLV infection [113]. In case animals with insertions of BLV proviral DNA into the genome and the expression of BLV reverse transcriptase (*pol*) are treated together as one group, regardless of the level of leukocytosis, then a statistically significant correlation between the expression of *dc1* and *ago* is also observed. The expression of both genes correlates positively ($p < 0.5$) with an increase in the number of leukocytes and lymphocytes and with the pol expression. If we conditionally divide the group of animals with proviral DNA insertion and *pol* expression into subgroups of individuals with moderate (less than 20×10^9/l) and high (more than 20×10^9/l) leukocytosis, it turned out that in cows with moderate leukocytosis, correlations between *dc* and *ago2* disappear while negative correlation appears between *ago2* expression and thrombocytosis. At that, in animals with high leukocytosis, a correlation between *dc1* and *pol* expression is preserved ($p < 0.5$) and correlations of *dc1* and *pol* expression with the number of eosinophils is detercted [113]. In the group of BLV-infected cows with microRNA expression, there are correlations between the number of leukocytes, lymphocytes, monocytes, *pol* and *dc1* expression, but there is no correlation between *dc1* and *ago2* expression [113].

The complexity and variability of the correlation relationships between the expression of the studied genes and the leukocyte blood count that we revealed are obviously due to high quantitative dynamics of cells of different differentiation types and various maturation levels in the peripheral blood in vivo, especially at various stages of the infectious process. Gene expression available for analysis changes in a significantly smaller number of cells, from which total mRNA is isolated at the first stages of the study. This problem becomes especially clear for the dynamics of the *dc1* gene product (aviDicer1) isoform with deletions of exons 7 and 8 in embryonic stem and differentiated somatic cells [112]. In our studies [113], the activity of *dc1* expression was assessed for the terminal region of the transcript (exon 29). Therefore, it is obvious that in the blood cell population we could type the expression of both the full transcript and the aviDicer1 isoform.

Thus, intracellular protection against the integration of the sequence of DNA copies of retroviruses and, apparently, retrotranspositions in the host genome also depends on the set of genes. The end result may turn out to be similar

with the contribution of the activity of different genes, depending on the network relationships between them.

Another reason for the variability in the relationship between the virus and the host is the increased mutability of the nucleotide sequences of the virus. In some studies, the estimated mutation rate in retroviruses was on average about 10^{-3}-10^{-5}per nucleotide in one transcription cycle [114]. Such variability is primarily due to the predisposition of some parts of the nucleotide sequence of the viral RNA itself to the formation of secondary structures. In particular, these motifs include sequences with increased ability to form G4 quadruplexes [115]. We assessed [116, 117] the distribution of nucleotide motifs predisposed to the formation of secondary structures (G4 quadruplexes, triplexes, inverted repeats) both in RNA and in BLV proviral DNA. Our findings showed that the number and density of sequences capable of forming G4 quadruplexes increased in the BLV *env* gene compared to the *pol* gene. We found [116, 117] that the *pol* gene contains sequences with G4 quadruplex motifs on the flanks, which have a certain homology (> 70%) with regions of retroviruses belonging to other groups of retroviruses and found in other species, which coincides to some extent with reported data [118]. We also revealed an increased density of nucleotide motifs with a predisposition to the formation of secondary structures in the long terminal repeats (LTR) of the BLV genome when comparing the results of their sequencing presented in GenBank (National Center for Biotechnology Information, https://www.ncbi.nlm.nih. gov/). Analysis of polymorphic regions in the sequenced LTR BLV from GenBank performed separately for primary infected animals and for lymphomas [119] found only partial matches. This may indicate different clonal selection in populations of BLV-infected cells and in lymphomas undergoing stages of tumor progression. The greatest polymorphism appears in both cases in the regions where the regulatory sequences are located. Of the three sequences of potential G4 quadruplexes, the first one (positions 49-68 bp of LTR BLV) coincides with the most polymorphic region in sequences both in different isolates and in lymphomas, as well as with the TxRE regulatory region [117]. The other two are characterized by a relatively increased polymorphism in lymphomas and are located at positions 469-508 bp, i.e., on the 5′-flank of the site of homology to the primate endogenous retrovirus (positions 505-531 bp). This is consistent with our previous assumption of a relationship between the localization of potential G4 quadruplexes and recombination events, in particular, in the *pol* BLV gene [117].

In addition, two non-overlapping inverted repeats were identified which differed in complexity and unequal polymorphism of localization sites [117]. In sequences from GenBank and from lymphomas [119], the first inverted repeat is localized in the regulatory sequences in the U3 region with relatively low polymorphism, the second inverted repeat found in the U5 region in sequences with high polymorphism overlaps with purine-pyrimidine tracks predisposed to formation of intramolecular triplexes. Another purine-pyrimidine sequence predisposed to the formation of DNA-RNA triplexes, is localized in a relatively conserved region and overlaps with the second regulatory region of TxRE [117].

It can be expected that the detected genetic heterogeneity of LTR regulatory motifs is associated with an increased density of noncanonical nucleic acid structures localized in them (in particular, G4 quadruplexes), which contributes to the interaction of viral proteins with various host defense systems. It should be noted that G4 quadruplexes are present in the genomes of both DNA and RNA viruses and control the critical stages of their replication. For example, G4 quadruplexes in the HIV-1 genome regulate reverse transcription and proviral DNA transcription steps, which require their interaction with cellular proteins

and/or RNA [120]. A search is under way for ligands for G4 quadruplexes that could provide antiviral protection, and some encouraging results have been obtained [121].

The significance of G4 quadruplexes in mutagenesis and regulation of viral gene expression together with the preferential localization of G4 quadruplexes in certain regions of the genome of both viruses and their hosts, suggest close coevolution of the virus with the host and mutual "mimicry" in the genomic distribution of such noncanonical sequences [122].

It is important to note that in viruses of different origins, all three critical stages of interaction with host cells have certain similarities. The penetration in all cases is associated with host proteins that provide transport across the cell membrane (and, consequently, with glycosylation of viral proteins and modifications of host protease targets on these proteins). Suppression of innate immunity is associated with an increase in the production of growth-transforming factor TGF-β by regulatory T-cells of mammals [123, 124]. The interaction of virus and host genome involves molecular genetic systems for recombination, including reverse transcription, with influence on the host's regulatory networks.

Thus, in the interaction of retroviruses with mammalian cells, as exemplified by the processes during infection of cattle with the bovine leukemia virus (BLV), virus reception by host cell proteins, protective reaction of the host's innate and adaptive immunity, and integration of proviral DNA into the host genome can be distinguished as critical events. Many host genes are involved in the processes occurring at all stages of the interaction between the virus and the host cell, and such polygenicity is characteristic of various viral infections. For the first stage, two levels of sources of variability can be distinguished, i.e., the interaction itself with host proteins (reception, membrane fusion) and modification of viral proteins during its reproduction in host cells (glycosylation, methylation). The interaction with the host immune system involves viral proteins and microRNAs in the metabolic pathways that provide innate and adaptive immunity. Post-translational modifications of viral proteins can contribute to and modulate the processes occurring at this stage. The third stage is also characterized by the interaction of virus and host gene products, apparently involved in metabolic pathways that are directly related to the proviral DNA integration and retrotranspositions in the host genome. All these processes are accompanied by a high rate of mutation and even recombination between viral sequences. The polygenicity of the relationship between the pathogen and the host (in fact, the formation of networks of their interacting genes) leads to the severity of individual manifestations of the disease. The effective prediction of its development can be based on the simultaneous assessment of the expression of a set of genes that are critical for the pathogen—host relationships at different stages of this process.

REFERENCES

1. Aida Y., Murakami H., Takahashi M., Takeshima S.N. Mechanisms of pathogenesis induced by bovine leukemia virus as a model for human T-cell leukemia virus. *Front Microbiol.*, 2013, 4: 328 (doi: 10.3389/fmicb.2013.00328).
2. Kosovskii G.Yu., Glazko V.I., Andreichenko I.A., Koval'chuk S.N., Glazko T.T. The infection hazard of carriers of proviral bovine leukemia virus and its evaluation with regard to leukocytosis. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2016, 51(4): 475-482 (doi: 10.15389/agrobiology.2016.4.475eng).
3. Glazko V.I., Kosovskii G.Yu., Glazko T.T., Donnik I.M. Cellular and extracellular levels of retrovirus—host interactions on the example of the bovine leukose virus. 1. Cell penetration and integration into the host genome (review) *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2018, 53(6): 1093-1106 (doi: 10.15389/agrobiology.2018.6.1093eng).
4. Kosovskii G.Yu., Glazko V.I., Koval'chuk S.N., Arkhipova A.L., Glazko T.T. Expression of *NK*-

lysin, *blvr*, *ifn-a* and blood cell populations in cows infected by bovine leukemia virus. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52(4): 785-794 (doi: 10.15389/agrobiology.2017.4.785eng).
5. Bai L., Sato H., Kubo Y., Wada S., Aida Y. CAT1/SLC7A1 acts as a cellular receptor for bovine leukemia virus infection. *FASEB J.,* 2019, 33(12): 14516-14527 (doi: 10.1096/fj.201901528R).
6. Sato H., Bai L., Borjigin L., Aida Y. Overexpression of bovine leukemia virus receptor SLC7A1/CAT1 enhances cellular susceptibility to BLV infection on luminescence syncytium induction assay (Lu-SIA). *Virol J.*, 2020, 17(1): 57 (doi: 10.1186/s12985-020-01324-y).
7. Gillet N., Florins A., Boxus M., Burteau C., Nigro A., Vandermeers F., Balon H., Bouzar A.B., Defoiche J., Burny A., Reichert M., Kettmann R., Willems L. Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. *Retrovirology*, 2007, 4: 18 (doi: 10.1186/1742-4690-4-18).
8. Manel N., Kim F.J., Kinet S., Taylor N., Sitbon M., Battini J.L. The ubiquitous glucose transporter GLUT-1 is a receptor for HTLV. *Cell*, 2003, 115(4): 449-459 (doi: 10.1016/s0092-8674(03)00881-x).
9. Ghezzi P.C., Dolcini G.L., Gutiérrez S.E., Bani P.C., Torres J.O., Arroyo G.H., Esteban E.N. Virus de la leucosis bovina (BLV): prevalencia en la Cuenca Lechera Mar y Sierras entre 1994 y 1995 [Bovine leukemia virus (BLV): prevalence in the Cuenca Lechera Mar y Sierras from 1994 to 1995]. *Revista Argentina de Microbiologia*, 1997, 29(3): 37-146 (in Span.).
10. Jones K.S., Petrow-Sadowski C., Bertolette D.C., Huang Y., Ruscetti F.W. Heparan sulfate proteoglycans mediate attachment and entry of human T-cell leukemia virus type 1 virions into CD4+ T cells. *J. Virol.*, 2005, 79(20): 12692-12702 (doi: 10.1128/JVI.79.20.12692-12702.2005).
11. Mueckler M. Facilitative glucose transporters. *European Journal of Biochemistry*, 1994, 219(3): 713-725 (doi: 10.1111/j.1432-1033.1994.tb18550.x).
12. Albritton L.M., Tseng L., Scadden D., Cunningham J.M. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. *Cell*, 1989, 57(4): 659-666 (doi: 10.1016/0092-8674(89)90134-7).
13. Kim J.W., Closs E.I., Albritton L.M., Cunningham J.M. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature*, 1991, 352(6337): 725-728 (doi: 10.1038/352725a0).
14. Wang H., Kavanaugh M.P., North R.A., Kabat D. Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. *Nature*, 1991, 352(6337): 729-731 (doi: 10.1038/352729a0).
15. Albritton L.M., Kim J.W., Tseng L., Cunningham J.M. Envelope-binding domain in the cationic amino acid transporter determines the host range of ecotropic murine retroviruses. *J. Virol.*, 1993, 67(4): 2091-2096 (doi: 10.1128/JVI.67.4.2091-2096.1993).
16. Yoshimoto T., Yoshimoto E., Meruelo D. Identification of amino acid residues critical for infection with ecotropic murine leukemia retrovirus. *J. Virol.*, 1993, 67(3): 1310-1314 (doi: 10.1128/JVI.67.3.1310-1314.1993).
17. Bae E.H., Park S.H., Jung Y.T. Role of a third extracellular domain of an ecotropic receptor in Moloney murine leukemia virus infection. *J. Microbiol.*, 2006, 44(4): 447-452.
18. Kakoki K., Shinohara A., Izumida M., Koizumi Y., Honda E., Kato G., Igawa T., Sakai H., Hayashi H., Matsuyama T., Morita T., Koshimoto C., Kubo Y. Susceptibility of muridae cell lines to ecotropic murine leukemia virus and the cationic amino acid transporter 1 viral receptor sequences: implications for evolution of the viral receptor. *Virus Genes*, 2014, 48(3): 448-456 (doi: 10.1007/s11262-014-1036-1).
19. Matsuura R., Inabe K., Otsuki H., Kurokawa K., Dohmae N., Aida Y. Three YXXL sequences of a bovine leukemia virus transmembrane protein are independently required for fusion activity by controlling expression on the cell membrane. *Viruses*, 2019, 11(12): 1140 (doi: 10.3390/v11121140).
20. Vonèche V., Callebaut I., Kettmann R., Brasseur R., Burny A., Portetelle D. The 19-27 amino acid segment of gp51 adopts an amphiphilic structure and plays a key role in the fusion events induced by bovine leukemia virus. *Journal of Biological Chemistry*, 1992, 267(21): 15193-15197 (doi: 10.1016/S0021-9258(18)42164-3).
21. Johnston E.R., Radke K. The SU and TM envelope protein subunits of bovine leukemia virus are linked by disulfide bonds, both in cells and in virions. *J. Virol.*, 2000, 74: 2930-2935 (doi: 10.1128/JVI.74.6.2930-2935.2000).
22. Reth M. Antigen receptor tail clue. *Nature*, 1989, 338: 383-384 (doi: 10.1038/338383b0).
23. Inabe K., Nishizawa M., Tajima S., Ikuta K., Aida Y. The YXXL sequences of a transmembrane protein of bovine leukemia virus are required for viral entry and incorporation of viral envelope protein into virions. *J. Virol.*, 1999, 73(2): 1293-1301 (doi: 10.1128/JVI.73.2.1293-1301.1999).
24. Ohno H., Stewart J., Fournier M.C., Bosshart H., Rhee I., Miyatake S., Saito T., Gallusser A., Kirchhausen T., Bonifacino J.S. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science*, 1995, 269: 1872-1875 (doi: 10.1126/science.7569928).
25. Mettlen M., Chen P.H., Srinivasan S., Danuser G., Schmid S.L. Regulation of clathrin-mediated endocytosis. *Annu. Rev. Biochem.*, 2018, 87: 871-896 (doi: 10.1146/annurev-biochem-062917-012644).

26. Boge M., Wyss S., Bonifacino J.S., Thali M. A membrane-proximal tyrosine-based signal mediates internalization of the HIV-1 Envelope glycoprotein via interaction with the AP-2 clathrin adaptor. *J. Biol. Chem.,* 1998, 273: 15773-15778 (doi: 10.1074/jbc.273.25.15773).
27. Yuste E., Reeves J.D., Doms R.W., Desrosiers R.C. Modulation of Env content in virions of simian immunodeficiency virus: correlation with cell surface expression and virion infectivity. *J. Virol.,* 2004, 78: 6775-6785 (doi: 10.1128/JVI.78.13.6775-6785.2004).
28. Delamarre L., Pique C., Rosenberg A.R., Blot V., Grange M.P., Le Blanc I., Dokhélar M.C. The Y-S-L-I tyrosine-based motif in the cytoplasmic domain of the human T-cell leukemia virus type 1 envelope is essential for cell-to-cell transmission. *J. Virol.,* 1999, 73(11): 9659-9663 (doi: 10.1128/JVI.73.11.9659-9663.1999).
29. Lambele M., Labrosse B., Roch E., Moreau A., Verrier B., Barin F., Roingeard P., Mammano F., Brand D. Impact of natural polymorphism within the gp41 cytoplasmic tail of human immunodeficiency virus type 1 on the intracellular distribution of Envelope glycoproteins and viral assembly. *J. Virol.*, 2007, 81: 125-140 (doi: 10.1128/JVI.01659-06).
30. Willems L., Gatot J.S., Mammerickx M., Portetelle D., Burny A., Kerkhofs P., Kettmann R. The YXXL signalling motifs of the bovine leukemia virus transmembrane protein are required for in vivo infection and maintenance of high viral loads. *J. Virol.,* 1995, 69(7): 4137-4141 (doi: 10.1128/JVI.69.7.4137-4141.1995).
31. De Brogniez A., Mast J., Willems L. Determinants of the bovine leukemia virus envelope glycoproteins involved in infectivity, replication and pathogenesis. *Viruses*, 2016, 8: 88 (doi: 10.3390/v8040088).
32. De Brogniez A., Bouzar A.B., Jacques J.-R., Cosse J.-P., Gillet N., Callebaut I., Reichert M., Willems L. Mutation of a single envelope N-linked glycosylation site enhances the pathogenicity of bovine leukemia virus. *J. Virol.*, 2015, 89: 8945-8956 (doi: 10.1128/JVI.00261-15).
33. Rizzo G., Forti K., Serroni A., Cagiola M., Baglivo S., Scoccia E., De Giuseppe A. Single N-glycosylation site of bovine leukemia virus SU is involved in conformation and viral escape. *Vet. Microbiol.,* 2016, 197: 21-26 (doi: 10.1016/j.vetmic.2016.10.024).
34. Mamoun R.Z., Morisson M., Rebeyrotte N., Busetta B., Couez D., Kettmann R., Hospital M., Guillemain B. Sequence variability of bovine leukemia virus env gene and its relevance to the structure and antigenicity of the glycoproteins. *J. Virol.*, 1990, 64: 4180-4188 (doi: 10.1128/JVI.64.9.4180-4188.1990).
35. Pikora C. Glycosylation of the ENV spike of primate immunodeficiency viruses and antibody neutralization. *Curr. HIV Res.*, 2004, 2: 243-254 (doi: 10.2174/1570162043351264).
36. Aguilar H.C., Matreyek K.A., Filone C.M., Hashimi S.T., Levroney E.L., Negrete O.A., Bertolotti-Ciarlet A., Choi D.Y., McHardy I., Fulcher J.A., Su S.V., Wolf M.C., Kohatsu L., Baum L.G., Lee B. N-glycans on Nipah virus fusion protein protect against neutralization but reduce membrane fusion and viral entry. *J. Virol.*, 2006, 80: 4878-4889 (doi: 10.1128/JVI.80.10.4878-4889.2006).
37. Wei X., Decker J.M., Wang S., Hui H., Kappes J.C., Wu X., Salazar-Gonzalez J.F., Salazar M.G., Kilby J.M., Saag M.S., Komarova N.L., Nowak M.A., Hahn B.H., Kwong P.D., Shaw G.M. Antibody neutralization and escape by HIV-1. *Nature,* 2003, 422: 307-312 (doi: 10.1038/nature01470).
38. Assi W., Hirose T., Wada S., Matsuura R., Takeshima S.N., Aida Y. PRMT5 is required for bovine leukemia virus infection in vivo and regulates BLV gene expression, syncytium formation, and glycosylation in vitro. *Viruses*, 2020, 12(6): 650 (doi: 10.3390/v12060650).
39. Hoffmann M., Kleine-Weber H., Schroeder S., Krüger N, Herrler T., Erichsen S., Schiergens T.S., Herrler G., Wu N.H., Nitsche A., Müller M.A., Drosten C., Pцhlmann S. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell*, 2020, 181(2): 271-280.e8. (doi: 10.1016/j.cell.2020.02.052).
40. Lee I.H., Lee J.W., Kong S.W. A survey of genetic variants in SARS-CoV-2 interacting domains of ACE2, TMPRSS2 and TLR3/7/8 across populations. *Infect. Genet. Evol.*, 2020, 85: 104507 (doi: 10.1016/j.meegid.2020.104507).
41. Lei W., Liang Q., Jing L., Wang C., Wu X., He H. BoLA-DRB3 gene polymorphism and FMD resistance or susceptibility in Wanbei cattle. *Mol. Biol. Rep.*, 2012, 39: 9203-9209 (doi: 10.1007/s11033-012-1793-7).
42. Yoshida T., Mukoyama H., Furuta H., Kondo Y., Takeshima S.N., Aida Y., Kosugiyama M., Tomogane H. Association of the amino acid motifs of BoLA-DRB3 alleles with mastitis pathogens in Japanese Holstein cows. *Anim. Sci. J.,* 2009, 80: 510-519 (doi: 10.1111/j.1740-0929.2009.00664.x).
43. Morales J.P.A., López-Herrera A., Zuluaga J.E. Association of BoLA DRB3 gene polymorphisms with BoHV-1 infection and zootechnical traits. *Open Vet. J.*, 2020, 10: 331-339 (doi: 10.4314/ovj.v10i3.12).
44. Takeshima S.-N., Ohno A., Aida Y. Bovine leukemia virus proviral load is more strongly associated with bovine major histocompatibility complex class II DRB3 polymorphism than with DQA1 polymorphism in Holstein cow in Japan. *Retrovirology*, 2019, 16: 1-6 (doi: 10.1186/s12977-019-0476-z).

45. Miyasaka T., Takeshima S.-N., Jimba M., Matsumoto Y., Kobayashi N., Matsuhashi T., Sentsui H., Aida Y. Identification of bovine leukocyte antigen class II haplotypes associated with variations in bovine leukemia virus proviral load in Japanese Black cattle. *Tissue Antigens*, 2012, 81: 72-82 (doi: 10.1111/tan.12041).
46. Udina I.G., Karamysheva E.E., Turkova S.O., Orlova A.R., Sulimova G.E. Genetic mechanisms of resistance and susceptibility to leukemia in Ayrshire and Black Pied cattle breeds determined by allelic distribution of gene Bola-DRB3. *Russ. J. Genet.*, 2003, 39: 306-317 (doi: 10.1023/a:1023279818867).
47. Lewin H.A. Bernoco D. Evidence for BoLA-linked resistance and susceptibility to subclinical progression of bovine leukaemia virus infection. *Anim. Genet.*, 1986, 17(3): 197-207 (doi: 10.1111/j.1365-2052.1986.tb03191.x).
48. Lewin H.A. Disease resistance and immune response genes in cattle: strategies for their detection and evidence of their existence. *J. Dairy Sci.*, 1989, 72(5): 1334-1348 (doi: 10.3168/jds.S0022-0302(89)79241-9).
49. Forletti A., Lützelschwab C.M., Cepeda R., Esteban E.N., Gutiérrez S.E. Early events following bovine leukaemia virus infection in calves with different alleles of the major histocompatibility complex DRB3 gene. *Vet. Res.*, 2020, 51: 4 (doi: 10.1186/s13567-019-0732-1).
50. Jimba M., Takeshima S.N., Murakami H., Kohara J., Kobayashi N., Matsuhashi T., Ohmori T., Nunoya T., Aida Y. BLV-CoCoMo-qPCR: a useful tool for evaluating bovine leukemia virus infection status. *BMC Vet. Res.*, 2012, 8: 167 (doi: 10.1186/1746-6148-8-167).
51. Lo C.-W., Borjigin L., Saito S., Fukunaga K., Saitou E., Okazaki K., Mizutani T., Wada S., Takeshima S.-n., Aida Y. BoLA-DRB3 polymorphism is associated with differential susceptibility to bovine leukemia virus-induced lymphoma and proviral load. *Viruses*, 2020, 12: 352 (doi: 10.3390/v12030352).
52. Rosewick N., Durkin K., Artesi M.M., Marçais A.A., Hahaut V.V., Griebel P., Arsic N.N., Avettand-Fenoel V., Burny A., Charlier C.C., Hermine O., Georges M., Van den Broeke A. Cis-perturbation of cancer drivers by the HTLV-1/BLV proviruses is an early determinant of leukemogenesis. *Nat. Commun.*, 2017, 8: 15264 (doi: 10.1038/ncomms15264).
53. Gillet N.A., Gutiérrez G., Rodriguez S.M., De Brogniez A., Renotte N., Alvarez I., Trono K., Willems L. Massive depletion of bovine leukemia virus proviral clones located in genomic transcriptionally active sites during primary infection. *PLoS Pathog.*, 2013, 9: e1003687 (doi: 10.1371/journal.ppat.1003687).
54. Safari R., Hamaidia M., de Brogniez A., Gillet N., Willems L. Cis-drivers and trans-drivers of bovine leukemia virus oncogenesis. *Current Opinion in Virology*, 2017, 26: 15-19 (doi: 10.1016/j.coviro.2017.06.012).
55. Ohnuki N., Kobayashi T., Matsuo M., Nishikaku K., Kusama K., Torii Y., Inagaki Y., Hori M., Imakawa K., Satou Y. A target enrichment high throughput sequencing system for characterization of BLV whole genome sequence, integration sites, clonality and host SNP. *Sci. Rep.*, 2021, 11(1): 4521 (doi: 10.1038/s41598-021-83909-3).
56. Murakami H., Yamada T., Suzuki M., Nakahara Y., Suzuki K., Sentsui H. Bovine leukemia virus integration site selection in cattle that develop leukemia. *Virus Res.*, 2011, 156(1-2): 107-112 (doi: 10.1016/j.virusres.2011.01.004).
57. Miyasaka T., Oguma K., Sentsui H. Distribution and characteristics of bovine leukemia virus integration sites in the host genome at three different clinical stages of infection. *Arch. Virol.*, 2015, 160: 39-46 (doi: 10.1007/s00705-014-2224-y).
58. Lo C.W., Takeshima S.N., Okada K., Saitou E., Fujita T., Matsumoto Y., Wada S., Inoko H., Aida Y. Association of bovine leukemia virus-induced lymphoma with BoLA-DRB3 polymorphisms at DNA, amino acid, and binding pocket property levels. *Pathogens*, 2021, 10(4): 437 (doi: 10.3390/pathogens10040437).
59. Miyazato P., Katsuya H., Fukuda A., Uchiyama Y., Matsuo M., Tokunaga M., Hino S., Nakao M., Satou Y. Application of targeted enrichment to next-generation sequencing of retroviruses integrated into the host human genome. *Sci. Rep.*, 2016, 6: 28324 (doi: 10.1038/srep28324).
60. Rosewick N., Hahaut V., Durkin K., Artesi M., Karpe S., Wayet J., Griebel P., Arsic N., Marçais A., Hermine O., Burny A., Georges M., Van den Broeke A. An improved sequencing-based bioinformatics pipeline to track the distribution and clonal architecture of proviral integration sites. *Front Microbiol.*, 2020, 11: 587306 (doi: 10.3389/fmicb.2020.587306).
61. Pluta A., Jaworski J.P., Douville R.N. Regulation of Expression and latency in BLV and HTLV. *Viruses*, 2020, 12(10): 1079 (doi: 10.3390/v12101079).
62. Arainga M., Takeda E., Aida Y. Identification of bovine leukemia virus tax function associated with host cell transcription, signaling, stress response and immune response pathway by microarray-based gene expression analysis. *BMC Genomics*, 2012, 13: 121 (doi: 10.1186/1471-2164-13-121).
63. Arainga M., Murakami H. Aida Y. Visualizing spatiotemporal dynamics of apoptosis after G1 arrest by human T cell leukemia virus type 1 Tax and insights into gene expression changes using microarray-based gene expression analysis. *BMC Genomics*, 2012, 13: 275 (doi: 10.1186/1471-2164-13-275).

64. Pierard V., Guiguen A., Colin L., Wijmeersch G., Vanhulle C., Van Driessche B., Dekoninck A., Blazkova J., Cardona C., Merimi M., Vierendeel V., Calomme C., Nguyên T.L., Nuttinck M., Twizere J.C., Kettmann R., Portetelle D., Burny A., Hirsch I., Rohr O., Van Lint C. DNA cytosine methylation in the bovine leukemia virus promoter is associated with latency in a lymphoma-derived B-cell line: potential involvement of direct inhibition of cAMP-responsive element (CRE)-binding protein/CRE modulator/activation transcription factor binding. *J. Biol. Chem.*, 2010, 285(25): 19434-19449 (doi: 10.1074/jbc.M110.107607).
65. Fu J., Qu Z., Yan P., Ishikawa C., Aqeilan R.I., Rabson A.B., Xiao G. The tumor suppressor gene WWOX links the canonical and noncanonical NF-κB pathways in HTLV-I Tax-mediated tumorigenesis. *Blood*, 2011, 117(5): 1652-1661 (doi: 10.1182/blood-2010-08-303073).
66. Huguet C., Crepieux P., Laudet V. Rel/NF-kappa B transcription factors and I kappa B inhibitors: evolution from a unique common ancestor. *Oncogene*, 1997, 15(24): 2965-2974 (doi: 10.1038/sj.onc.1201471).
67. Arainga M., Takeda E., Aida Y. Identification of bovine leukemia virus tax function associated with host cell transcription, signaling, stress response and immune response pathway by microarray-based gene expression analysis. *BMC Genomics*, 2012, 13: 121 (doi: 10.1186/1471-2164-13-121).
68. Lendez P.A., Passucci J.A., Poli M.A., Gutierrez S.E., Dolcini G.L., Ceriani M.C. Association of TNF-α gene promoter region polymorphisms in bovine leukemia virus (BLV)-infected cattle with different proviral loads. *Arch. Virol.*, 2015, 160(8): 2001-2007 (doi: 10.1007/s00705-015-2448-5).
69. Kosovskii G.Yu., Glazko V.I., Koval'chuk S.N., Glazko T.T. Changes in leukocyte and erythrocyte blood profile and parameters under a combined *Anaplasma marginale* and Bovine leukemia virus infection in cattle *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2017, 52(2): 391-400 (doi: 10.15389/agrobiology.2017.2.391eng).
70. Chang L.Y., Lin Y.C., Chiang J.M., Mahalingam J., Su S.H., Huang C.T., Chen W.T., Huang C.H., Jeng W.J., Chen Y.C., Lin S.M., Sheen I.S., Lin C.Y. Blockade of TNF-α signaling benefits cancer therapy by suppressing effector regulatory T cell expansion. *Oncoimmunology*, 2015, 4(10): e1040215 (doi: 10.1080/2162402X.2015.1040215).
71. Ohira K., Nakahara A., Konnai S., Okagawa T., Nishimori A., Maekawa N., Ikebuchi R., Kohara J., Murata S., Ohashi K. Bovine leukemia virus reduces anti-viral cytokine activities and NK cytotoxicity by inducing TGF-β secretion from regulatory T cells. *Immun. Inflamm. Dis.*, 2016, 4(1): 52-63 (doi: 10.1002/iid3.93).
72. Vlasova A.N., Saif L.J. Bovine Immunology: implications for dairy cattle. *Front. Immunol.*, 2021, 12: 643206 (doi: 10.3389/fimmu.2021.643206).
73. Tao S.C., Yuan T., Rui B.Y., Zhu Z.Z., Guo S.C., Zhang C.Q. Exosomes derived from human platelet-rich plasma prevent apoptosis induced by glucocorticoid-associated endoplasmic reticulum stress in rat osteonecrosis of the femoral head via the Akt/Bad/Bcl-2 signal pathway. *Theranostics*, 2017, 7(3): 733-750 (doi: 10.7150/thno.17450).
74. Safari R., Jacques J.R., Brostaux Y., Willems L. Ablation of non-coding RNAs affects bovine leukemia virus B lymphocyte proliferation and abrogates oncogenesis. *PLoS Pathog.*, 2020, 16(5): e1008502 (doi: 10.1371/journal.ppat.1008502).
75. Zhang X., Ma X., Jing S., Zhang H., Zhang Y. Non-coding RNAs and retroviruses. *Retrovirology*, 2018, 15(1): 20 (doi: 10.1186/s12977-018-0403-8).
76. Durkin K., Rosewick N., Artesi M., Hahaut V., Griebel P., Arsic N., Burny A., Georges M., Van den Broeke A. Characterization of novel Bovine Leukemia Virus (BLV) antisense transcripts by deep sequencing reveals constitutive expression in tumors and transcriptional interaction with viral microRNAs. *Retrovirology*, 2016, 13(1): 33 (doi: 10.1186/s12977-016-0267-8).
77. Pluta A., Blazhko N.V., Ngirande C., Joris T., Willems L., Kuzmak J. Analysis of nucleotide sequence of Tax, miRNA and LTR of bovine leukemia virus in cattle with different levels of persistent lymphocytosis in Russia. *Pathogens*, 2021, 10: 246 (doi: 10.3390/pathogens10020246).
78. Herbert K.M., Nag A. A tale of two RNAs during viral infection: how viruses antagonize mRNAs and small non-coding RNAs in the host cell. *Viruses*, 2016, 8(6): 154 (doi: 10.3390/v8060154).
79. Kincaid R.P., Chen Y., Cox J.E., Rethwilm A., Sullivan C.S. Noncanonical microRNA (miRNA) biogenesis gives rise to retroviral mimics of lymphoproliferative and immunosuppressive host miRNAs. *mBio*, 2014, 5(2): e00074-14 (doi: 10.1128/mBio.00074-14).
80. Ojha C.R., Rodriguez M., Dever S.M., Mukhopadhyay R., El-Hage N. Mammalian microRNA: an important modulator of host-pathogen interactions in human viral infections. *J. Biomed. Sci.*, 2016, 23(1): 74 (doi 10.1186/s12929-016-0292-x).
81. Te Pas M.F., Madsen O., Calus M.P., Smits M.A. The importance of endophenotypes to evaluate the relationship between genotype and external phenotype. *Int. J. Mol. Sci.*, 2017, 18(2): 472 (doi: 10.3390/ijms18020472).
82. Braud M., Magee D.A., Park S.D.E., Sonstegard T.S., Waters S.M., MacHugh D.E., Spillane C. Genome-wide microRNA binding site variation between extinct wild aurochs and modern cattle identifies candidate microRNA-regulated domestication genes. *Front. Genet.*, 2017, 8: 3 (doi: 10.3389/fgene.2017.00003).
83. Do D.N., Li R., Dudemaine P.L., Ibeagha-Awemu E.M. MicroRNA roles in signalling during

lactation: an insight from differential expression, time course and pathway analyses of deep sequence data. *Sci. Rep.*, 2017, 7: 44605 (doi: 10.1038/srep44605).
84. Wang D., Liang G., Wang B., Sun H., Liu J., Guan L.L. Systematic microRNAome profiling reveals the roles of microRNAs in milk protein metabolism and quality: insights on low-quality forage utilization. *Sci. Rep.*, 2016, 6: 21194 (doi: 10.1038/ srep21194).
85. Derse D., Casey J.W. Two elements in the bovine leukemia virus long terminal repeat that regulate gene expression. *Science*, 1986, 231: 1437-1440 (doi: 10.1126/science.3006241).
86. Van Driessche B., Rodari A., Delacourt N., Fauquenoy S., Vanhulle C., Burny A., Rohr O,. Van Lint C. Characterization of new RNA polymerase III and RNA polymerase II transcriptional promoters in the Bovine Leukemia Virus genome. *Sci. Rep.*, 2016, 6: 31125 (doi: 10.1038/srep31125).
87. Tan B.J., Sugata K., Reda O., Matsuo M., Uchiyama K., Miyazato P., Hahaut V., Yamagishi M., Uchimaru K., Suzuki Y., Ueno T., Suzushima H., Katsuya H., Tokunaga M., Uchiyama Y., Nakamura H., Sueoka E., Utsunomiya A., Ono M., Satou Y. HTLV-1 infection promotes excessive T cell activation and transformation into adult T cell leukemia/lymphoma. *J. Clin. Invest.*, 2021, 131(24): e150472 (doi: 10.1172/JCI150472).
88. Rosewick N., Momont M., Durkin K., Takeda H., Caiment F., Cleuter Y., Vernin C., Mortreux F., Wattel E., Burny A., Georges M., Van den Broeke A. Deep sequencing reveals abundant noncanonical retroviral microRNAs in B-cell leukemia/lymphoma. *Proceedings of the National Academy of Sciences*, 2013, 110(6): 2306-2311 (doi: 10.1073/pnas.1213842110).
89. Kincaid R.P., Burke J.M., Sullivan C.S. RNA virus microRNA that mimics a B-cell oncomiR. *Proceedings of the National Academy of Sciences*, 2012, 109(8): 3077-3082 (doi: 10.1073/pnas.1116107109).
90. Olive V., Jiang I., He L. mir-17-92, a cluster of miRNAs in the midst of the cancer network. *Int. J. Biochem .Cell Biol.*, 2010, 42(8): 1348-1354 (doi: 10.1016/j.biocel.2010.03.004).
91. Tajima S., Zhuang W.Z., Kato M.V., Okada K., Ikawa Y., Aida Y. Function and conformation of wild-type p53 protein are influenced by mutations in bovine leukemia virus-induced B-cell lymphosarcoma. *Virology*, 1998, 243: 235-246 (doi: 10.1006/viro.1998.9051).
92. Dequiedt F., Kettmann R., Burny A., Willems L. Mutations in the p53 tumor-suppressor gene are frequently associated with bovine leukemia virus-induced leukemogenesis in cattle but not in sheep. *Virology*, 1995, 209: 676-683 (doi: 10.1006/viro.1995.1303).
93. Konnai S., Usui T., Ikeda M., Kohara J., Hirata T.-I., Okada K., Ohashi K., Onuma M. Tumor necrosis factor-alpha genetic polymorphism may contribute to progression of bovine leukemia virus-infection. *Microbes Infect.,* 2006, 8: 2163-2171 (doi: 10.1016/j.micinf.2006.04.017).
94. Bai L., Hirose T., Assi W., Wada S., Takeshima S.-N., Aida Y. Bovine leukemia virus infection affects host gene expression associated with DNA mismatch repair. *Pathogens*, 2020, 9: 909 (doi: 10.3390/pathogens9110909).
95. Assi W., Hirose T., Wada S., Matsuura R., Takeshima S.-N., Aida Y. PRMT5 is required for bovine leukemia virus infection in vivo and regulates BLV gene expression, syncytium formation, and glycosylation in vitro. *Viruses*, 2020, 12: 650 (doi: 10.3390/v12060650).
96. Frie M.C., Droscha C.J., Greenlick A.E., Coussens P.M. MicroRNAs encoded by bovine leukemia virus (BLV) are associated with reduced expression of B cell transcriptional regulators in dairy cattle naturally infected with BLV. *Front. Vet. Sci.*, 2018, 4: 245 (doi: 10.3389/fvets.2017.00245).
97. Bello-Morales R., Andreu S., Ripa I., López-Guerrero J.A. HSV-1 and endogenous retroviruses as risk factors in demyelination. *Int. J. Mol. Sci.*, 2021, 22(11): 5738 (doi: 10.3390/ijms22115738).
98. Elsik C.G., Tellam R.L., Worley K.C. The genome sequence of taurine cattle: a window to ruminant biology and evolution. By the bovine genome sequencing and analysis consortium. *Science*, 2009, 324(5926): 522-528 (doi: 10.1126/science.1169588).
99. Gallus S., Kumar V., Bertelsen M.F., Janke A., Nilsson M.A. A genome survey sequencing of the Java mouse deer (*Tragulus javanicus*) adds new aspects to the evolution of lineage specific retrotransposons in *Ruminantia* (*Cetartiodactyla*). *Gene*, 2015, 571(2): 271-278 (doi: 10.1016/j.gene.2015.06.064).
100. Ivancevic A.M., Kortschak R.D., Bertozzi T., Adelson D.L. Horizontal transfer of BovB and L1 retrotransposons in eukaryotes. *Genome Biol.*, 2018, 19(1): 85 (doi: 10.1186/s13059-018-1456-7).
101. Glazko V.I., Gladyr E.A., Feofilov A.V., Bardukov N.V., Glazko T.T. ISSR-PCR and mobile genetic elements in genomes of farm mammalian species. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2013, 2: 71-76 (doi: 10.15389/agrobiology.2013.2.71eng).
102. Kosovsky G.Y., Glazko V.I., Arkhipov A.V., Petrova I.O., Glazko T.T. Dairy cattle population-specific genetic differentiation based on ISSR-PCR markers. *Russ. Agricult. Sci.*, 2014, 40: 463-466 (doi: 10.3103/S1068367414060135).
103. Glazko V.I., Kosovskii G.Yu., Tagmazyan A.A., Glazko T.T. *Zhivye i biokosnye sistemy*, 2017, 20: article 9 (in Russ.).
104. Glazko V.I., Kosovskii G.Yu., Koval'chuk S.N., Arkhipov A.V., Petrova I.O., Dedovich G.O., Glazko T.T. *Innovatsionnye tekhnologii v meditsine*, 2014, 2(3): 63-79 (in Russ.).
105. Glazko V.I., Kosovskii G.Yu., Koval'chuk S.N., Glazko T.T. *Vestnik RAEN*, 2015, 15(1): 75-81 (in Russ.).
106. Babii A.V., Koval'chuk S.N., Glazko T.T., Glazko V.I., Kosovskii G.I. Functional insights into

genic neighbourhood organization of helitron transposons in *Bos taurus* genomes. *Journal of Siberian Federal University. Biology*, 2018, 11(1): 60-74 (doi: 10.17516/1997-1389-0003).
107. Babii A., Kovalchuk S., Glazko T., Kosovsky G., Glazko V. Helitrons and retrotransposons are co-localized in *Bos taurus* genomes. *Curr. Genomics*, 2017, 18(3): 278-286 (doi: 10.2174/1389202918666161108143909).
108. Glazko V., Kosovsky G., Glazko T. High density of transposable elements in sequenced sequences in cattle genomes, associated with AGC microsatellites. *Global Advanced Research Journal of Agricultural Science*, 2018, 7(2): 034-045e.
109. Cohen J. Do coronavirus genes slip into human chromosomes? *Science*, 2021, 372(6543): 674-675 (doi: 10.1126/science.372.6543.674).
110. Yin Y., Liu X.Z., He X., Zhou L.Q. Exogenous coronavirus interacts with endogenous retrotransposon in human cells. *Front. Cell Infect. Microbiol.*, 2021, 11: 609160 (doi: 10.3389/fcimb.2021.609160).
111. Maillard P.V., van der Veen A.G., Poirier E.Z., Reis e Sousa C. Slicing and dicing viruses: antiviral RNA interference in mammals. *The EMBO Journal*, 2019, 38(8): e100941 (doi: 10.15252/embj.2018100941;
112. Poirier E.Z., Buck M.D., Chakravarty P., Carvalho J., Frederico B., Cardoso A., Healy L., Ulferts R., Beale R., Reis E Sousa C. An isoform of Dicer protects mammalian stem cells against multiple RNA viruses. *Science*, 2021, 373(6551): 231-236 (doi: 10.1126/science.abg2264).
113. Kosovsky G.Y., Glazko V.I., Glazko G.V., Zybaylov B.L., Glazko T.T. Leukocytosis and expression of bovine leukemia virus microRNAs in cattle. *Front. Vet. Sci.*, 2020, 7: 272 (doi: 10.3389/fvets.2020.00272).
114. Duffy S., Shackelton L.A., Holmes E.C. Rates of evolutionary change in viruses: patterns and determinants. *Nat. Rev. Genet.*, 2008, 9(4): 267-276 (doi: 10.1038/nrg2323).
115. Glazko V.I., Glazko T.T. *Nanotekhnologii i okhrana zdorov'ya*, 2013, 5(1): 40-54 (in Russ.).
116. Glazko V.I., Kosovsky G.Y. Structure of genes coding the envelope proteins of the avian influenza a virus and bovine leucosis virus. *Russ. Agricult. Sci.*, 2013, 39: 511-515 (doi: 10.3103/S1068367413060074).
117. Samuilenko A.Ya., Kosovskii G.Yu., Grin' S.A., Sinkovets S.M., Glazko T.T., Glazko V.I. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii, posvyashchennoi 45-letiyu instituta VNITIBP «Nauchnye osnovy proizvodstva i obespecheniya kachestva biologicheskikh preparatov dlya APK»* [Proc. Int. Conf. «Scientific basis for the production and quality of biological products for the agro-industrial complex»]. Moscow, 2014: 106-117 (in Russ.).
118. Henzy J.E., Johnson W.E. Pushing the endogenous envelope. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 2013, 368(1626): 20120506 (doi: 10.1098/rstb.2012.0506).
119. Moratorio G., Fischer S., Bianchi S., Tomé L., Rama G., Obal G., Carriyn F., Pritsch O., Cristina J. A detailed molecular analysis of complete bovine leukemia virus genomes isolated from B-cell lymphosarcomas. *Vet. Res.*, 2013, 44(1): 19 (doi: 10.1186/1297-9716-44-19).
120. Lavigne M., Helynck O., Rigolet P., Boudria-Souilah R., Nowakowski M., Baron B, Brülé S., Hoos S., Raynal B., Guittat L., Beauvineau C., Petres S., Granzhan A., Guillon J., Pratviel G., Teulade-Fichou M.P., England P., Mergny J.L., Munier-Lehmann H. SARS-CoV-2 Nsp3 unique domain SUD interacts with guanine quadruplexes and G4-ligands inhibit this interaction. *Nucleic Acids Res.*, 2021, 49(13): 7695-7712 (doi: 10.1093/nar/gkab571).
121. Ruggiero E., Richter S.N. Viral G-quadruplexes: new frontiers in virus pathogenesis and antiviral therapy. *Annu. Rep. Med. Chem.*, 2020, 54: 101-131 (doi: 10.1016/bs.armc.2020.04.001).
122. Bohálová N., Cantara A., Bartas M., Kaura P., Št'astný J., Pecinka P., Fojta M., Brázda V. Tracing dsDNA virus—host coevolution through correlation of their G-quadruplex-forming sequences. *Int. J. Mol. Sci.*, 2021, 22: 3433 (doi: 10.3390/ijms22073433).
123. Chen W. A potential treatment of COVID-19 with TGF-β blockade. *Int. J. Biol. Sci.*, 2020, 16(11): 1954-1955 (doi: 10.7150/ijbs.46891).
124. Glazko G.V., Kosovskii G.Yu., Zybailov B.L., Glazko V.I., Glazko T.T. *Vestnik RAEN*, 2020, 3: 18-38 (in Russ.).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1099-1110
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Genome structure and genome technologies

UDC 636.5:573.6.086.83:577.21

doi: 10.15389/agrobiology.2021.6.1099eng
doi: 10.15389/agrobiology.2021.6.1099rus

CREATION OF GENOME EDITING SYSTEMS BASED ON CRISPR-CAS9 FOR KNOCKOUT IN *FGF20* AND *HR* GENES OF EMBRYONIC AND GENERATIVE CELLS FROM CHICKEN AND QUAILS

A.N. VETOKH[1] ✉, P.V. SERGIEV[2, 3, 4], M.P. RUBTSOVA[2, 5], N.A. VOLKOVA[1], E.K. TOMGOROVA[1], L.A. VOLKOVA[1], N.A. ZINOVIEVA[1]

[1]*Ernst Federal Research Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail anastezuya@mail.ru (✉ corresponding author), natavolkova@inbox.ru, tomgorova@rambler.ru, ludavolkova@inbox.ru, n_zinovieva@mail.ru;
[2]*Faculty of Chemistry, Lomonosov Moscow State University,* GSP-1, 1-3, Leninskie Gory, Moscow, 119991 Russia, e-mail petya@genebee.msu.ru, mprubtsova@gmail.com;
[3]*Center of Life Sciences, Skolkovo Institute of Science and Technology,* 30-1, Bolshoi bulvar, Skolkovo, Moscow Province, 143026 Russia, e-mail petya@genebee.msu.ru;
[4]*Institute of Functional Genomics, Lomonosov Moscow State University,* 1, Leninskie Gory, Moscow, 119991 Russia, e-mail petya@genebee.msu.ru;
[5]*Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS,* 16/10, ul. Miklukho-Maklaya, Moscow, 117997 Russia, e-mail mprubtsova@gmail.com
ORCID:
Vetokh A.N. orcid.org/0000-0002-2865-5960
Sergiev P.V. orcid.org/0000-0001-8866-1863
Rubtsova M.P. orcid.org/0000-0002-4808-5416
Volkova N.A. orcid.org/0000-0001-7191-3550
Tomgorova E.K. orcid.org/0000-0001-5398-8815
Volkova L.A. orcid.org/0000-0002-9407-3686
Zinovieva N.A. orcid.org/0000-0003-4017-6863

The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Foundation for Basic Research, grant No. 18-29-07079
Received September 30, 2021

Abstract

Genome editing technologies using site-specific nucleases (ZNF, TALEN, CRISPR/Cas9) are used more and more in animal husbandry, including poultry farming. With the use of these technologies, scientists hope not only to speed up the process of creating breeds with improved economically useful traits, high resistance to infectious diseases, but also to create individuals carrying phenotypes, the introduction of which into animal and bird populations by traditional breeding methods is impossible or difficult. The creation of individuals devoid of plumage in order to improve the commercial qualities of poultry product is of interest for industrial poultry farming. For this, we selected the *FGF20* and *HR* genes associated with the development and growth of hair in mammals (F. Benavides et al., 2009) and feathers in birds (K.L. Wells et al., 2012). The aim of the study was to create a system for knocking out the *FGF20* and *HR* genes in chickens and *FGF20* in quails by genome editing techniques. We inactivated *FGF20* and *HR* genes in the region of the third exons based on the analysis of their structure. The optimal cutting regions of these genes and guide RNAs and primers for amplifying the *FGF20* and *HR* DNA fragments were selected bioinformatically and using internet resources (https://zlab.bio/guide-design-resources, https://www.ncbi.nlm.nih.gov/). To create genetic constructs for cutting in the regions encoding *FGF20* and *HR*, the vector pX458 was selected (F.A. Ran et al., 2013). The hybridized oligonucleotides 5′-CACCGAAAGATGGTACTCCCAGAGA-3′ and 3′-CTTTCTACCATGAGGGTCTCTCAAA-5′ (for *FGF20* gene in chicken), 5′-CACCGTCCATGTTTGTACACGTTGG-3′ and 3′-CAGGTACAAACATGTGCAACCCAAA-5′ (for *FGF20* gene in chicken and in quails); 5′-CACCGACGTGGCTGACGCGGCACT-3′ and 3′-CTGCACCGACTGCGCCGTGACAAA-5′ (for gene *HR*) were used for ligation. The effectiveness of cloning constructs was confirmed by sequencing. The plasmids that were obtained were used for edit the genome of embryonic (fibroblasts) and generative (primordial germ cells — PGCs, spermatogonia) chicken and quail cells in in vitro experiments. Target cells were transfected by electroporation. Efficiency of electroporation was evaluated on a high-performance fluorescent cell sorter BD FacsAria III («BD Biosciences», USA) by expression of the *eGFP* marker gene. The proportion of in vitro transfected embryonic fibroblasts, PGCs and spermatogonia from chickens with a knockout of the *FGF20* gene reached 5.7, 0.9, and 1.2 %, with a knockout of the *HR* gene — 7.4, 0.8, and 1.0 %, respectively. The percentage of embryonic fibroblasts, PGCs and spermatogonia from quails with a knockout of the *FGF20* gene was 6.3,

0.9, and 1.1 %, respectively. Genomic DNA was isolated from transformed chicken and quail cells and used for amplification and sequencing of the regions of the *FGF20* and *HR* genes in which deletions were introduced. The presence of multiple mutations in the amplified DNA regions was shown. The data obtained indicate the success of the knockout system creation for *FGF20* and *HR* genes in chickens and for *FGF20* gene in quails using genetic constructs based on the pX458 vector.

Keywords: genome editing, CRISPR/Cas9, chicken, quail, primordial germ cells (PGCs), spermatogonia cells, *FGF20*, *HR*

The development of genomic editing technologies using site-specific endonucleases (ZNF, TALEN, CRISPR/Cas9) has opened up new possibilities for introducing targeted genetic changes into animal and bird embryonic lines [1, 2]. These technologies are increasingly attracting attention due to their high efficiency and specificity [3]. In animal husbandry, including poultry, the researchers rely on genome editing to accelerate breeding for improved economically useful traits [4, 5], increased resistance to infectious diseases [6, 7] and also the creation of individuals with phenotypes which are diffecult or impossible to introduce into populations of animals and birds by traditional breeding methods [8, 9]. Obtaining lines of poultry that transmit the introduced genetic changes by inheritance requires manipulations with generative cells. However, the peculiarities of the embryonic development and reproductive physiology of birds do not allow application of methods used in mammals for genetic engineering manipulations, for example, microinjections and somatic cloning [10, 11]. Note that a significant part of the embryonic period in birds passes outside the body of females, which facilitates access to the embryo in the early stages of development. To date, a wide range of developed methods and approaches allow the introduction of genetic constructs into avian embryonic cells [12]. A number of research works confirm the successful production of genetically modified chickens and quails expressing the reporter genes *LacZ* [13] and *GFP* [14], the bacterial β-lactamase gene [15], human interferon α2b [16], human β-interferon [17], human granulocyte colony stimulating factor [18], monoclonal antibodies [19], β-interleukin receptor antagonist [20] and human growth hormone [21]. However, the technologies used to modify the genome of animals and birds until 2012 were non-specific, with the exception of model scientific systems. Since 2012, CRISPR/Cas9 technology has been consistently replacing other methods of industrial transgenesis. There are a number of works on the successful use of CRISPR/Cas9 for the modification of mammalian cells with the subsequent production of individuals with desired properties [22, 23]. In birds, until recently, the transformation of blastodermal cells at stage X using lentiviral and retroviral vectors was considered the most effective way to introduce hereditary changes into generative chicken cells [24]. In recent years, with the development of new methods for editing the genome, in particular TALEN and CRISPR, there has been an increasing interest in using generative cells, e.g., primordial germ cells (PGCs) and spermatogonia, as target cells for the introduction of genetic constructs. PGCs are embryonic cells characterized by pluripotency, that is, the ability to differentiate into both male and female germ cells. Spermatogonia refers to the stem cells of the testes of males. Spermatogonia are a small population of spermatogenic cells located on the basement membrane of the seminiferous tubules. They have the ability to self-renewal and differentiation with the formation of sperm, the male highly specialized mature germ cells.

The peculiarities of PGCs and spermatogonia open up wide opportunities for realizing their potential as targets for genome editing in poultry in order to create individuals with desired traits. The ability to manipulate cells of this type in vitro allows precise integration of expression constructs into a specific locus under a preselected endogenous promoter-enhancer system. Moreover, these technologies allow preservation of the endogenous gene expression. The use of PGCs as a

vector involves their isolation from donor embryos, transformation in in vitro culture, and introduction into recipient embryos. When working with spermatogonia, donor spermatogonia are subjected to transformation in vitro, selection of the transformed cells and their transplantation into the testes of sterile male recipients followed by the production of sperm for insemination of females to obtain genetically modified offspring.

TALEN and CRISPR technologies are applicable for genetic modification of chicken cells in vitro [25, 26]. A number of works have been published on the successful production of chickens and quails with desired properties using various genomic editing systems [27, 28]. One of the possible directions of genome editing, which is of practical interest for improving the commercial qualities of poultry products, is the creation of individuals devoid of plumage. The genes (*FGF20* and *HR*) required for the development and growth of hair in mammals [29] and feathers in birds [30] have been identified.

This report submits the results of studies on the creation of an editing system based on CRISPR/Cas9 for knockout of the *FGF20* and *HR* genes that control the development of plumage in birds. Here, on embryonic fibroblasts and generative cells (PCG and spermatogonia), we revealed the effectiveness of using the created constructs for introducing deletions in the *FGF20* and *HR* genes of chickens and quails under in vitro conditions.

Our goal was to develop a genome editing system for knockout of the *FGF20* and *HR* genes in chickens and quails, suitable for obtaining genetically modified poultry.

Materials and methods. Embryonic fibroblasts were isolated from 5-day-old chicken embryos (*Gallus gallus domesticus,* Russian White breed) or 4-day-old quail embryos (*Coturnix coturnix*, Japanese quail breed). Embryos were removed under aseptic conditions. For disaggregation, the embryos were first mechanically crushed with scissors, then subjected to enzymatic treatment by incubating tissue pieces in 0.15% trypsin solution (Gibco, Thermo Scientific, USA) for 15 min at 37 °C. Primordial germ cells were isolated from 6-day-old chicken embryos and 4-day-old quail embryos. Dissociation was carried out by successive mechanical and enzymatic treatments, as described above, but at a trypsin concentration of 0.05%. Spermatogenic cells were isolated by sequential mechanical and enzymatic treatment of the testis tissue of 1-week-old males. For enzymatic treatment, a 0.25% trypsin solution was used, incubated for 30 min at 37 °C.

The proportion of viable cells in the resulting cell suspension after mechanical and enzymatic treatment of embryos and testicular tissue was assessed by staining with 0.4% trypan blue for 10 min at 37 °C. Stained cells were counted in a Countess cell counter (Thermo Fisher Scientific, USA).

Suspensions of embryonic fibroblast cells and PGCs obtained after enzymatic treatment of embryos were transferred to Petri dishes and cultured in DMEM HG growth medium (Gibco, Thermo Scientific, USA) with a high glucose content (4.5 g/l), 10% fetal bovine serum (FBS, HyClone, USA), glutamine (2 mM), 2-mercaptoethanol (10^{-6} mM), and gentamicin (50 μg/ml). For the primary culture of testis cells, DMEM HG medium with a glucose content of 4.5 g/l supplemented with 20% FBS, alpha-glutamine (2 mM), MEM (100×), antimycotic antibiotic (100×) and ITS (100×) was used as a growth medium). Spermatogonia were cultured in DMEM HG supplemented with 5% FBS, 2 mM alpha-glutamine, MEM (10 μl/ml), antibiotic antimycotic (100×), ITS (10 μl/ml), mercaptoethanol (5×10^{-5} M), albumin (5 mg/ml), DL-lactic acid (1 μl/ml), EGF (20 ng/ml), bFGF (10 ng/ml), LIF (2 ng/ml).

Growth additives, amino acids, and antibiotics used in the culture media

are produced by Gibco (Thermo Scientific, USA) and Sigma (USA).

Chicken and quail cells were cultured at 37 °C and 5% CO_2. For passage and molecular genetic studies, the cells were removed from the substrate with a 0.25% trypsin solution.

Microscopy of the obtained cultures of embryonic fibroblasts, PGCs, and spermatogonia of chickens and quails was performed (an inverted microscope Nikon Eclipse TS100, Nikon, Japan).

Guide RNA sequences were designed using https://zlab.bio/guide-design-resources, https://www.ncbi.nlm.nih.gov/ tools, the GalGal5 genome assembly variant and gene names FGF20 (Gene ID: 428779) and HR (Gene ID: 107049623) for chicken and Coturnix japonica 2.0 and the gene name FGF20 (Gene ID: 107313688) for quail. Optimal cutting sites for the *FGF20* gene of chickens and the *HR* gene of quail were selected. To create genetic constructs for cutting selected genome regions, pairs of the following oligonucleotides were hybridized: 5'-CACCGAAAGAT-GTACTCCCAGAGA-3' and 3'-CTTTCTACCA-TGAGGGTCTCTCAA-5' (for the *FGF20* gene in chickens), 5'-CACCGTCCA-TGTTTGTACACGTTGG-3' and 3'- CAGGTACAAACATGTGCAACCCAAA-5′ (for the *FGF20* gene in chickens and quails); 5'-CA-CCGACGTGGCTGAC-GCGGCACT-3' and 3'-CTGCACCGACTGCGCCA-5' (for the *HR* gene of chicken). Hybridized oligonucleotides were ligated with plasmid pX458 (Addgene #48138) linearized with BbsI restriction endonuclease (ER1011, Thermo Scientific, USA) as described [31]. After ligation and transformation of *Escherichia coli* cells, the grown colonies were subcultured in liquid LB medium with ampicillin and used to isolate plasmids. Analysis of the results of cloning was performed by sequencing. Successfully cloned constructs were used to transfect cells.

The commercial QuickExtract™ DNA Extraction Solution kit (Lucigen Corporation, USA) was used as per the manufacturer's recommendations to isolate genomic DNA when creating constructs and evaluating the efficiency of cutting the target genome region. Amplification was carried out in a PCR mixture PCR MM based on Taq DNA polymerase (K0171, Thermo Scientific, USA) in a 25-μl mix volume at 60 °C for hybridization and at 72 °C for 1 min for elongation. Amplification of the *FGF20* gene region was performed with primers F20_CHK2F 5'-TGTTCCTTTGTGCAGGAGAAACT-2' and F20_CHK2R 5'-TCCCTCTCT-CCTCAGCTGTATC-3'.

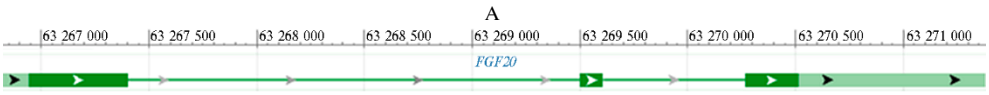
Editing systems were introduced into embryonic fibroblasts, PGCs, and spermatogonia by electroporation (a Neon™ Transfection System, Invitrogen, USA). Transfected cells were selected using a high-throughput BD FACSAria III cell sorter (BD Biosciences, USA).

Efficiency of cutting the target genome region was evaluated by amplification and sequencing of the corresponding genomic DNA fragments with the designed primers in the above modes (the sequences of the amplified fragments are shown in Figures 1 and 2). Sequencing (performed by OOO Sintol, Moscow) was performed by Sanger method with a direct primer for amplification.

Results. Development of the CRISPR/Cas9 genomic editing system for knockout of the *FGF20* and *HR* genes started with a bioinformatic search for the *HR* gene homologue in birds and guide RNA sequences to inactivate the *FGF20* and *HR* genes.

The *FGF20* gene of the chicken *G. gallus domesticus* is located on chromosome 4 (Fig. 1, A). To inactivate this gene, we chose exon III. The figure shows cut sites in the *FGF20* gene of chickens and quails, which are optimal for introducing target mutations into the genome with the CRISPR/Cas9 system, hybridization sites for two guide RNAs and primers for amplification of the selected gene

FGF20 regions. (see Fig. 1, B). One of the guide RNAs was universal for chicken and quail, the second one was complementary only for chicken. The primers for amplification of target regions of the *FGF20* gene corresponded to the genome of both chicken and quail.



B

Quail (*Coturnix japonica*)

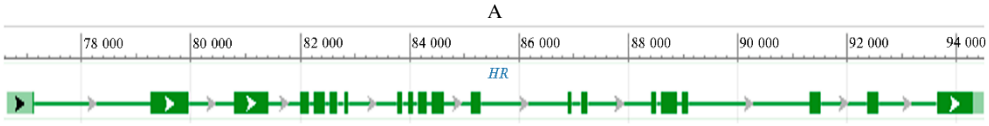
TGAGTGCATCTTCAGGGAACAGTTTGAGGAAAACTGGTACAACACTTACTCCTCCAA**CGTG**TACAAACATGGAGAT
TCTGGGCGGCGATACTTCGTAGCACTTAACAAGGACGGTACTCCC**AGAG**ATGGTGCAAGGTCCAAAAGACACCAGA
AATTCACACATTTCCTGCCCAGACCTGTGGATCCTGAAAGAGTTCCAGACT

Rooster (*Gallus gallus domesticus*)

GAGTGCATCTTCAGGGAACAGTTTGAGGAAAACTGGTACAACACTTACTCCTCCAA**CGTG**TACAAACATGGAGATT
CTGGGCGGCGATACTTCGTAGCACTTAACAAAGATGGTACTCCC**AGAG**ATGGAGCAAGGTCCAAAAGACACCAGAA
GTTCACACATTTCCTGCCCAGACCTGTGGATCCTGAAAGAGTTCCAGA

Fig. 1. Scheme of the *FGF20* gene (A) **and regions of hybridization of guide RNAs** (B) **in *Gallus gallus domesticus* and *Coturnix japonica*.** The genomic coordinate of chromosome 4 of *G. gallus domesticus* is shown with the location of exon (thick lines) and intron (thin lines) regions of the gene. Non-coding regions are marked in light green, coding regions in dark green. The arrows show the direction of transcription. Alignment of genome regions of quail *C. japonica* and chicken *G. gallus domesticus* with hybridization regions of the designed primers (marked in green) and guide RNA (marked in turquoise) is presented. PAM (protospacer adjacent motive) sites are shown in purple.

The *HR* gene is located on chicken chromosome 22 (Fig. 2, A). For its inactivation, exon III was chosen, encoding the amino acids of the active center of the HR enzyme, which is a lysine demethylase. Figure 2, B shows a fragment of the chicken *G. gallus domesticus* genome with selected regions for hybridization of primers and guide RNA.



B

GTCGGACGGTGAGAGCCCGGCGGGGGCGCACGGGGGCGTCCAGAAGCTGAAGAAGACGTGGCTGACGCGGCACTCGGA
GCAGTCGGGGCCGCGCTGTAGGGCGGCGCGGAGGGACGGAGCCGAAGAGGGCTTCAAACGCGCG

Fig. 1. Scheme of the gene *HR* (A) **and regions of hybridization of guide RNAs** (B) **in *Gallus gallus domesticus*.** The genomic coordinate of chromosome 22 of *G. gallus domesticus* is shown with the location of exon (thick lines) and intron (thin lines) regions of the gene *HR*. Non-coding regions are marked in light green, coding regions in dark green. The arrows show the direction of transcription. A genome region of chicken *G. gallus domesticus* with hybridization regions of the designed primers (marked in green) and guide RNA (marked in turquoise) is presented. PAM (protospacer adjacent motive) sites are shown in purple.

Oligonucleotides for the guide RNAs were synthesized and used to make genetic constructs for the inactivation of the *FGF20* and HR *genes*. Plasmid pX458 was treated with restriction endonuclease BbsI and ligated at the cleavage site with hybridized oligonucleotides F20C, F20U, and HR (Fig. 3).

F20C
5′-CACCGAAAGATGGTACTCCCAGAGA
CTTTCTACCATGAGGGTCTCTCAAA-5′

F20U
5′-CACCGTCCATGTTTGTACACGTTGG
CAGGTACAAACATGTGCAACCCAAA-5′

HR
5′-CACCGACGTGGCTGACGCGGCACT
CTGCACCGACTGCGCCGTGACAAA-5′

Fig. 3. Oligonucleotides providing specificity of guide RNAs and used to create plasmids for the *FGF20* and *HR* inactivation by the CRISPR/Cas9 technoogy.

After ligation and transformation of competent *E. coli* JM109 cells [32], and the clones were used for plasmid isolation. Sequencing of the obtained plasmids confirmed the success of cloning constructs which were further

used for transfection of chicken and quail cells.

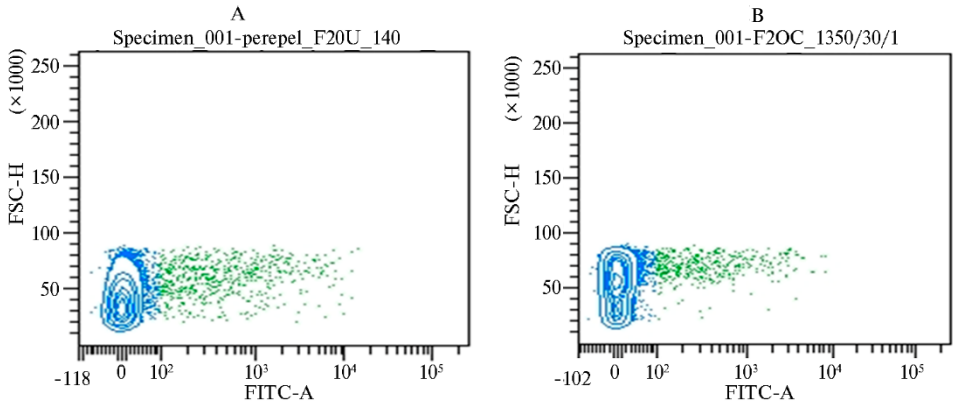


Fig. 4. An example of sorting a population of quail (A) **and chicken** (B) **embryonic fibroblast cells after transfection with pX458 plasmid-based constructs.** The X-axis shows the intensity of fluorescence in the green range, the Y-axis shows light scattering. Green indicates cells with green fluorescence.

The effectiveness of editing systems designed to knock out the *FGF20* and *HR* genes was first evaluated on embryonic fibroblasts due to the simplicity and availability of their production. Chicken and quail embryonic fibroblasts with a plasmid encoding the components of the CRISPR/Cas9 genomic editing system were separated from non-transfected cells using a cell sorter (Fig. 4). pX458 contains regions corresponding to the *Cas9* and *GFP* genes the coding regions of which are separated from each other by the sequence encoding the P2A peptide. Thereof, cells which synthesize Cas9 also contain GFP and can be separated from non-transfected cells due to fluorescence (see Fig. 4). The proportion of successfully transfected chicken embryonic cells (sample size of 10,000 cells) using systems for knocking out the *FGF20* and *HR* genes reached 5.7 and 7.4%, respectively. According to the distribution of cells by fluorescence intensity, the efficiency of transfection of quail embryonic cells using the editing system for the *FGF20* gene knockout was 6.3%.

Sorted transfected chicken and quail embryonic fibroblasts were used to isolate genomic DNA in order to evaluate the efficiency of editing the *FGF20* and *HR* genes. The isolated DNA was used for PCR amplification of loci containing regions of complementarity with guide RNA (see Fig. 1, 2). The isolated PCR products were analyzed by Sanger sequencing. The analysis of transfected chicken (Fig. 5, 6) and quail embryonic fibroblasts detected multiple microdeletions.

The data obtained (see Fig. 5, 6) allow us to conclude that our constructs are highly efficient to introduce microdeletions into the *FGF20* and *HR* genes. Of the two constructs targeting the *FGF20* gene, the F20U proved to be more effective and was used together with the HR construct.

When generating genetically modified birds, a directed modification of gonadal cells with the aim to further obtain offspring with a modified genome is of greatest interest. For these purposes, both mature germ cells and their precursors, PGCs and spermatogonia are suitable.

With disaggregation of chicken and quail embryos by mechanical and enzymatic treatment, we obtained a suspension of dissociated cells (the proportion of non-viable cells did not exceed 5%). The suspension contained different types of embryonic cells the separation of which by adhesion [33] maximally enriched population of embryonic cells with PGCs. The proportion of PGCs from the total number of other cell types in the culture of chicken and quail embryonic cells reached 88 and 81%, respectively. A small population of fibroblasts remaining

after cell separation served as a feeder layer on which PGCs were attached and cultured, forming colonies (Fig. 7).

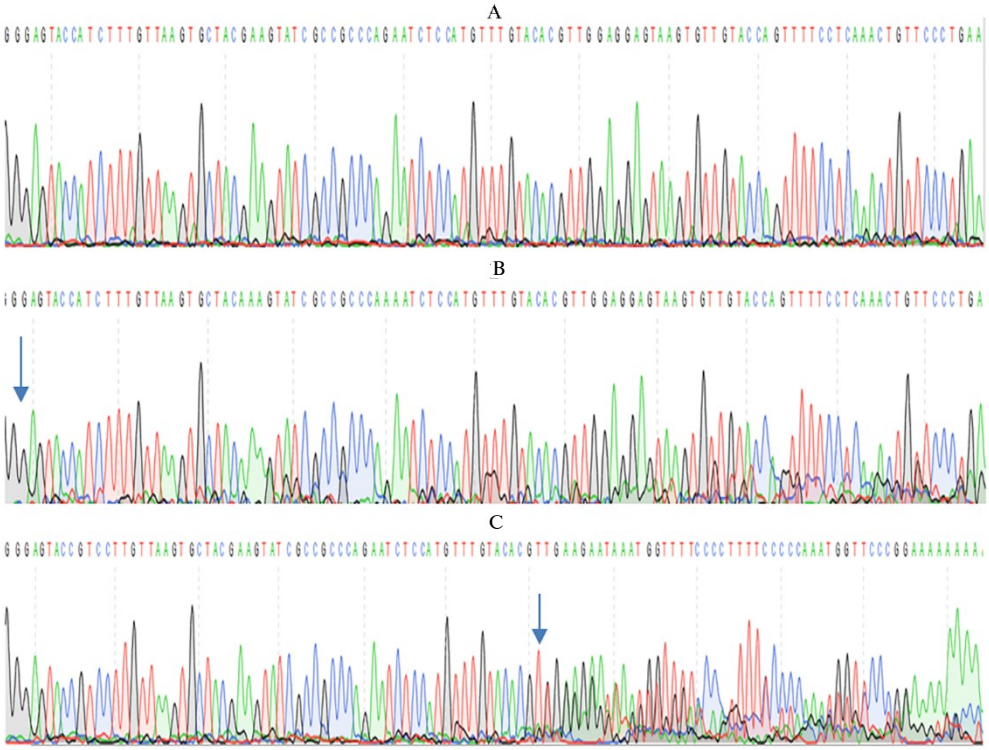


Fig. 5. Analysis of the CRISPR/Cas9 editing system efficiency for the *FGF20* gene by Sanger sequencing-based assay: A — unedited chicken *FGF20* gene; B — chicken *FGF20* gene after editing with Cas9 and F20C guide RNA; C — chicken *FGF20* gene after editing with Cas9 and F20U guide RNA. The arrows show the sites of gene cutting. You can see the overlap of the results of sequencing the products of multiple microdeletions. When sequencing heterogeneous microdeletion products, in contrast to a homogeneous wild-type amplicon, there is an overlap of peaks corresponding to different nucleotides (green for A, black for G, blue for C, and red for T).

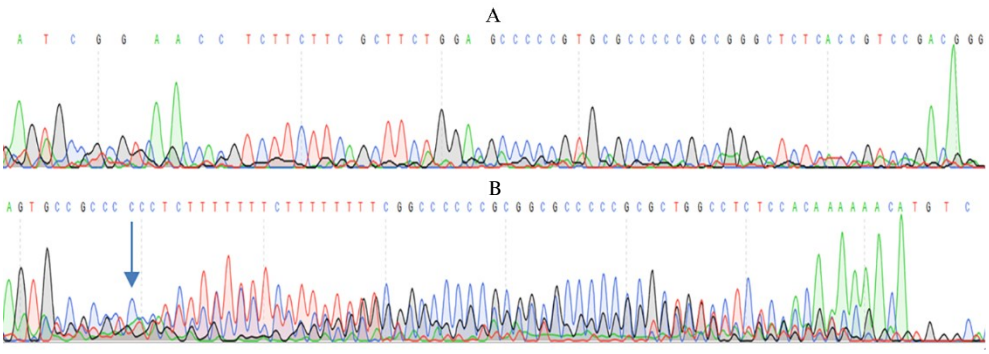


Fig. 6. Analysis of the CRISPR/Cas9 editing system efficiency for the *HR* gene by Sanger sequencing-based assay: A — unedited chicken *HR* gene; B — chicken *HR* gene after editing with Cas9 and HR guide RNA. The arrows show the sites of gene cutting. You can see the overlap of the results of sequencing the products of multiple microdeletions. When sequencing heterogeneous microdeletion products, in contrast to a homogeneous wild-type amplicon, there is an overlap of peaks corresponding to different nucleotides (green for A, black for G, blue for C, and red for T).

Disaggregation of the testis tissue of roosters and quails by trypsin created suspensions consisting mainly of Sertoli cells and spermatogonia. In culturiing, Sertoli cells spread flat on the surface of Petri dishes. Spermatogonia attached to Sertoli cells, forming colonies on days 7-8 of culture (Fig. 8).

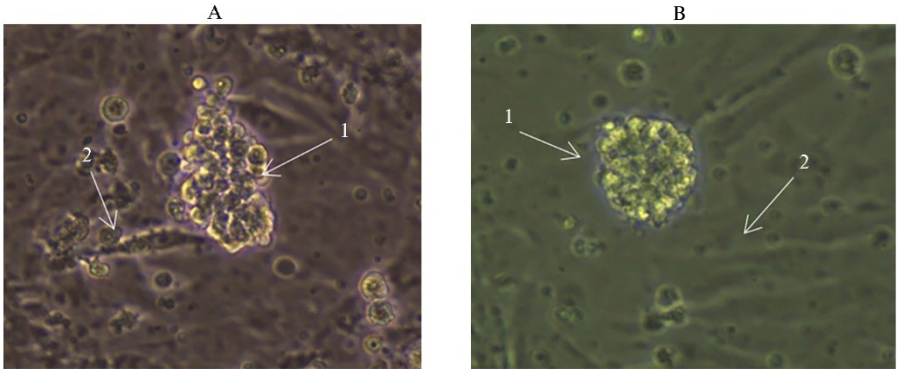


Fig. 7. Colonies of primordial germ cells (PGCs) **of chickens** (A) **and quails** (B) **used for transfection with constructs for knockout of the *FGF20* and *HR* genes:** 1 — PGCs, 2 — fibroblasts (feeder layer). Native preparation, light microscopy (Nikon Eclipse TS100, Nikon Co., Japan; magnification ×400).

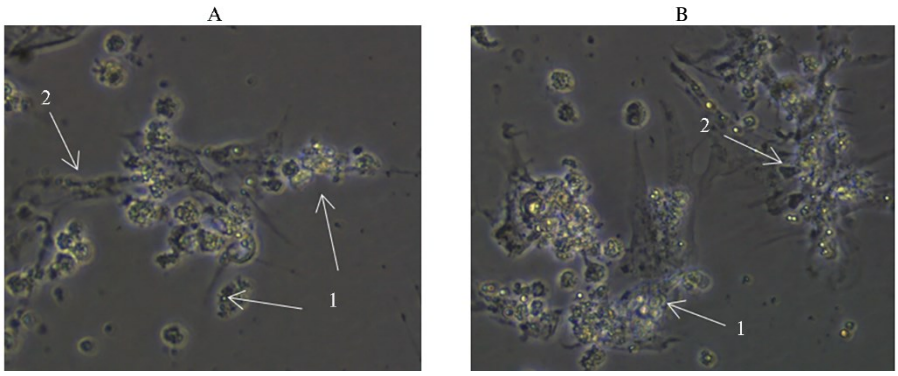


Fig. 7. Cultures of spermatogenic cells from testis of chickens (A) **and quails (B) used for transfection with constructs for knockout of the *FGF20* and *HR* genes:** 1 — spermatogonia, 2 — Sertoli cells. Native preparation, light microscopy (Nikon Eclipse TS100, Nikon Co., Japan; magnification ×400).

The resulting cultures of PZK and spermatogonia of chickens and quails were transfected with the created constructs for knockout of the *FGF20* and *HR* genes. The share of in vitro transfected PZK and spermatogonia of chickens with *FGF20* gene knockout reached 0.9 and 1.2%, respectively, with *HR* gene knockout 0.8 and 1.0%. The proportion of PGCs and quail spermatogonia with *FGF20* gene knockout was 0.9 and 1.1%, respectively. Note that the efficiency of transfection of these target cells was relatively low. However, we obtained a pure population of transfected cells by sorting and multiplied them in vitro to the required abundance.

Several research groups have studied the effectiveness of PGCs as target cells for gene editing to obtain birds with knockout of various genes, in particular, chickens with CRISPR/Cas9-mediated [5, 6, 8, 34] and TALEN-mediated [35] knockout of genes for myostatin [5], immunoglobulin heavy chain [34], DDX4 [35], ovomucin [8], and NHE1 [6]. In these works, two methods were used for PGC transfection, the electroporation [6, 34, 35] and lipofection [5, 8]. With electroporation, in most cases, the transfected cells were selected in growth media with a selective antibiotic. When the authors selected transfected PGCs by sorting without preliminary culture in a selective medium, the transfection efficiency was low (1%), which is consistent with our data.

We did not find information on the use of spermatogonia as targets for the introduction of gene editing systems in the available sources of information.

Thus, we developed gene editing systems for knockout of the *FGF20* and *HR* genes in chickens and the *FGF20* gene in quails. To find optimal cutting sites of these genes and to design the sequences of guide RNAs and primers for amplification of selected target DNA segments, we used bioinformatics tools. To introduce deletions into the regions encoding FGF20 and HR, gene constructs based on the pX458 vector were created. Our research results confirm the effectiveness of introducing microdeletions into these genes of chickens and quails with the constructs created. The transfection frequency is 5.7 and 6.3% for chicken and quail embryonic fibroblasts, respectively, 0.9 and 0.9% for primordial germ cells, 1.2 and 1.1% for spermatogonia. These findings indicate the successful creation of gene editing systems in poultry using gene constructs based on the pX458 vector.

REFERENCES

1. Bahrami S., Amiri-Yekta A., Daneshipour A., Jazayeri S.H., Mozdziak P.E., Sanati M.H., Gourabi H. Designing a transgenic chicken: applying new approaches toward a promising bioreactor. *Cell Journal*, 2020, 22(2): 133-139 (doi: 10.22074/cellj.2020.6738).
2. Cooper C. A., Doran T.J., Challagulla A., Tizard M.L.V., Jenkins K.A. Innovative approaches to genome editing in avian species. *Journal of Animal Science and Biotechnology*, 2018, 9: 15 (doi: 10.1186/s40104-018-0231-7).
3. Petersen B. Basics of genome editing technology and its application in livestock species. *Reproduction in Domestic Animals*, 2017, 52(S3): 4-13 (doi: 10.1111/rda.13012).
4. Bhattacharya T.K., Shukla R., Chatterjee R.N., Bhanja S.K. Comparative analysis of silencing expression of myostatin (MSTN) and its two receptors (ACVR2A and ACVR2B) genes affecting growth traits in knock down chicken. *Scientific Reports*, 2019, 9: 7789 (doi: 10.1038/s41598-019-44217-z).
5. Kim G-D., Lee J.H., Song S., Kim S.W., Han J.S., Shin S.P., Park B.-C., Park T.S. Generation of myostatin-knockout chickens mediated by D10A-Cas9 nickase. *FASEB*, 2020, 34: 5688-5696 (doi: 10.1096/fj.201903035R).
6. Hellmich R., Sid H., Lengyel K., Flisikowski K., Schlickenrieder A., Bartsch D., Thoma T., Bertzbach L.D., Kaufer B.B., Nair V., Preisinger R., Schusser B. Acquiring resistance against a retroviral infection via CRISPR/Cas9 targeted genome editing in a commercial chicken line. *Frontiers in Genome Editing*, 2020, 2: 3 (doi: 10.3389/fgeed.2020.00003).
7. Koslová A., Trefil P., Mucksová J., Reinišová M., Plachý J., Kalina J., Kučerová D., Geryk J., Krchlнková V., Lejčková B., Hejnar J. Precise CRISPR/Cas9 editing of the NHE1 gene renders chickens resistant to the J subgroup of avian leukosis virus. *PNAS*, 2020, 117(4): 2108-2112 (doi: 10.1073/pnas.1913827117).
8. 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).
9. Oishi I., Yoshii K., Miyahara D., Tagami T. Efficient production of human interferon beta in the white of eggs from ovalbumin gene-targeted hens. *Scientific Reports*, 2018, 8: 10203 (doi: 10.1038/s41598-018-28438-2).
10. Stern C.D. The marginal zone and its contribution to the hypoblast and primitive streak of the chick embryo. *Development*, 1990, 109: 667-682 (doi: 10.1242/dev.109.3.667).
11. Mozdziak P.E., Petitte J.N. Status of transgenic chicken models for developmental biology. *Developmental Dynamics*, 2004, 229: 414-421 (doi: 10.1002/dvdy.10461).
12. Korshunova L.G., Karapetyan R.V., Ziadinova O.F., Fisinin V.I. Transgenic poultry: derivation and areas of application (review). *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(6): 1080-1094 (doi: 10.15389/agrobiology.2019.6.1080rus).
13. Mozdziak P.E., Borwornpinyo S., Mccoy D.W., Petitte J.N. Development of transgenic chickens expressing bacterial beta-galactosidase. *Developmental Dynamics*, 2003, 226: 439-445 (doi: 10.1002/dvdy.10234).
14. Byun S.J., Kim S.W., Kim K.W., Kim J.S., Hwang I.S., Chung H.K., Kan I.S., Jeon I.S., Chang W.K., Park S.B., Yoo J.G. Oviduct-specific enhanced green fluorescent protein expression in transgenic chickens. *Bioscience, Biotechnology, and Biochemistry*, 2011, 75(4): 646-649 (doi: 10.1271/bbb.100721).
15. Harvey A.J., Speksnijder G., Baugh L.R., Morris J.A., Ivarie R. Expression of exogenous protein in the egg white of transgenic chickens. *Nature Biotechnology*, 2002, 20: 396 (doi: 10.1038/nbt0402-396).
16. Rapp J.C., Harvey A.J., Speksnijder G.L., Hu W., Ivarie R. Biologically active human interferon a-2b produced in the egg white of transgenic hens. *Transgenic Research*, 2003, 12(5): 569-575

(doi: 10.1023/A:1025854217349).
17. Lillico S.G., Sherman A., McGrew M.J., Robertson C.D., Smith J., Haslam C., Barnard P., Radcliffe P.A., Mitrophanous K.A., Elliot E.A., Sang H.M. Oviduct-specific expression of two therapeutic proteins in transgenic hens. *PNAS*, 2007, 104(6): 1771-1776 (doi: 10.1073/pnas.0610401104).
18. 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).
19. Kamihira M., Ono K., Esaka K., Nishijima K., Kigaku R., Komatsu H., Yamashita T., Kyogoku K., Iijima S. High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector. *Journal of Virology*, 2005, 79(17): 10864-10874 (doi: 10.1128/JVI.79.17.10864-10874.2005)
20. Kwon S.C., Choi J.W., Jang H.J., Shin S.S., Lee S.K., Park T.S., Choi I.Y., Lee G.S., Song G., Han J.Y. Production of biofunctional recombinant human interleukin 1 receptor antagonist (rhIL1RN) from transgenic quail egg white. *Biology of Reproduction*, 2010, 82: 1057-1064 (doi: 10.1095/biolreprod.109.081687).
21. Kodama D., Nishimiya D., Nishijima K., Okino Y., Inayoshi Y., Kojima Y., Ono K., Motono M., Miyake K,. Kawabe Y., Kyogoku K., Yamashita T., Kamihira M., Iijima S. Chicken oviduct-specific expression of transgene by a hybrid ovalbumin enhancer and the Tet expression system. *Journal of Bioscience and Bioengineering*, 2012, 113(2): 146-153 (doi: 10.1016/j.jbiosc.2011.10.006).
22. Li W.R., Liu C.X., Zhang X.M., Chen L., Peng X.R., He S.G., Lin J.P., Han B, Wang L.Q., Huang J.C., Liu M.J. CRISPR/Cas9-mediated loss of FGF5 function increases wool staple length in sheep. *FEBS Journal*, 2017 284(17): 2764-2773 (doi: 10.1111/febs.14144).
23. Wang X., Niu Y., Zhou J., Zhu H., Ma B., Yu H., Yan H., Hua J., Huang X., Qu L., Chen Y. CRISPR/Cas9-mediated MSTN disruption and heritable mutagenesis in goats causes increased body mass. *Animal Genetics*, 2018, 49(1): 43-51 (doi: 10.1111/age.12626).
24. Scott B.B., Velho T.A., Sim S., Lois C. Applications of avian transgenesis. *ILAR Journal*, 2010, 51(4): 353-361 (doi: 10.1093/ilar.51.4.353).
25. 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).
26. Park T.S., Lee H.J., Kim K.H., Kim J.-S., Han J.Y. Targeted gene knockout in chickens mediated by TALENs. *PNAS*, 2014, 111(35): 12716-12721 (doi: 10.1073/pnas.1410555111).
27. 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: 331-347 (doi: 10.1007/s11248-016-0003-0).
28. Lee J., Kim D. H., Lee K. Muscle hyperplasia in Japanese quail by single amino acid deletion in MSTN propeptide. *International Journal of Molecular Sciences*, 2020, 21: 1504 (doi: 10.3390/ijms21041504).
29. Benavides F., Oberyszyn T.M., Van Buskirk A.M., Reeved V.E., Kusewit D.F. The hairless mouse in skin research. *Journal of Dermatological Science*, 2009, 53(1): 10-18 (doi: 10.1016/j.jdermsci.2008.08.012).
30. Wells K.L., Hadad Y., Ben-Avraham D., Hillel J., Cahaner A., Headon D.J. Genome-wide SNP scan of pooled DNA reveals nonsense mutation in *FGF20* in the scaleless line of featherless chickens. *BMC Genomics*, 2012, 13: 257 (doi: 10.1186/1471-2164-13-257).
31. Ran F., Hsu P., Wright J., Agarwala V., Scott D.A., Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, 2013, 8: 2281-2308 (doi: 10.1038/nprot.2013.143).
32. Inoue H., Nojima H., Okayama H. High efficiency transformation of Escherichia coli with plasmids. *Gene,* 1990, 96(1): 23-28 (doi: 10.1016/0378-1119(90)90336-P).
33. 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. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2017, 52(2): 261-267 (doi: 10.15389/agrobiology.2017.2.261eng).
34. Dimitrov L., Pedersen D., Ching K.H., Yi H., Collarini E. J., Izquierdo S., van de Lavoir M.-C., Leighton P.A. Germline gene editing in chickens by efficient CRISPR-mediated homologous recombination in primordial germ cells. *PLoS ONE*, 2016, 11: e0154303 (doi: 10.1371/journal.pone.0154303).
35. Taylor L., Carlson D.F., Nandi S., Sherman A., Fahrenkrug S.C., McGrew M.J. Efficient TALEN-mediated gene targeting of chicken primordial germ cells. *Development*, 2017, 144: 928-934 (doi: 10.1242/dev.145367).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1111-1122
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 636.2:577.2

doi: 10.15389/agrobiology.2021.6.1111eng
doi: 10.15389/agrobiology.2021.6.1111rus

WHOLE GENOME STUDY OF SINGLE NUCLEOTIDE POLYMORPHISMS' ASSOCIATIONS WITH WITHERS HEIGHT IN LOCAL AND TRANSBOUNDARY BREEDS IN RUSSIA

A.S. ABDELMANOVA ✉, M.S. FORNARA, N.V. BARDUKOV, A.A. SERMYAGIN, A.V. DOTSEV, N.A. ZINOVIEVA

Ernst Federal Science Center for Animal Husbandry, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail preevetic@mail.ru (✉ corresponding author), margaretfornara@gmail.com, bardukv-nikolajj@mail.ru, alex_sermyagin85@mail.ru, asnd@mail.ru, n_zinovieva@mail.ru
ORCID:
Abdelmanova A.S. orcid.org/0000-0003-4752-0727
Fornara M.S. orcid.org/0000-0002-8844-177X
Bardukov N.V. orcid.org/0000-0002-5497-2409
Sermyagin A.A. orcid.org/0000-0002-1799-6014
Dotsev A.V. orcid.org/0000-0003-3418-2511
Zinovieva N.A. orcid.org/0000-0003-4017-6863
The authors declare no conflict of interests
Acknowledgements:
The equipment of the Center for Biological Resources and Bioengineering of Agricultural Animals (Ernst Federal Research Center for Animal Husbandry) was used.
Supported financially by the Russian Science Foundation (No. 21-66-00007)
Received September 14, 2021

Abstract

The stature of an animal is a classic quantitative trait that affects the predisposition to certain diseases and is associated with the productivity of farm animals. Currently, many quantitative trait loci (QTL) have been mapped that affect the cattle's growth constituents, which confirms its polygenic determinism. An assessment of the frequencies of SNPs alleles associated with withers height in cattle populations bred in Russia has been carried out firstly in this work. The prevalence of alleles associated with high stature of animals in three out of four identified single nucleotide polymorphisms in Russian local breeds was revealed. The aim of the work was to identify loci that are under selection pressure and associated with body size in populations of Russian local breeds and transboundary breeds bred in the territory of the Russian Federation, belonging to different types of productivity, as well as with an unequal degree of pressure of artificial selection and distribution in the world. Thirteen cattle breeds (n = 670) subjected to our study including Angus (n = 39), Ayrshire (n = 144), Black-and-White (n = 50), Holstein (n = 184), Istoben (n = 22), Jersey (n = 32), Kalmyk (n = 27), Kholmogor (n = 26), Kyrgyz (n = 24), Mongolian (n = 26), Tagil (n = 26), Yakut (n = 29) and Yaroslavl (n = 41). Samples of blood, tissue and sperm stored in UNU "Genetic material bank of domestic and wild animal species and birds" of the Ernst Federal Research Center for Animal Husbandry were used as a source of DNA for this study. The samples were genotyped using DNA arrays GGP Bovine 150K and BovineHD BeadChip (Illumina Inc., USA) with the different density. In the course of data processing, SNPs common for the two arrays were determined and were used for further analysis. The genome-wide study of the associations of genotyping data with measurements of physical development of animals was carried out by the PLINK 1.9 program using filters (--geno 0.1), (--mind 0.2), (--maf 0.05). Height at withers for the studied breeds was obtained from the FAO database. All studied breeds were divided into groups according to the following criteria: growth (tall, short), type of productivity (dairy, meat), the degree of pressure of artificial selection (primitive, commercial) and distribution in the world (local, transboundary). Four SNPs were identified in total. Three of them were localized on chromosome 4 (ARS-BFGL-NGS-116590, Hapmap53144-ss46525999, BovineHD0400021479), and one on chromosome 14 (BovineHD1400007259). The alternative alleles in the detected SNPs significantly differ in their frequency in different groups of breeds, and also have significant positive or negative correlations with the height at the withers. The diversity and heterogeneity of the breeds presented in the study allows us to consider the identified traces of selection not as characteristic of one breed, region or type of productivity, but as for a group of breeds of the species *Bos taurus taurus*, the distribution of which from the center of domestication proceeded along the Danube Route. Thus, the identified SNPs can be used as genetic markers in breeding programs in order to increase the stature of animals and their productivity.

Keywords: *Bos taurus*, cattle, local breeds, transboundary breeds, QTL, SNP markers, DNA arrays, GWAS, *PLAG1*, withers' height

Animal breeding aimed at improving the characteristics of cattle is key for sustainable animal husbandry; well-balanced animals are highly productive and are in demand in the livestock market [1].

Whole genome association studies (GWAS) have enabled the identification of many genomic variations associated with the quantitative traits of livestock [2]. Over the past decade, numerous studies have mapped the quantitative trait loci (QTLs) responsible for the dairy yield [3], disease resistance [4, 5], reproductive performance [6], growth [7-9], beef quality [10-13], and carcass weight [14-16] of cattle.

Animal stature is a classic quantitative trait that is of interest to geneticists [17-19]. Apart from providing information on the overall genetic architecture of quantitative traits, a comprehensive understanding of inter-individual growth variability can also elucidate the mechanisms which govern the growth of an organism. Growth influences the predisposition of livestock to certain diseases, and thus, their overall productivity. Although sensitive to environmental factors, humans too are generally influenced by this indicator (~ 85%) [20].

The domestication of cattle was highly influenced by this indicator. The growth of the species *Bos taurus* decreased by approximately 1.5 times from the Neolithic to Middle Ages, and it increased again only in the Early Modern period [21]. The extinct auroch (*Bos primigenius*) was much larger than its domestic descendants (height at the withers ~ 2 m versus ~ 1.1-1.5 m in modern cattle). The heritability coefficient of the indicator varies within 25-85% depending on the population [22, 23]. If the mechanisms of genetic variation have aided the recovery of growth since the Early Modern period, their selection fingerprints should be determined based on the genomic data of modern cattle breeds.

Currently, many QTLs that influence the growth components of cattle have been mapped, which confirms its polygenic determinism. Illustratively, 38 and 52 QTLs have been reported to account for more than 60% of the variation in adult height and weight, respectively, in a population of the American Aberdeen Angus breed [24]. L. Karim et al. [25] examined the Holstein-Friesian and Jersey breeds to identify the QTLs that influence traits responsible for augmenting dairy yield. More than 500 traits were measured, six of which were related to body size: birth weight, weight at 6, 8, 12, 18 and 24 months (body weight), and height at withers at 18 months.

I. Randhawa et al. [26] compared single nucleotide polymorphism (SNP) data across the genome in several European and African breeds of *B. taurus* with high (145-155 cm) and low (105-133 cm) withers, and they found that the most significant selection signal associated with growth is mapped on chromosome 14 (BTA14) within the 24.79-28.25 Mbp region [27]. This selection signature was recently confirmed by full genome sequencing of four *B. taurus* breeds, and it is limited to a smaller region spanning positions within 24.80-25.08 Mbp [28], where the pleomorphic adenoma 1 (*PLAG1*) gene is located.

The *PLAG1* gene, consisting of five exons and four introns, with five transcripts, was first discovered through positional mapping, during the investigation of pleomorphic adenomas of the human salivary glands [29]. *PLAG1* initiates the transcription of insulin-like growth factor 2 (*IGF2*), a mitogenic hormone important for fetal growth and development, and it influences the genetic variability of growth in both humans and cattle [25, 30-32]. Several studies have examined the association of the *PLAG1* gene with various traits, such as calving ease [33], body size [25], birth and yearling weight [14, 35], carcass quality [34] and other traits related to the growth of cattle [36]. However, in Russian local breeds, which are of great interest for biodiversity conservation [37, 38], the role of the *PLAG1*

gene has not been investigated, despite previous genome-wide studies [39, 40].

In this study for the first time the allele frequencies of SNPs associated with withers height in populations of cattle bred in Russia were investigated. In the Russian local breeds, the predominance of alleles associated with high growth of cattle was revealed in 3 out of 4 identified SNPs.

The aim of this study was to identify the loci and characterize allelic variants that are associated with body size, which are under selection pressure in the populations of Russian local breeds and transboundary species bred in the Russian Federation. These breeds have different types of productivity, as well as unequal degrees of artificial selection pressure and global distribution.

Materials and methods. Totally 670 animals of 13 cattle breeds were included in this study: Aberdeen Angus (ANG, $n = 39$], Ayrshire (AYR, $n = 144$), Black and White (BLW, $n = 50$), Holstein (HLS, $n = 184$), Eastoben (IST, $n = 22$), Jersey (JRS, $n = 32$), Kalmyk (KLM, $n = 27$), Kholmogorsk (KHL, $n = 26$), Kyrgyz (KRG, $n = 24$), Mongolian (MNG, $n = 26$), Tagil (TAG, $n = 26$), Yakut (YKT, $n = 29$) and Yaroslavl (YRS, $n = 41$).

The biological material represented whole blood, semen and ear's tissue stored in the Bioresource collection «Bank of genetic materials of domestic and wild animals and birds» of the Ernst Federal Research Center for Animal Husbandry. DNA was isolated using commercial DNA-Extran-1 and DNA-Extran-2 kits (OOO Sintol, Russia) in accordance with the manufacturer's recommendations. The concentrations of dsDNA solutions were determined using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Wilmington, DE, USA). To check the purity of extracted DNA, OD260/280 ratios were determined using NanoDrop-2000 (Thermo Fisher Scientific, Wilmington, DE, USA).

The genotyping of the samples of 11 breeds was carried out in the Ernst Federal Research Center for Animal Husbandry. The genotypes of two breeds, the Aberdeen Angus and Jersey were downloaded from the publicly available WIDDE database (http://widde.toulouse.inra.fr/widde/widde/main.do?module=cattle).

The genotyping of the samples was conducted on SNP chips of different densities, GGP Bovine 150 K and BovineHD BeadChip (Illumina, Inc., USA). During the data processing, SNPs common to the two chips were determined and used for further analysis. Quality control genotyping was performed in PLINK 1.9 [41], using the following filters. At least 90% of loci (--geno 0.1) were successfully genotyped in at least 80% of cattle (--mind 0.2) and the frequency of minor alleles was at least 5% (--maf 0.05). After combining the datasets and quality control of genotyping, 115237 SNPs were included in the analysis.

Employing PLINK 1.9, a GWAS of SNPs associated with withers height was performed. The positions of SNPs were assigned according to the *Bos taurus* genome assembly UMD_3.1.1 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000003055.6).

Withers height data for the studied breeds were obtained from the FAO public database (DAD-IS, https://www.fao.org/dad-is/data/ru/, accessed 09/10/2021). Since the values of indicators vary among different countries, the average values were calculated for each breed irrespective of their country and sexuality. Consequently, the average value of the height at withers was calculated for the entire array of the studied breeds. Calculations and visualization of the results were performed using the R software.

Based on the obtained body size data of the cattle, all studied breeds were divided into two groups. Breeds for which the average withers height did not exceed the average values calculated for the entire array were classified as the low group. In contrast, breeds for which the average values of the indicator exceeded the average values of the sample were classified as the high group. Furthermore, the studied breeds were subdivided into groups according to the following criteria:

the type of productivity (dairy, meat), degree of artificial selection pressure (primitive, commercial), and prevalence in the world (local Russian, transboundary).

The significance of the difference in the frequencies of alternative alleles for SNPs that showed significant associations with withers height in different groups of breeds was determined using the ^{2} test in R. The authors excluded Mongolian and Kyrgyz cattle breeds from the analysis because these groups are not bred within Russia in commercial or breeding herds.

Results. The characteristics of the studied cattle breeds are shown in Table 1.

1. Characteristics of the studied Russian local and transboundary breeds of cattle (*Bos taurus taurus*)

Breed	Acronym	Group	Geographic classification (by FAO)	Type of productivity	Degree of artificial selection pressure
Aberdeen Angus	ANG	High	transboundary	meat	commercial
Ayrshire	AYR	High	transboundary	dairy	commercial
Black-and-White	BLW	High	transboundary	dairy	commercial
Kholmogor	KHL	High	local Russian	dairy	commercial
Holstein	HLS	High	transboundary	dairy	commercial
Yaroslavl	YRS	High	local Russian	dairy	commercial
Tagil	TAG	High	local Russian	dairy	commercial
Istoben	IST	High	local Russian	dairy	commercial
Kalmyk	KLM	Low	transboundary	meat	commercial
Jersey	JRS	Low	transboundary	dairy	commercial
Kyrgyz	KRG	Low	–	meat	primitive
Mongolian	MNG	Low	–	meat	primitive
Yakut	YKT	Low	local Russian	meat	primitive

N o t e. Low and high breeds are designated to cattle for which their average height at withers fall behind and exceed the average values calculated for the entire array, respectively. Dashes mean that the breeds are excluded from the group, since they are not bred in Russia.

Genome-wide association studies using single nucleotide polymorphism markers for withers height (Fig. 1) were conducted for 13 breeds of cattle. Statistically significant (p-value < 1e-80) associations were found for some SNPs (Fig. 2).

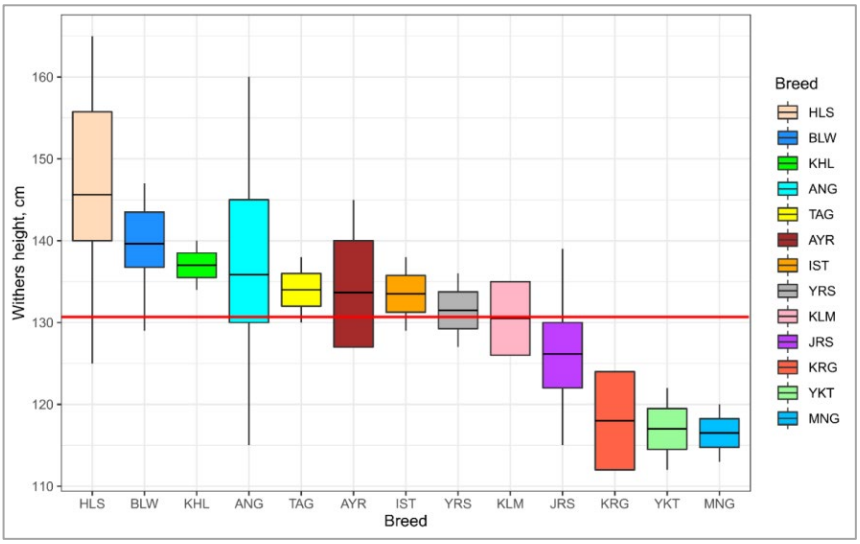


Fig. 1. Average values of the height at the withers for the studied Russian local and transboundary breeds of cattle (*Bos taurus taurus*): HLS — Holstein, BLW — Black-and-White, KHL — Kholmogor, ANG — Aberdeen-Angus, TAG — Tagil, AYR — Ayrshire, IST — Istoben, YRS — Yaroslavl, KLM — Kalmyk, JRS — Jersey, KRG — Kyrgyz cattle, YKT — Yakut, MNG — Mongolian cattle. On the range charts for each breed, the values of the lower quartile, the average value and upper quartile are given. The red line is the average value calculated for the entire array of studied breeds.

In total, four SNPs were identified. Three of these SNPs were located on chromosome 4, and the remaining was located on chromosome 14.

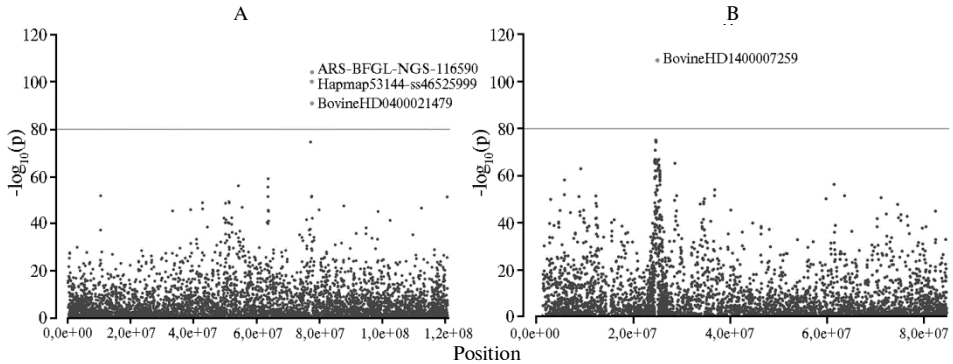


Fig. 2. Localization of single nucleotide polymorphisms significantly associated with height at the withers in Russian local and transboundary breeds of cattle (*Bos taurus taurus*): A — chromosome 4, B — chromosome 14.

We searched for genes located within ±200 Kb of the detected SNPs (Table 2).

2. Genes found in the region of localization of single nucleotide polymorphisms significantly associated with height at the withers in Russian local and transboundary breeds of cattle (*Bos taurus taurus*)

SNP name	Chromosome	Position	Region, Mb	Genes	Traits
Hapmap53144-ss46525999	4	77555681	77.3-77.8	*PURB, MIR4657, H2AFV, PPIA, ZMIZ2, OGDH, TMED4, DDX56, NPC1L1, NUDCD3, CAMK2B, YKT6*	Duration of pregnancy, synthesis of milk components, protein and fat metabolism, average daily weight gain
BovineHD0400021479	4	77613816			
ARS-BFGL-NGS-116590	4	77635835			
BovineHD1400007259	14	25015640	24.8-25.2	*LYN, RPS20, MOS, PLAG1, CHCHD7, SDR16C5, SDR16C6*	Growth, constitution, feed intake, fertility

The region 24.8-25.2 Mb located on chromosome 14 contains genes which are associated with body size [42, 43], food intake [44, 45], and fertility [46]. On chromosome 4 within the 77.3-77.8 Mb region, genes associated with metabolism [47], milk composition [48, 49] and milk synthesis [50], and average daily body weight gain [51], were identified. S.M. Ghoreishifar et al. [52] examined breeding patterns in a group of five cattle breeds originating from northern, middle and southern parts of Sweden. The genome regions where the most significant selection signals were found on BTA5 (105.75-106.52), BTA1 (2.25-2.52) and BTA14 (24.42-25.11 and 25.35-25.73). Annotation of genes in these regions made possible to identify both already known and new candidate genes associated with high-altitude hypoxia (*DCAF8, PPP1R12A, SLC16A3, UCP2, UCP3, TIGAR*), cold acclimatization (*AQP3, AQP7, HSPB8*), body size and growth (*PLAG1, KCNA6, NDUFA9, AKAP3, C5H5112orf1, RAD51AP1, FGF6, TIGAR, CCND2, CSMD3*), resistance to diseases and bacterial infections (*CHI3L2, GBP6, PPFIBP1, REP15, CYP4F2, TIGD2, PYURF, SLC10A2, FCHSD2, ARHGEF17, RELT, PRDM2, KDM5B*), reproductive qualities (*PPP1R12A, ZFP36L2, CSPP1*), milk yield and milk composition (*NPC1L1, NUDCD3, ACSS1, FCHSD2*).

Our data correlates with those of previously published papers. Meanwhile, some regions located on other chromosomes and described in the previous studies [53-55] were not identified in our sample. Interestingly, studies conducted by other researchers, including those using contrast breeds, did not reveal the associations of SNPs located in the region of 77.3-77.8 Mb on BTA4 with the withers height [56-58].

The frequencies of alternative alleles for the SNPs detected in each breed

were calculated (Table 3).

3. Allele frequencies at loci significantly associated with height at withers in Russian local and transboundary breeds of cattle (*Bos taurus taurus*)

Breed	Group	Chromosome 4						Chromosome 14	
		ARS-BFGL-NGS-116590		Hapmap53144-ss46525999		BovineHD0400021479		BovineHD1400007259	
		T	*G* [a]	*T* [a]	*C*	*A*	*G* [a]	*T*	*G* [a]
HLS	High	0.82	99.18	99.18	0.82	0.82	99.18	0.54	99.46
BLW	High	1.00	99.00	99.00	1.00	1.00	99.00	0.00	100.00
KHL	High	23.08	76.92	76.92	23.08	23.08	76.92	13.46	86.54
ANG	High	62.82	37.18	36.84	63.16	62.82	37.18	0.00	100.00
TAG	High	26.92	73.08	73.08	26.92	26.92	73.08	9.62	90.38
AYR	High	28.47	71.53	71.53	28.47	28.47	71.53	21.88	78.13
IST	High	45.00	55.00	54.55	45.45	36.36	63.64	9.52	90.48
YRS	High	45.12	54.88	55.00	45.00	45.12	54.88	17.50	82.50
KLM	Low	95.83	4.17	3.85	96.15	96.15	3.85	94.23	5.77
JRS	Low	95.31	4.69	4.69	95.31	95.31	4.69	100.00	0.00
KRG	Low	72.92	27.08	29.17	70.83	52.27	47.73	85.42	14.58
YKT	Low	98.28	1.72	0.00	100.00	87.50	12.50	98.28	1.72
MNG	Low	100.00	0.00	6.25	93.75	89.58	10.42	100.00	0.00

N o t e. HLS — Holstein, BLW — Black and White, KHL — Kholmogor, ANG — Aberdeen Angus, TAG — Tagil, AYR — Ayrshire, IST — Istoben, YRS — Yaroslavl, KLM — Kalmyk, JRS — Jersey, KRG — Kyrgyz cattle, YKT — Yakut, MNG — Mongolian cattle; Low — breeds that have the average height at the withers did not exceed the average values calculated for the entire array; High — breeds that have the average height at the withers exceeded the average values of the sample: [a] — alleles associated with high stature in cattle.

The most significant differences between the frequencies of alternative alleles were observed in the low-breed group. Illustratively, in the ARS-BFGL-NGS-116590 locus, the frequencies of G/T alleles varied from 0 to 27.08% and from 72.92 to 100%, respectively; at the Hapmap53144-ss46525999 locus, T/C allele frequencies ranged from 0 to 29.17% and from 70.83 to 100%; at the BovineHD1400007259 locus, the G/T allele frequencies ranged from 0 to 14.58% and from 85.42 to 100%. While in the high-breed group, the most significant difference in the frequencies of T/G alleles (0 and 100%, respectively) was observed in the BovineHD1400007259 locus in Black-and-White and Aberdeen Angus breeds.

One of the alternative alleles was absent in some breeds; therefore, the differences between the frequencies of alternative alleles were not assessed for each breed separately, but for all the groups formed according to different traits (Table 4).

4. The differences between the frequencies of alternative alleles at the loci associated with the withers height in the different groups of Russian local and transboundary cattle breeds (*Bos taurus taurus*)

Group	Chromosome 4									Chromosome 14		
	ARS-BFGL-NGS-116590			Hapmap53144-ss46525999			BovineHD0400021479			BovineHD1400007259		
	T	*G* [a]	p	*T* [a]	*C*	p	*A*	*G* [a]	p	*T*	*G* [a]	p
High	0.204	0.796	***	0.795	0.205	***	0.201	0.799	***	0.09	0.91	***
Low	0.349	0.651	***	0.082	0.918	**	0.309	0.647	***	0.959	0.041	ns
Meat	0.839	0.161	ns	0.168	0.832	ns	0.77	0.230	ns	0.687	0.313	***
Milk	0.218	0.782	***	0.781	0.219	***	0.215	0.785	***	0.152	0.848	***
Commercial	0.276	0.724	***	0.721	0.279	***	0.275	0.725	***	0.177	0.823	***
Primitive	0.909	0.091	***	0.112	0.888	***	0.777	0.223	***	0.948	0.052	ns
Transboundary	0.256	0.744	***	0.740	0.260	***	0.259	0.741	***	0.187	0.813	***
Local Russian	0.486	0.514	*	0.514	0.486	*	0.448	0.552	ns	0.306	0.694	***

N o t e. [a] — alleles associated with high stature in cattle.
* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, ns — not significant.

Generally, the frequencies of alternative alleles in the studied groups were significantly different. The exception was the group of beef cattle in all three SNPs found on BTA4. In addition, the frequencies of the SNP BovineHD1400007259 did not differ from those theoretically expected from the primitive cattle. A similar situation was observed in the undersized cattle group. This may be attributed to

the fact that three out of their five breeds belong to primitive cattle (Kyrgyz and Mongolian cattle, Yakut breed).

In the Aberdeen Angus breed, similar trends in the distribution of allele frequencies on chromosome 4 were observed as that of the low-breed group, even though they belong to the high-breed group.

The G allele at the BovineHD0400021479 locus, the G allele at the ARS-BFGL-NGS-116590 locus, the G allele at the BovineHD1400007259 locus, and the T allele at the Hapmap53144-ss46525999 locus are associated with large height at the withers. For the SNP BovineHD1400007259, located within the PLAG1 gene, the G allele frequencies associated with high stature correlated with those from the previous studies on Holstein-Friesian and Jersey breeds [25]. Previously, Hou J. et al. [58] studied the association of SNP rs109815800 (AC_000171.1: g.25015640, G > T, designated on the chip as BovineHD1400007259) in the PLAG1 gene with cattle growth. The results of the association analysis showed that cattle breeds with the T/T genotype were shorter than those with T/G or G/G genotypes ($p < 0.001$).

A statistically significant correlation was determined between the frequencies of alternative alleles and withers height for all detected SNPs (Table 5).

5. Correlation between the withers height and the frequency of alternative alleles in the loci associated with the height at the withers in different groups of Russian local and transboundary cattle breeds (*Bos taurus taurus*)

Group	Chromosome 4						Chromosome 14	
	ARS-BFGL-NGS-116590		Hapmap53144-ss46525999		BovineHD0400021479		BovineHD1400007259	
	T/*G* [a]	p	*T* [a]/*C*	p	*A*/*G* [a]	p	*T*/*G* [a]	p
High	0.722	***	0.719	***	0.708	***	0.678	***
Low	–0.172	ns	–0.281	ns	–0.498	**	–0.063	ns
Meat	0.530	***	0.456	*	0.090	ns	0.767	***
Milk	0.908	***	0.908	***	0.907	***	0.751	***
Commercial	0.829	***	0.827	***	0.821	***	0.729	***
Primitive	0.962	***	0.859	***	0.96	***	0.975	***
Transboundary	0.876	***	0.875	***	0.876	***	0.84	***
Local Russian	0.981	***	0.982	***	0.989	***	0.971	***
All samples	0.838	***	0.826	***	0.752	**	0.854	***

N o t e. [a] — alleles associated with high stature in cattle. Correlation coefficients are shown for the "high" allele.
* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, ns — not significant.

Significant correlations were found between high allele frequencies and the withers height in almost all breed groups. Interestingly, for the low-breed group, contrary to the expectation, no significant negative correlation was found at the BovineHD1400007259 locus.

Thus, we identified that SNPs ARS-BFGL-NGS-116590, Hapmap53144-ss46525999, BovineHD0400021479, and BovineHD1400007259, localized, according to the assembly of the *Bos taurus* UMD 3.1.1 genome, on chromosomes 4 and 14, were associated with cattle withers height. The frequencies of the identified SNPs among breeds differed significantly from that of a randomly possible outcome. Alternative alleles in the detected SNPs were determined, and they had statistically significant positive or negative correlations with withers height. Thus, it can be inferred that the selection pressure on these loci is not the same for different breeds. The diversity and heterogeneity of the breeds shown by the sample allows us to consider that the identified traces of selection aren't the characteristic of one breed, region, or productivity type, but are the characteristic for a group of breeds of the species *Bos taurus taurus* that migrated from the center of domestication along the Danube route. Thus, the identified SNPs can be used as genetic markers in breeding programs to improve cattle stature and their overall productivity.

REFERENCES

1. Petrova M.Yu., Chernigov Yu.V., Kuznetsova T.Sh. *Vestnik OmGAU*, 2019, 2(34): 120-125 (in Russ.).
2. Goddard M., Hayes B. Mapping genes for complex traits in domestic animals and their use in breeding programmes. *Nature Reviews Genetics*, 2009, 10: 381-391 (doi: 10.1038/nrg2575).
3. Sermyagin A.A., Bykova O.A., Loretts O.G., Kostyunina O.V., Zinov'eva N.A. Genomic variability assess for breeding traits in Holsteinizated Russian Black-and-White cattle using GWAS analysis and ROH patterns. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2020, 55(2): 257-274 (doi: 10.15389/agrobiology.2020.2.257rus).
4. Matukumalli L.K., Lawley C.T., Schnabel R.D., Taylor J.F., Allan M.F., Heaton M.P., O'Connell J., Moore S.S., Smith T.P.L., Sonstegard T.S., Van Tassell C.P. Development and characterization of a high density SNP genotyping assay for cattle. *PLoS ONE*, 2009, 4(4): e5350 (doi: 10.1371/journal.pone.0005350).
5. Pant S.D., Schenkel F.S., Verschoor C.P., You Q., Kelton D.F., Moore S.S., Karrow N.A. A principal component regression based genome wide analysis approach reveals the presence of a novel QTL on BTA7 for MAP resistance in Holstein cattle. *Genomics*, 2010, 95(3): 176-182 (doi: 10.1016/j.ygeno.2010.01.001).
6. Crispim A.C., Kelly M.J., Guimarães S.E., e Silva F.F., Fortes M.R., Wenceslau R.R., Moore S. Multi-trait GWAS and new candidate genes annotation for growth curve parameters in Brahman cattle. *PLoS ONE*, 2015, 10(10): e0139906 (doi: 10.1371/journal.pone.0139906).
7. Hoshiba H., Setoguchi K., Watanabe T., Kinoshita A., Mizoshita K., Sugimoto Y., Takasuga A. Comparison of the effects explained by variations in the bovine *PLAG1* and *NCAPG* genes on daily body weight gain, linear skeletal measurements and carcass traits in Japanese Black steers from a progeny testing program. *Animal Science Journal*, 2013, 84(7): 529-534 (doi: 10.1111/asj.12033).
8. Snelling W.M., Allan M.F., Keele J.W., Kuehn L.A., McDaneld T., Smith T.P.L., Sonstegard T.S., Thallman R.M., Bennett G.L. Genome-wide association study of growth in crossbred beef cattle. *Journal of Animal Science*, 2010, 88(3): 837-848 (doi: 10.2527/jas.2009-2257).
9. Bolormaa S., Hayes B.J., Savin K., Hawken R., Barendse W., Arthur P.F., Herd R.M., Goddard M.E. Genome-wide association studies for feedlot and growth traits in cattle. *Journal of Animal Science*, 2011, 89(6): 1684-1697 (doi: 10.2527/jas.2010-3079).
10. Barendse W. Haplotype analysis improved evidence for candidate genes for intramuscular fat percentage from a genome wide association study of cattle. *PLoS ONE*, 2011, 6(12): e29601 (doi: 10.1371/journal.pone.0029601).
11. Dang C.G., Cho S.H., Sharma A., Kim H.C., Jeon G.J., Yeon S.H., Hong S.K., Park B.Y., Kang H.S., Lee S.H. Genome-wide association study for Warner-Bratzler shear force and sensory traits in Hanwoo (Korean cattle). *Asian-Australasian Journal of Animal Sciences*, 2014, 27(9): 1328-1335 (doi: 10.5713/ajas.2013.13690).
12. Wu Y., Fan H., Wang Y., Zhang L., Gao X., Chen Y., Li J., Ren H., Gao H. Genome-wide association studies using haplotypes and individual SNPs in Simmental cattle. *PLoS ONE*, 2014, 9(10): e109330 (doi: 10.1371/journal.pone.0109330).
13. Weng Z.Q., Su H.L., Saatchi M., Lee J., Thomas M.G., Dunkelberger J.R., Garrick D.J. Genome-wide association study of growth and body composition traits in Brangus beef cattle. *Livestock Science*, 2016, 183: 4-11 (doi: 10.1016/j.livsci.2015.11.011).
14. Littlejohn M., Grala T., Sanders K., Walker C., Waghorn G., Macdonald K., Coppieters W., Georges M., Spelman R., Hillerton E., Davis S., Snell R. Genetic variation in *PLAG1* associates with early life body weight and peripubertal weight and growth in *Bos taurus*. *Animal Genetics*, 2012, 43(5): 591-594 (doi: 10.1111/j.1365-2052.2011.02293.x).
15. Bolormaa S., Pryce J.E., Kemper K., Savin K., Hayes B.J., Barendse W. Zhang Y., Reich C. M., Mason B.A., Bunch R.J., Harrison B.E., Reverter A., Herd R.M., Tier B., Graser H.-U., Goddard M.E. Accuracy of prediction of genomic breeding values for residual feed intake and carcass and meat quality traits in *Bos taurus*, *Bos indicus*, and composite beef cattle. *Journal of Animal Science*, 2013, 91(7): 3088-3104 (doi: 10.2527/jas.2012-5827).
16. Sasaki S., Ibi T., Matsuhashi T., Takeda K., Ikeda S., Sugimoto M., Sugimoto Y. Genetic variants in the upstream region of activin receptor IIA are associated with female fertility in Japanese Black cattle. *BMC Genetics*, 2015, 16: 123.
17. Galton F. Regression towards mediocrity in hereditary stature. *The Journal of the Anthropological Institute of Great Britain and Ireland*, 1886, 15: 246-263. Available: http://www.jstor.org/stable/2841583. Accessed: 10.10.2021.
18. Fisher R.A. The correlation between relatives on the supposition of Mendelian inheritance. *Earth and Environmental Science Transactions of the Royal Society of Edinburgh*, 1918, 52(2): 399-433 (doi: 10.1017/S0080456800012163).

19. Deniskova T.E., Petrov S.N., Sermyagin A.A., Dotsev A.V., Fornara M.S., Reyer H., Wimmers K., Bagirov V.A., Brem G., Zinov'eva N.A. A search for genomic variants associated with body weight in sheep based on high density SNP genotypes analysis. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2021, 56(2): 279-291 (doi: 10.15389/agrobiology.2021.2.279eng).
20. Visscher P.M. , Macgregor S., Benyamin B., Zhu G., Gordon S., Medland S., Hill W.G., Hottenga J.-J., Willemsen G., Boomsma D.I., Liu Y.-Z., Deng H.-W., Montgomery G.W., Martin N.G. Genome partitioning of genetic variation for height from 11,214 sibling pairs. *The American Journal of Human Genetics*, 2007, 81: 1104-1110 (doi: 10.1086/522934).
21. Ajmone-Marsan P., Garcia J.F., Lenstra J.A. On the origin of cattle: how aurochs became cattle and colonized the world. *Evolutionary Anthropology*, 2010, 19(4): 148-157 (doi: 10.1002/evan.20267).
22. Nelsen T.C., Short R.E., Urick J.J., Reynolds W.L. Heritabilities and genetic correlations of growth and reproductive measurements in Hereford bulls. *Journal of Animal Science*, 1986, 63(2): 409-417 (doi: 10.2527/jas1986.632409x).
23. Northcutt S.L., Wilson D.E. Genetic parameter estimates and expected progeny differences for mature size in Angus cattle. *Journal of Animal Science*, 1993, 71(5): 1148-1153 (doi: 10.2527/1993.7151148x).
24. McClure M.C., Morsci N.S., Schnabel R.D., Kim J.W., Yao P., Rolf M.M., McKay S.D., Gregg S.J., Chapple R.H., Northcutt S.L., Taylor J.F. A genome scan for quantitative trait loci influencing carcass, post-natal growth and reproductive traits in commercial Angus cattle. *Animal Genetics*, 2010, 41(6): 597-607 (doi: 10.1111/j.1365-2052.2010.02063.x).
25. Karim L., Takeda H., Lin L., Druet T., Arias J.A., Baurain D., Cambisano N., Davis S.R., Farnir F., Grisart B., Harris B.L., Keehan M.D., Littlejohn M.D., Spelman R.J., Georges M., Coppieters W. Variants modulating the expression of a chromosome domain encompassing *PLAG1* influence bovine stature. *Nature Genetics*, 2011, 43: 405-413 (doi: 10.1038/ng.814).
26. Randhawa I.A.S., Khatkar M.S., Thomson P.C., Raadsma H.W. Composite selection signals for complex traits exemplified through bovine stature using multibreed cohorts of European and African *Bos taurus*. *G3 Genes|Genomes|Genetics*, 2015, 5(7): 1391-1401 (doi: 10.1534/g3.115.017772).
27. Zimin A.V., Delcher A.L., Florea L., Kelley D.R., Schatz M.C., Puiu D., Hanrahan F., Pertea G., Van Tassell C.P., Sonstegard T.S., Marçais G., Roberts M., Subramanian P., Yorke J.A., Salzberg S.L. A whole-genome assembly of the domestic cow, *Bos taurus*. *Genome Biology*, 2009, 10: R42 (doi: 10.1186/gb-2009-10-4-r42).
28. Boitard S., Boussaha M., Capitan A., Rocha D., Servin B. Uncovering adaptation from sequence data: lessons from genome resequencing of four cattle breeds. *Genetics*, 2016, 203(1): 433-450 (doi: 10.1534/genetics.115.181594).
29. Kas K., Voz M.L., Rцijer E., Estrцm A.K., Meyen E., Stenman G., Van de Ven W.J. Promoter swapping between the genes for a novel zinc finger protein and β-catenin in pleiomorphic adenomas with t(3;8)(p21;q12) translocations. *Nature Genetics*, 1997, 15: 170-174 (doi: 10.1038/ng0297-170).
30. Wood A., Esko T., Yang J. et al. Defining the role of common variation in the genomic and biological architecture of adult human height. *Nature Genetics*, 2014, 46: 1173-1186 (doi: 10.1038/ng.3097).
31. Pryce J.E., Hayes B.J., Bolormaa S., Goddard M.E. Polymorphic regions affecting human height also control stature in cattle. *Genetics*, 2011, 187(3): 981-984 (doi: 10.1534/genetics.110.123943).
32. Fortes M.R.S., Kemper K., Sasazaki S., Reverter A., Pryce J.E., Barendse W., Bunch R., McCulloch R., Harrison B., Bolormaa S., Zhang Y.D., Hawken R.J., Goddard M.E., Lehnert S.A. Evidence for pleiotropism and recent selection in the *PLAG1* region in Australian Beef cattle. *Animal Genetics*, 2013, 44: 636-647 (doi: 10.1111/age.12075).
33. Pausch H., Flisikowski K., Jung S., Emmerling R., Edel C., Götz K.U., Fries R. Genome-wide association study identifies two major loci affecting calving ease and growth-related traits in cattle. *Genetics*, 2011, 187(1): 289-297 (doi: 10.1534/genetics.110.124057).
34. Nishimura S., Watanabe T., Mizoshita K., Tatsuda K., Fujita T., Watanabe N., Sugimoto Y., Takasuga A. Genome-wide association study identified three major QTL for carcass weight including the *PLAG1-CHCHD7* QTN for stature in Japanese Black cattle. *BMC Genetics*, 2012, 13: 40 (doi: 10.1186/1471-2156-13-40).
35. Utsunomiya Y.T., Do Carmo A.S., Carvalheiro R., Neves H.H., Matos M.C., Zavarez L.B., Pérez O'Brien A.M., Sölkner J., McEwan J.C, Cole J.B., Van Tassell C.P., Schenkel F.S., da Silva M.V.G.B., Porto Neto L.R., Sonstegard T.S., Garcia J.F. Genome-wide association study for birth weight in Nellore cattle points to previously described orthologous genes affecting human and bovine height. *BMC Genetics*, 2013, 14: 52 (doi: 10.1186/1471-2156-14-52).
36. Zhong J.-L., Xu J.-W., Wang J., Wen Y.-F., Niu H., Zheng L., He H., Peng K., He P., Shi S.Y., Huang Y.-Q., Lei C.-Z., Dang R.-H., Lan X.-Y., Qi X.-L., Chen H., Huang Y.-Z. A novel SNP of *PLAG1* gene and its association with growth traits in Chinese cattle. *Gene*, 2019, 689: 166-171 (doi: 10.1016/j.gene.2018.12.018).
37. Abdelmanova A.S., Kharzinova V.R., Volkova V.V., Mishina A.I., Dotsev A.V., Sermyagin A.A., Boronetskaya O.I., Petrikeeva L.V., Chinarov R.Y., Brem G., Zinovieva N.A. Genetic diversity of historical and modern populations of russian cattle breeds revealed by microsatellite analysis. *Genes*, 2020, 11(8): 940 (doi: 10.3390/genes11080940).

38. Abdelmanova A.S., Kharzinova V.R., Volkova V.V., Dotsev A.V., Sermyagin A.A., Chinarov R.Y., Zinovieva N.A., Boronetskaya O.I., Lutshikhina E.M., Sölkner J., Brem G. Comparative study of the genetic diversity of local steppe cattle breeds from Russia, Kazakhstan and Kyrgyzstan by microsatellite analysis of museum and modern samples. *Diversity*, 2021, 13(8): 351 (doi: 10.3390/d13080351).
39. Zinov'eva N.A., Sermyagin A.A., Dotsev A.V., Boronetskaya O.I., Petrikeeva L.V., Abdel'manova A.S., Brem G. Animal genetic resources: developing the research of allele pool of Russian cattle breeds — minireview. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2019, 54(4): 631-641 (doi: 10.15389/agrobiology.2019.4.631eng).
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. *Genetics Selection Evolution*, 2018, 50(1): 37 (doi: 10.1186/s12711-018-0408-8).
41. 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).
42. Zinovieva N.A., Dotsev A.V., Sermyagin A.A., Deniskova T.E., Abdelmanova A.S., Kharzinova V.R., Sölkner J., Reyer H., Wimmers K., Brem G. Selection signatures in two oldest Russian native cattle breeds revealed using high-density single nucleotide polymorphism analysis. *PLoS ONE*, 2020, 15(11): e0242200 (doi: 10.1371/journal.pone.0242200).
43. Pryce J.E., Arias J., Bowman P.J., Davis S.R., Macdonald K.A., Waghorn G.C., Wales W.J., Williams Y.J., Spelman R.J., Hayes B.J. Accuracy of genomic predictions of residual feed intake and 250-day body weight in growing heifers using 625,000 single nucleotide polymorphism markers. *Journal of Dairy Science*, 2012, 95(4): 2108-2119 (doi: 10.3168/jds.2011-4628).
44. de Las Heras-Saldana S., Clark S.A., Duijvesteijn N., Gondro C., van der Werf J.H.J., Chen Y. Combining information from genome-wide association and multi-tissue gene expression studies to elucidate factors underlying genetic variation for residual feed intake in Australian Angus cattle. *BMC Genomics*, 2019, 20(1): 939 (doi: 10.1186/s12864-019-6270-4).
45. Cheruiyot E.K., Bett R.C., Amimo J.O., Zhang Y., Mrode R., Mujibi F.D.N. Signatures of Selection in admixed dairy cattle in Tanzania. *Frontiers in Genetics*, 2018, 9: 607 (doi: 10.3389/fgene.2018.00607).
46. Taye M., Yoon J., Dessie T., Cho S., Oh S.J., Lee H.K., Kim H. Deciphering signature of selection affecting beef quality traits in Angus cattle. *Genes & Genomics*, 2018, 40(1): 63-75 (doi: 10.1007/s13258-017-0610-z).
47. Lee S.H., Zhu C., Peng Y., Johnson D. T., Lehmann L., Sun Z. Identification of a novel role of ZMIZ2 protein in regulating the activity of the Wnt/β-catenin signaling pathway. *The Journal of biological chemistry*, 2013, 288(50): 35913-35924 (doi: 10.1074/jbc.M113.529727).
48. Sanchez M.-P., Ramayo-Caldas Y., Wolf V, Laithier C., El Jabri M., Michenet A., Boussaha M., Taussat S., Fritz S., Delacroix-Buchet A., Brochard M., Boichard D. Sequence-based GWAS, network and pathway analyses reveal genes co-associated with milk cheese-making properties and milk composition in Montbéliarde cows. *Genetics Selection Evolution*, 2019, 51: 34 (doi: 10.1186/s12711-019-0473-7).
49. Sanchez M.P., Govignon-Gion A., Croiseau P., Fritz S., Hozé C., Miranda G., Martin P., Barbat-Leterrier A., Letaïef R., Rocha D., Brochard M., Boussaha M., Boichard D. Within-breed and multi-breed GWAS on imputed whole-genome sequence variants reveal candidate mutations affecting milk protein composition in dairy cattle. *Genetics Selection Evolution*, 2017, 49(1): 68 (doi: 10.1186/s12711-017-0344-z).
50. Huo N., Yu M., Li X., Zhou C., Jin X., Gao X. PURB is a positive regulator of amino acid-induced milk synthesis in bovine mammary epithelial cells. *Journal of Cellular Physiology*, 2019, 234(5): 6992-7003 (doi: 10.1002/jcp.27452).
51. Lu D., Miller S., Sargolzaei M., Kelly M., Vander Voort G., Caldwell T., Wang Z., Plastow G., Moore S. Genome-wide association analyses for growth and feed efficiency traits in beef cattle. *Journal of Animal Science*, 2013, 91(8): 3612-3633 (doi: 10.2527/jas.2012-5716).
52. Ghoreishifar S.M., Eriksson S., Johansson A.M., Khansefid M., Moghaddaszadeh-Ahrabi S., Parna N., Davoudi P., Javanmard A. Signatures of selection reveal candidate genes involved in economic traits and cold acclimation in five Swedish cattle breeds. *Genetics Selection Evolution*, 2020, 52(1): 52 (doi: 10.1186/s12711-020-00571-5).
53. Setoguchi K., Furuta M., Hirano T., Nagao T., Watanabe T., Sugimoto Y., Takasuga A. Cross-breed comparisons identified a critical 591-kb region for bovine carcass weight QTL (CW-2) on chromosome 6 and the Ile-442-Met substitution in NCAPG as a positional candidate. *BMC Genetics*, 2009, 10: 43 (doi: 10.1186/1471-2156-10-43).
54. Vanvanhossou S., Scheper C., Dossa L.H., Yin T., Brügemann K., König S. A multi-breed GWAS for morphometric traits in four Beninese indigenous cattle breeds reveals loci associated with conformation, carcass and adaptive traits. *BMC Genomics*, 2020, 21(1): 783 (doi: 10.1186/s12864-020-07170-0).

55. Bouwman A.C., Daetwyler H.D., Chamberlain A.J., Ponce C.H., Sargolzaei M., Schenkel F.S., Sahana G., Govignon-Gion A., Boitard S., Dolezal M., Pausch H., Brшndum R.F., Bowman P.J., Thomsen B., Guldbrandtsen B., Lund M.S., Servin B., Garrick D.J., Reecy J., Vilkki J., Bagnato A., Wang M., Hoff J.L., Schnabel R.D., Taylor J.F., Vinkhuyzen A.A.E., Panitz F., Bendixen C., Holm L.E., Gredler B., Hozé C., Boussaha M., Sanchez M.P., Rocha D., Capitan A., Tribout T., Barbat A., Croiseau P., Drögemüller C., Jagannathan V., Vander Jagt C., Crowley J.J., Bieber A., Purfield D.C., Berry D.P., Emmerling R., Götz K.U., Frischknecht M., Russ I., Sölkner J., Van Tassell C.P., Fries R., Stothard P., Veerkamp R.F., Boichard D., Goddard M.E., Hayes B.J. Meta-analysis of genome-wide association studies for cattle stature identifies common genes that regulate body size in mammals. *Nature Genetics*, 2018, 50: 362-367 (doi: 10.1038/s41588-018-0056-5).
56. Doyle J.L., Berry D.P., Veerkamp R.F., Carthy T.R., Walsh S.W., Evans R.D., Purfield D.C. Genomic regions associated with skeletal type traits in beef and dairy cattle are common to regions associated with carcass traits, feed intake and calving difficulty. *Frontiers in Genetics*, 2020, 11: 20 (doi: 10.3389/fgene.2020.00020).
57. An B., Xu L., Xia J., Wang X., Miao J., Chang T., Song M., Ni J., Xu L., Zhang L., Li J., Gao H. Multiple association analysis of loci and candidate genes that regulate body size at three growth stages in Simmental beef cattle. *BMC Genetics*, 2020 21(1): 32 (doi: 10.1186/s12863-020-0837-6).
58. Hou J., Qu K., Jia P., Hanif Q., Zhang J., Chen N., Dang R., Chen H., Huang B., Lei C. A SNP in *PLAG1* is associated with body height trait in Chinese cattle. *Animal Genetics*, 51(1): 87-90 (doi: 10.1111/age.12872).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1123-1133
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 636.2:577.21:575.174:575.2

doi: 10.15389/agrobiology.2021.6.1123eng
doi: 10.15389/agrobiology.2021.6.1123rus

THE POPULATION-GENETIC STRUCTURE OF NATIVE TAGIL CATTLE BY STR- AND SNP-MARKERS

Yu.A. **STOLPOVSKY[1] ✉, S.V. BEKETOV[1], E.V. SOLODNEVA[1], V.M. ABSALIKOV[2], A.S. ABDELMANOVA[3], E.A. GLADYR[3], N.A. ZINOVIEVA[3]**

[1]*Vavilov Institute of General Genetics RAS,* 3, ul. Gubkina, Moscow, 119333 Russia, e-mail stolpovsky@mail.ru (✉ corresponding author), svbeketov@gmail.com, eugenia.575.2012@yandex.ru;
[2]*Department of Agriculture, Oktyabrsky District of Perm Krai,* 57, ul. Lenina, Oktyabrsky District, Perm Krai, 617860 Russia, e-mail vabs1966@gmail.com;
[3]*Ernst Federal Research Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail preevetic@mail.ru, elenagladyr@mail.ru, n_zinovieva@mail.ru
ORCID:
Stolpovsky Yu.A. orcid.org/0000-0003-2537-1900
Beketov S.V., orcid.org/0000-0001-7947-8688
Solodneva E.V. orcid.org/0000-0002-7178-4012
Abdelmanova A.S. orcid.org/0000-0003-4752-0727
Gladyr E.A. orcid.org/0000-0002-5210-8932
Zinovieva N.A. orcid.org/0000-0003-4017-6863
The authors declare no conflict of interests
Acknowledgements:
Supported financially by the grant 19-76-20061 of the Russian Science Foundation (http://rscf.ru)
Received September 1, 2021

Abstract

Rearing specialized cattle breeds or several intra-breed lines reduces the breed and genetic diversity and creates a real threat of extinction of native livestock. Microsatellite analysis and genome-wide SNP (single nucleotide polymorphism) genotyping are common methods to study the population genetic structure of local breeds with unique adaptive traits and diseases resistance. The history of the Tagil breed is more than 200 years old. Currently, in Russia and the world, there is the only herd of Tagil cattle of about 600 animals molecular genetic characteristics of which remain insufficiently poor studied. Here we present the first results of identification of STR and SNP genotypes of the unique local Tagil breed. The work aimed to assess genetic diversity and survey the population structure of the modern population of indigenous Tagil cattle by microsatellite analysis (STR) and genome-wide analysis of single nucleotide polymorphism (SNPs). Genotypes of the Tagil animals (TAGIL, $n = 98$; SPK Shorokhov, Perm Territory, 2021) were studied by multiplex analysis using 11 microsatellites (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA122, INRA0623, TGL1812 ETH225, BM1824). For interbreed differentiation by STR markers in PCA, we used a set of breeds that could be potentially involved in the formation of the modern population of Tagil cattle (TAGIL) — Holstein (HLST), Kholmogory Holsteinized (Tatarstan type) (TAT), Kholmogorsk purebred (Pechora type) (PECH), black-and-white (old type) (Ch_P_OLD), Tagil (TAG) (samples from the ONIS BioTechZh database, 2020, https://www.vij.ru/2-obshchaya/226-infrastruktura-test). To cover maximum genetic diversity in genotyping of TAGIL by SNP markers, the most unrelated animals ($n = 48$) were selected based on the results of analysis of STR genotypes. Genome-wide genotyping for SNP markers was performed using a high density GGP Bovine HD 150K BeadChip DNA chip (150,000 SNPs, Illumina, Inc., USA) (10,8432 SNPs before and 62,809 SNPs after LD filtration). A database of genome-wide SNP genotypes of Tagil cattle (TAGIL) was formed to analyze the results of SNP genotyping (population genetic and phylogenetic studies). Holstein animals (HLST) ($n = 45$) were the reference group. We clearly differentiated the Tagil (TAGIL) and Holstein animals by PCA method. Cluster analysis based on genetic distances F_{ST} divided the Tagil and Holstein animals into two separate groups. Genome-wide SNP genotyping revealed genomic regions in which allelic variants are specific for the Tagil cattle (TAGIL). The hapFLK analysis showed five regions ($p < 0.01$) (chromosomes 4, 5, 8, 11, and 15, from 1.20 Mb on BTA8 to 9.61 Mb on BTA5, the number of SNPs within the regions from 24 to 92) under selection pressure in the Tagil animals (TAGIL). The STR genotyping data showed the participation of the Kholmogory cattle, Black-and-White and Holstein breeds in the Tagil breed formation with the greatest introgression of Holstein cattle which most likely was used to improve Tagil cattle in recent decades. We reveled that more than 50 % of the Tagil animals (TAGIL) have the ROH (BTA14, positions 24437778-25098364, 0.661 Mb) previously identified in the Yaroslavl and Kholmogor breeds as a region under selection pressure. This ROH region may be an element of the adaptive genetic system in indigenous Russian breeds. In 40 % Tagil animals, we additionally identified five ROH islands.

The findings of the research will be used to identify genes and their variants that determine adaptive and commercial traits of the Tagil breed, study the formation of its genetic structure, develop monitoring regulations to preserve the Tagil cattle breed specificity and biodiversity.

Keywords: dairy cattle, Tagil breed, microsatellites, SNP genotyping, biodiversity

The intensification of world dairy cattle breeding leads to the predominance of Holstein cows in the structure of cattle breeds. According to statistics, Holstein cattle (purebred or crossbred) in the aggregate account for 61% of the 3.47 million dairy cows in the UK [1], in the USA the proportion of cattle with Holstein breed in the pedigree is even higher and reaches 90% of the total number of dairy cows (2). In Russia, the number of Holstein cattle is still not so large (22.95%), but the black-and-white breed prevails (49.98%) [3], including partially Holsteinized. Breeding of specialized breeds or several intrabreed lines reduces the pedigree and genetic diversity of the livestock, and, as a result, a real threat of extinction of aboriginal livestock is created. In this regard, the study of the population genetic structure of local breeds, which have unique adaptive traits and resistance to a number of diseases, is attracting more and more attention [4]. Most often, such studies use microsatellite analysis or a more informative method of whole genome SNP (single nucleotide polymorphism) genotyping, which has become actively used in population genetics and breeding of cattle after deciphering its genome [5].

Using STR (short tandem repeat) markers, we studied the genetic structure of populations of local breeds of Indian zebu Ongole, Deoni, Gir, Kankrei [6], Mexican Criollo [7], as well as Red Steppe [8], Suksun, Istoben, Yaroslavl, Kholmogory, gray Ukrainian and Kholmogory (Pechora type) breeds of cattle [9]. Genome-wide SNP screening has been used to establish genetic diversity and interbreed differentiation of native South African cattle (Afrikaner, Drakensberger, Nguni) [10], five indigenous cattle breeds in Bangladesh (Chitagon Red, Pabna and Zebu Munshiganj, Northern Bengal Gray, Deshi) [11], six breeds of cattle from the Sichuan province in China (Ba Shan, Xuanhan, Pingwu, Sanjiang, Ganzi, Langshan) [12], the Irish Kerry breed [13] and Russian ancient breeds Bestuzhev, Kholmogory, Kostroma, Red Gorbatov and Yaroslavl [14, 15].

Tagil cattle is one of the oldest domestic breeds of dairy cattle in the Russian Federation. The history of the creation of this breed has more than 200 years. The formation of the main group of Tagil cattle took place in the Ural region in the Nizhny Tagil region. There is no exact information about the origin of the Tagil cattle, but the participation in its formation of the English shorthorned and Kholmogory cattle, imported from the Arkhangelsk province in 1842, was discussed [16]. There is an opinion that the Tagil breed is a product of crossing local Ural cattle with imported animals of the Kholmogory, Yaroslavl and Dutch breeds [17] and the use of Black-and-White bulls [18]. Planned selection and breeding work to improve the Tagil cattle began after the decree of the Council of People's Commissars dated July 19, 1918 "On breeding livestock" was issued. Then a strict selection of purebred Tagil sires was applied, followed by breeding "in itself", which made it possible to significantly increase the productivity of Tagil cows, and in 1930 a new Tagil breed of cattle was approved [18, 19]. Unpretentious to the conditions of feeding and keeping, Tagil cattle, when bred in the Urals, were not much inferior in terms of milk yield to black-and-white, surpassing it in fat milk content. In addition, Tagil cows, due to the specific structure of the pelvis, are distinguished by the ease of calving.

Subsequently, the massive Holsteinization of cattle led to the displacement of not only the Tagil, but also the Black-and-White breed. As a result, the only gene pool herd of Tagil cattle in Russia and the world (about 600 heads) is currently left, formed at the Shorokhov SPK in the Oktyabrsky District of the Perm

region. This gene pool, limited in number, is of undoubted interest as a source of valuable biological and economic traits; however, its molecular genetic characteristics remain insufficiently studied.

This report presents for the first time the results of identification of the STR and SNP genotypes of the unique local Tagil breed of cattle, which will allow the identification of genes and their variants that determine the biological, adaptive and productive qualities of animals of economic importance.

The purpose of this work is to carry out microsatellite analysis (STR) and genome-wide analysis of single nucleotide polymorphisms (SNPs) to assess genetic diversity and establish the population structure of the modern population of aboriginal Tagil cattle.

Materials and methods. DNA was isolated from blood samples ($n = 98$) of Tagil animals (TAGIL). Samples were taken at the SPK them. Shorokhov (Perm Territory, 2021), the QIAmp® DNA Mini Kit (QIAGEN, Germany) was used for DNA extraction according to the attached protocol. The purity and concentration of the resulting DNA preparations were determined ($OD_{260/280}$, ultraviolet microspectrophotometer Implen Nano-Photometer®, Implen GmbH, Germany), the concentration of double-stranded DNA was measured using a Qubit™ (1.0) fluorimeter (Life Technologies, USA).

Animal genotypes were analyzed by multiplex analysis for 11 microsatellites (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA122, INRA023, TGLA126, BM1818, ETH225, BM1824) using the STR panel developed at the V.I. OK. Ernst based on the recommendations of the International Society for Animal Genetics (ISAG).

Interbreed differentiation by STR-markers was carried out by the method of principal components (PCA) with the involvement of genotypes of cattle breeds that could potentially take part in the formation of the studied modern population of Tagil cattle (TAGIL): Holstein (HLST), Kholmogory Holsteinized (Tatarstan type) (TAT) , Kholmogory purebred (Pechora type) (PECH), black-and-white (old type) (Ch_P_OLD), Tagil (TAG) (samples from the ONIS BioTechJ databank, 2020, https://www.vij.ru/2-obshchaya /226-infrastruktura-test).

For genotyping by SNP markers, samples ($n = 48$) were selected based on the results of the analysis of STR genotypes. In the Structure 2.3.4 program (https://web.stanford.edu/group/pritchardlab/structure.html), the similarity coefficient Q was used to preliminarily evaluate the purebred and in the ML-Relate program (https://www.montana.edu/kalinowski/software/ml-relate/index.html) the degree of relatedness of individuals in the studied population of Tagil cattle.

Whole genome genotyping for SNP markers was performed using a GGP Bovine HD 150K BeadChip high-density DNA chip (~ 150,000 SNPs, Illumina, Inc., USA). Quality control and filtering of genotyping data for each sample and SNP was performed using the PLINK 1.9 [20] software package (https://www.co-genomics.org/plink/). The following filters were applied (corresponding commands in the PLINK program are given in parentheses): call-rate for all studied SNPs for an individual sample is not lower than 90% (--mind); call-rate for each of the studied SNPs for all genotyped samples is not less than 90% (--geno); the frequency of occurrence of minor alleles (MAF) more than 0.01 or 0.05 (--maf 0.01); deviation of SNP genotypes from the Hardy-Weinberg distribution in the totality of tested samples with a p-value $< 10^{-6}$ (--hwe). Linkage disequilibrium (LD) was also assessed using the Pearson correlation coefficient ($r^2 < 0.2$) with a step of 50 kb (--indep-pairwaise).

To analyze the results of genotyping of SNP markers (population genetic and phylogenetic studies), a database of whole genome SNP genotypes

of Tagil cattle (TAGIL) was formed. Animals of the Holstein breed (HLST) (n = 45) were introduced into the data set as a comparison group.

Observable heterozygosity (H_o), unbiased expected heterozygosity (uH_e), allelic diversity (Ar), and inbreeding coefficient F_{IS} (with 95% confidence interval) were calculated based on the obtained SNP genotypes for each data set in the R package diveRsity [21].

Principal component analysis (PCA) was performed in the PLINK 1.9 program followed by plotting in the R package ggplot2 [22]. For phylogenetic studies in the R package diveRsity [21], pairwise F_{ST} values were calculated [23]. The matrix of pairwise F_{ST} values was visualized as a Neighbor-net group genetic network in the SplitsTree 4.14.5 [24] program (https://splitstree4.software.informer.com/). The population structure and genetic homogeneity of the studied cattle breeds were determined using the Admixture 1.3 program [25] with a graphical representation using the pophelper R package [26]. The most probable number of ancestral clusters (K) was determined by calculating the cross-validation error values (CV error) for K from 1 to 5 in the Admixture 1.3 program.

To search for loci under selection pressure, selection of 0.1% SNPs with the highest F_{ST} values in pairwise comparison of breeds was used, as well as hapFLK analysis and detection of ROH islets overlapping in some individuals [15]. For islets, the minimum ROH size was taken to be 0.5 Mb with 50% of animals carrying overlapping ROHs and an ROH overlap length of at least 0.1 Mb.

Bioinformatic data processing and plotting were performed using the R Project for Statistical Computing software environment [27].

In hapFLK analysis, a threshold of significance was set $p < 0.01$. Confidence intervals (CI, 95%) are given for F_{IS} values. When determining the mean values (M), their standard errors (±SEM) were calculated.

Results. The results of principal component analysis (PCA) (Fig. 1) were obtained from the data of our microsatellite profiling of Tagil animals in comparison with the data of the previously performed STR genotyping of populations of the Holstein, Kholmogory (Holsteinized and purebred), Black-and-White and Tagil cattle breeds. It can be seen that a significant part of the Tagil cows differs from animals of other breeds, but the arrays of both populations of Tagil cattle overlap with the Holstein breed. On this basis, we further considered the Holstein breed as a comparison group.

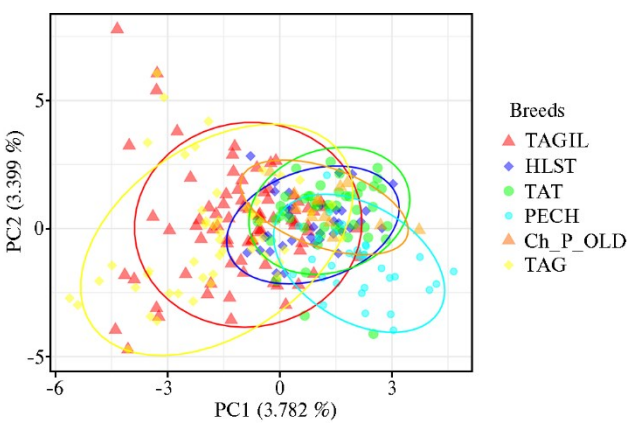


Fig. 1. Distribution of the Tagil breed animals based on genotypes by STR markers in the principal components analysis method: the modern population of Tagil cattle (TAGIL, n = 98) (Shorokhov SPK, Perm Territory, 2021), Holstein breed (HLST), Kholmogory Holsteinized breed (Tatarstan type) (TAT), Kholmogory purebred cattle (Pechora type) (PECH), Black-and-White breed (old type) (Ch_P_OLD), Tagil cattle (TAG) (samples from the ONIS BioTechJ databank, 2020).

To cover the maximum range of genetic diversity of the Tagil breed, the most unrelated animals were selected for SNP genotyping. Based on the calculation

of the similarity coefficient (Q) and the assessment of the degree of kinship (individuals with Q ≥ 0.35 were considered relatives), 51 animals (48 main and 3 spare) were selected. The selected individuals were divided into five groups: 1st with Q ≥ 90%, relatives not > 2 for each animal (although there are relatives with Q = 0.50; 37 cows); 2nd with 90% > Q ≥ 80%, no relatives > 2 for each animal (although there are relatives with Q = 0.50; 7 cows); 3rd with 80% > Q ≥ 70%, each animal has > 1 relative (none closer than with Q = 0.49; 5 cows); 4th with 70% > Q ≥ 60%, no relatives (2 cows); 5th (other) are undesirable for genotyping.

Whole genome genotyping of Tagil cattle for SNP markers was performed using the GGP Bovine HD 150K BeadChip DNA chip; the results were obtained for 48 animals (genotyping efficiency was more than 90%). The efficiency of genotyping (call rate) of the studied Tagil cows varied from 0.9900 to 0.9982. A total of 108432 SNPs were selected for analysis after quality control.

Table 1 summarizes the data of our population genetic study of the Tagil breed by SNP markers in comparison with the Holstein breed. One can see a significantly higher genetic diversity (in terms of Ar allelic diversity, observed Ho heterozygosity, and unbiased expected uHe heterozygosity) of Tagil cattle compared to Holstein. This may be the result of both less stringent selection for economically useful traits, and the participation of several breeds in the creation of Tagil cattle. The excess of heterozygotes in both populations should also be noted (see Table 1).

_1. Comparative characterization of the genetic diversity of the Tagil cattle population (Shorokhov SPK, Perm Territory, 2021) **and Holstein cattle** (ONIS BioTechJ databank, 2020) **by SNP markers** (*M*±SEM, GGP Bovine HD 150K BeadChip, Illumina, Inc., USA)

Breed	*n*	Ar	Ho	uHe	FIS [CI 95 %]
Tagil	48	1,999±0	0,408±0,001	0,398±0,000	–0,023 [–0,024; –0,022]
Holstein	45	1,989±0	0,366±0,001	0,360±0,001	–0,014 [–0,015; –0,013]

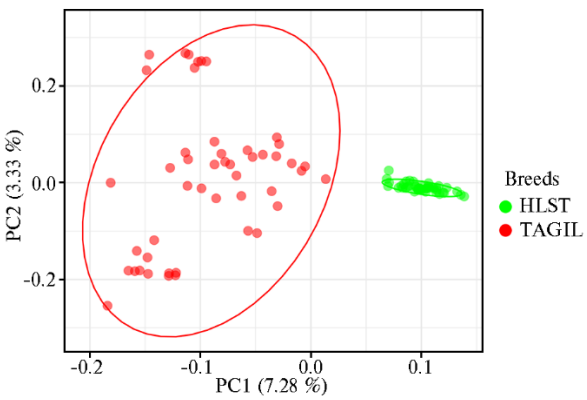


Fig. 2. Distribution of individuals of the Tagil (TAGIL, *n* = 48) (Shorokhov SPK, Perm Territory, 2021) **and Holstein breeds** (HLST, ONIS BioTechJ databank, 2020) **based on genotypes for 108432 SNPs.**

We clearly differentiated the breeds of selected animals (Tagil and Holstein) using the PCA method (Fig. 2). At the same time, unlike the Tagil Holstein breed, it turned out to be more consolidated (see Fig. 2), the first component (PC1) was responsible for 7.28% of genotypic variability and separated the Tagil cattle from the Holstein cattle.

Cluster analysis based on FST genetic distances (Fig. 3) assigned animals of the Tagil and Holstein breeds into two large groups in accordance with the breed. Animals belonging to the same breed were grouped on neighboring branches of the corresponding clusters.

When performing a structural analysis, the calculation of the cross-validation error (CV) showed the minimum value of this indicator for the number of clusters K = 4. At K = 2 (Fig. 4), each of the two compared breeds (TAGIL and HLST) exhibits a specific cluster structure, while the formation of the Tagil breed shows the presence of specific Holstein ancestral genomic components. An analysis of the genetic structure at K = 3 and K = 4 indicates the participation in the

formation of the Tagil breed of three more different ancestral breeds in addition to the Holstein breed. Taking into account that specific Holstein genomic components are manifested in most of the Tagil animals, and the components of the other three ancestral breeds of Tagil cattle have only minor traces of admixture in the Holstein breed, the resulting data set can be considered suitable for searching for loci that are under selection pressure.

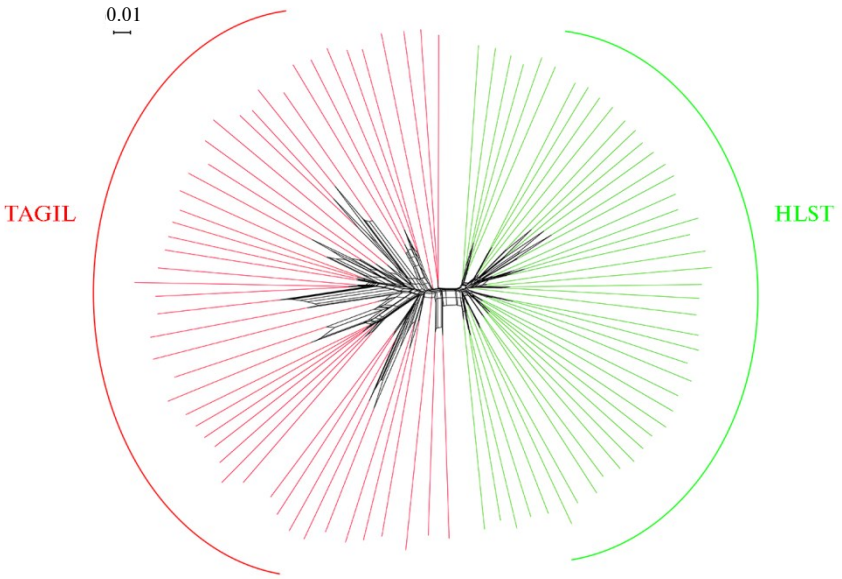


Fig. 3. Neighbor-net dendrogram based on genotypes of 108432 SNPs for selected animals of the Tagil (TAGIL, *n* = 48) (Shorokhov SPK, Perm Territory, 2021) **and Holstein breeds (**HLST, *n* = 45, ONIS BioTechJ databank, 2020) (visualization in SplitsTree 4.14.5).

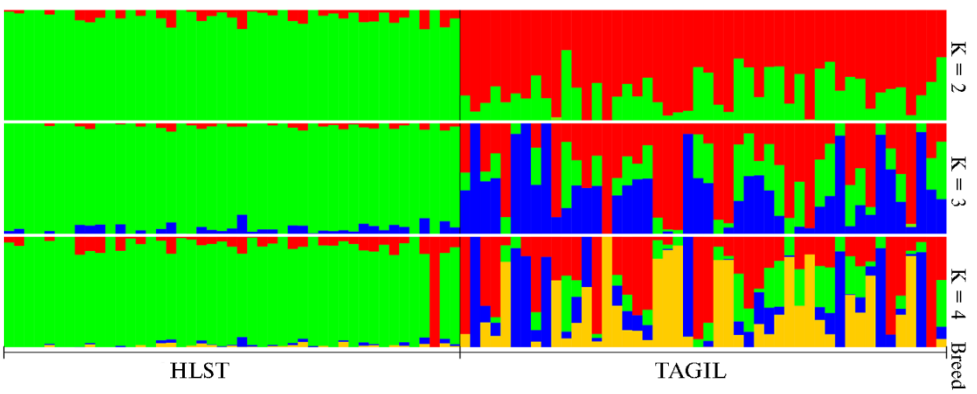


Fig. 4. Genetic structure of the Tagil breed population (TAGIL, *n* = 48, Shorokhov SPK, Perm Territory, 2021) **compared to the Holstein breed population** (HLST, *n* = 45, ONIS BioTechJ databank, 2020) **according to the Admixture analysis for 62809 SNPs** (Admixture 1.3 program, K is the number of clusters). Blue and yellow colors are genomic components of ancestral breeds identified in the Tagil breed.

By selecting the 0.1% SNPs with the highest FST values in a pairwise comparison of breeds, 109 SNPs located on 24 chromosomes were identified (excluding BTA8, BTA23, BTA27, BTA28, and BTA29).

Analysis of hapFLK showed the presence of five areas ($p < 0.01$) under selection pressure in the studied groups of Tagil cattle (Table 2) in comparison with Holstein. The identified regions were localized on chromosomes 4, 5, 8, 11, and 15, while a higher level of identification reliability was established for two

regions ($p < 0.001$). The length of the regions varied from 1.20 Mb (BTA8) to 9.61 Mb (BTA5), the number of SNPs localized within these regions varied from 24 to 92.

2. Characterization of chromosome (BTA) **regions under selection pressure in Tagil cattle** (TAGIL, $n = 48$, analysis by hapFLK method, Shorokhov SPK, Perm territory, 2021)

BTA	Position		Length, Mb	Most significant SNPs		Number of SNPs
	start	end		позиция	p	
4	6,842.949	10,648,384	3.81	8,495,236	7.08E-04	63
5	16,952.114	26,561,662	9.61	24,064,770	8.42E-04	92
8	50,695.489	51,899,568	1.20	51,101,318	5.14E-03	24
11	86,926.025	89,081,028	2.16	88,186,796	6.16E-03	31
15	48,905.274	54,898,376	5.99	54,218,907	5.08E-03	59

As a result of the study of the genomes of Tagil and Holstein cattle for the presence of runs of homozygosity (ROH), we identified 37 ROH islets that were found in more than 50% of animals, and 36 such regions were found in the genome of Holstein cattle, and only one is in Tagil (BTA14, positions 24437778-25098364, length 0.661 Mb). Interestingly, this region almost completely overlapped with the identical region identified in the genome of Holstein cattle (positions 24437778-25175950, length 0.750 Mb). It should be noted that the region on BTA14 in the region of 24.4-25.1 cm was previously identified as being under selection pressure in the Yaroslavl and Kholmogory breeds of cattle [15]. The structural annotation performed revealed the localization of eight genes in this region, the *XKR4*, *TMEM68*, *TGS1*, *LYN*, *RPS20*, *MOS*, *PLAG1*, and *CHCHD7*. Previously, in studies on different breeds of cattle (Holstein, Simmental, Wagyu, Hanu, etc.), it was shown that the listed genes are associated with height, exterior tallness, live weight, and feed intake [28-33].

Based on the fact that, when improving the Tagil cattle, it was crossed not only with the Holstein, but also with other black-and-white breeds of cattle (Kholmogory and Black-and-White), we lowered the threshold for the proportion of animals of the Tagil breed to 40%, whose genome contains common ROH. This made it possible to additionally identify five ROH islands in the Tagil breed (Table 3).

3. Characterization of ROH islets identified in the genome of Tagil cattle (TAGIL, $n = 48$, the threshold for the proportion of animals with total ROH is 40%, Shorokhov SPK, Perm territory, 2021)

Breed	BTA	Number of SNP	Position		Length, Mb	Proportion, %
			start	end		
TAGIL	2	15	65,513,882	65,946,493	0.433	41.7
TAGIL	14	15	33,026,716	33,348,218	0.322	41.7
TAGIL	16	4	44,372,045	44,552,678	0.181	41.7
TAGIL	20	17	71,433,871	71,720,853	0.287	41.7
TAGIL	23	18	479,600	936,645	0.457	41.7

Thus, as a result of the studies, a characterization of the population genetic characteristics of modern Tagil cattle was given and a database of full genome SNP genotypes was created that meets the established quality criteria (the number of genotyped animals with a genotyping efficiency of more than 90%). According to STR-marking, the participation of Kholmogory cattle, as well as Black-and-White and Holstein breeds in the formation of the Tagil breed was established. The greatest introgression of Holstein cattle was noted, which in the last decades, most likely, was used as an improving breed for Tagil cattle. In the analyzed population, animals of the Tagil breed are differentiated from Holstein cattle and represent a genotypically less consolidated and less structured group. In contrast to the Holstein cattle, the Tagil cattle were characterized by significantly ($p < 0.05$) higher genetic diversity and an excess of heterozygotes. Whole genome SNP genotyping revealed

genomic regions in which allelic variants are specific for the Tagil breed. When comparing the Tagil and Holstein breeds, 109 SNPs with the highest FST values were identified on 24 chromosomes in pairwise comparison. Five regions under selection pressure ($p < 0.01$) were identified in Tagil and Holstein cattle on chromosomes 4, 5, 8, 11 and 15. In more than 50% of the animals of the Tagil breed, an ROH islet (BTA14, positions 24437778-25098364, length 0.661 Mb) was found, previously identified in the Yaroslavl and Kholmogory breeds as a region under selection pressure. This ROH region may be an element of an adaptive genetic system in aboriginal breeds. In 40% of animals of the Tagil breed, five additional ROH islets are present. The data obtained by us will be used to identify genes and their variants that determine the adaptive and economically significant features of the Tagil breed, to study the history of the formation of its genetic structure, and to develop monitoring regulations to preserve the breed specificity and biodiversity of the Tagil cattle.

REFERENCES

1. *Most common of cattle in GB (NUTS 1 areas) in 01 April 2005*. London, 2005, Version 1.
2. *Holstein Association USA*. Available: http://holsteinusa.com. No date.
3. *Ezhegodnik po plemennoi rabote v molochnom skotovodstve v khozyaistvakh Rossiiskoi Federatsii (2019 g.)* [Yearbook on pedigree work in dairy cattle breeding on the farms of the Russian Federation (2019)]. Moscow, 2020 (in Russ.).
4. Mwai O., Hanotte O., Kwon Y.-J., Cho S. African indigenous cattle: unique genetic resources in a rapidly changing world. *Asian Australas. J. Anim. Sci.*, 2015, 28(7): 911-921 (doi: 10.5713/ajas.15.0002R)
5. Elsik C.G., Bovine Genome Sequencing and Analysis Consortium. The genome sequence of taurine cattle: a window to ruminant biology and evolution. *Science*, 2009, 324(5926): 522-528 (doi: 10.1126/science.1169588).
6. Kale D.S., Rank D.N., Joshi C.G., Yadav B.R., Koringa P.G., Thakkar K.M., Tolenkhomba T.C., Solanki J.V. Genetic diversity among Indian Gir, Deoni and Kankrej cattle breeds based on microsatellite markers. *Indian Journal of Biotechnology*, 2010, 9: 126-130.
7. Ulloa-Arvizu R., Gayosso-Vazquez A., Ramos-Kuri M., Estrada F.J., Montano M., Alonso R.A. Genetic analysis of Mexican Criollo cattle populations. *Journal of Animal Breeding and Genetics*, 2008, 125: 351-359 (doi: 10.1111/j.1439-0388.2008.00735.x).
8. Kramarenko A.S., Gladyr E.A., Kramarenko S.S., Pidpala T.V., Strikha L.A., Zinovieva N.A. Genetic diversity and bottleneck analysis of the Red Steppe cattle based on microsatellite markers. *Ukrainian Journal of Ecology*, 2018, 8(2): 12-17 (doi: 10.15421/2018_303).
9. Kiseleva T.Yu., Kantanen J., Vorobyov N.I., Podoba B.E., Terletsky V.P. Linkage disequilibrium analysis for microsatellite loci in six cattle breeds. *Russian Journal of Genetics*, 2014, 50(4): 406-414 (doi: 10.1134/S1022795414040048).
10. Zwane A.A., Schnabel R.D., Hoff J., Choudhury A., Makgahlela M.L., Maiwashe A., Van Marle-Koster E., Taylor J.T. Genome-Wide SNP discovery in indigenous cattle breeds of South Africa. *Front. Genet.*, 2019, 10(273): 1-16 (doi: 10.3389/fgene.2019.00273).
11. Bhuiyan M.S.A., Lee S.-H., Hossain S.M.J., Deb G.K., Afroz M.F., Lee, S.-H., Bhuiyan A.K.F.H. Unraveling the genetic diversity and population structure of Bangladeshi indigenous cattle populations using 50K SNP markers. *Animals*, 2021, 11(2381): 1-15 (doi: 10.3390/ani1108238).
12. Wang W., Gan J., Fang D., Tang H., Wang H., Yi J., Fu M. Genome-wide SNP discovery and evaluation of genetic diversity among six Chinese indigenous cattle breeds in Sichuan. *PLoS ONE*, 2018, 13(8): e0201534 (doi: 10.1371/ journal.pone.0201534).
13. Browett S., McHugo G., Richardson I.W., Magee D.A., Park S.D.E., Fahey A.G., Kearney J.F., Correia C.N., Randhawa I.A.S., MacHugh D.E. Genomic characterisation of the indigenous Irish Kerry Cattle breed. *Front. Genet.*, 2018, 9(51): 1-17 (doi: 10.3389/fgene.2018.00051).
14. Zinov'eva N.A., Dotsev A.V., Sermyagin A.A., Vimmers K., Reier Kh., Solkner I., Deniskova T.E., Brem G. Study of genetic diversity and population structure of five Russian cattle breeds using whole-genome SNP analysis. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2016, 51(6): 788-800 (doi: 10.15389/agrobiology.2016.6.788eng).
15. Zinovieva N.A., Dotsev A.V., Sermyagin A.A., Deniskova T.E., Abdelmanova A.S., Kharzinova V.R., Sölkner J., Reyer H., Wimmers K., Brem G. Selection signatures in two oldest Russian native cattle breeds revealed using high-density single nucleotide polymorphism analysis. *PLoS ONE*, 2020, 15(11): e0242200 (doi: 10.1371/journal.pone.0242200).
16. Romanov A.I. V sbornike: *Trudy soveshchaniya veterinarnykh vrachei i predstavitelei zemstv* [In: Proceedings of the meeting of veterinarians and representatives of zemstvos]. Perm', 1913: 11-129

(in Russ.).
17. *Puti i formy sozdaniya i sokhraneniya genofonda tsennykh lokal'nykh porod* [Ways and forms of creating and preserving the gene pool of valuable local breeds]. Leningrad, 1979 (in Russ.).
18. Kremer L.A. *Tagil'skii skot* [Tagil cattle]. Moscow-Leningrad, 1931 (in Russ.).
19. Moiseeva I.G., Ukhanov S.V., Stolpovskii Yu.A. et al. *Genofondy sel'skokhozyaistvennykh zhivotnykh: geneticheskie resursy zhivotnovodstva Rossii* /Pod red. I.A. Zakharova [Gene pools of farm animals: genetic resources of animal husbandry in Russia. I.A. Zakharov (ed.)]. Moscow, 2006 (in Russ.).
20. *Plink: Whole genome association analysis toolse*t. Available: http://zzz.bwh.harvard.edu/plink/. Accessed 25.01.2017.
21. 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).
22. Wickham H. *ggplot2: elegant graphics for data analysis.* N.-Y., 2009.
23. 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).
24. 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).
25. 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).
26. Francis R.M. pophelper: An R package and web app to analyse and visualise population structure. *Molecular Ecology Resources*, 2017, 17(1): 27-32 (doi: 10.1111/1755-0998.12509).
27. R Core Team. *R: a language and environment for statistical computing. R foundation for statistical computing*, Vienna, 2018. Available: https://www.R-project.org/. No date.
28. Lindholm-Perry A.K., Kuehn L.A., Smith T.P., Ferrell C.L., Jenkins T.G., Freetly H.C., Snelling W.M. A region on BTA14 that includes the positional candidate genes LYPLA1, XKR4 AND TMEM68 is associated with feed intake and growth phenotypes in cattle. *Anim Genet.*, 2012, 43: 216-219 (doi: 10.1111/j.1365-2052.2011.02232.x).
29. Pryce J.E., Arias J., Bowman P.J., Davis S.R., Macdonald K.A., Waghorn G.C., Wales W.J., Williams Y.J., Spelman R.J., Hayes B.J. Accuracy of genomic predictions of residual feed intake and 250-day body weight in growing heifers using 625,000 single nucleotide polymorphism markers. *J. Dairy Sci.*, 2012, 95(4): 2108-2119 (doi: 10.3168/jds.2011-4628).
30. Taye M., Yoon J., Dessie T., Cho S., Oh S.J., Lee H.K., Kim H. Deciphering signature of selection affecting beef quality traits in Angus cattle. *Genes Genomics*, 2018, 40(1): 63-75 (doi: 10.1007/s13258-017-0610-z).
31. Cheruiyot E.K., Bett R.C., Amimo J.O., Zhang Y., Mrode R., Mujibi F.D.N. Signatures of selection in admixed dairy cattle in Tanzania. *Front. Genet.*, 2018, 9(607): 1-15 (doi: 10.3389/fgene.2018.00607).
32. De Las Heras-Saldana S., Clark S.A., Duijvesteijn N., Gondro C., van der Werf J.H.J., Chen Y. Combining information from genome-wide association and multi-tissue gene expression studies to elucidate factors underlying genetic variation for residual feed intake in Australian Angus cattle. *BMC Genomics*, 2019, 20(1): 939 (doi: 10.1186/s12864-019-6270-4).
33. Grigoletto L., Ferraz J.B.S., Oliveira H.R., Eler J.P., Bussiman F.O., Abreu Silva B.C., Baldi F., Brito L.F. Genetic architecture of carcass and meat quality traits in Montana tropical composite beef cattle. *Front. Genet.*, 2020, 11(123): 1-13 (doi: 10.3389/fgene.2020.00123).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1134-1147
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 636.32/.38:575.162:577.2

doi: 10.15389/agrobiology.2021.6.1134eng
doi: 10.15389/agrobiology.2021.6.1134rus

A STUDY OF MATERNAL VARIABILITY OF RUSSIAN LOCAL SHEEP BREEDS BASED ON ANALYSIS OF CYTOCHROME b GENE POLYMORPHISM

O.A. KOSHKINA[1], T.E. DENISKOVA[1] ✉, A.V. DOTSEV[1], E. KUNZ[2], M. UPADHYAY[2], S. KREBS[3], A.D. SOLOVIEVA[1], I. MEDUGORAC[2], N.A. ZINOVIEVA[1]

[1]*Ernst Federal Research Center for Animal Husbandry*, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail olechka1808@list.ru, horarka@yandex.ru (✉ corresponding author), asnd@mail.ru, anastastasiya93@mail.ru, n_zinovieva@mail.ru;
[2]*Population Genomics Group, Department of Veterinary Sciences*, *Ludwig Maximilian University (LMU) Munich*, Germany, 80539 Munich, Veterinaerstr. 13, e-mail elisabeth.kunz@gen.vetmed.uni-muenchen.de, u.maulik@gen.vetmed.uni-muenchen.de, ivica.medjugorac@gen.vetmed.uni-muenchen.de;
[3]*Gene Center, Ludwig-Maximilians-University, (LMU) Munich*, Germany, 80539 Munich, Veterinaerstr. 13, e-mail krebs@genzentrum.lmu.de
ORCID:
Koshkina O.A. orcid.org/0000-0003-4830-6626
Deniskova T.E. orcid.org/0000-0002-5809-1262
Dotsev A.V. orcid.org/0000-0003-3418-2511
Kunz E. orcid.org/0000-0002-6949-4120
Upadhyay M. orcid.org/0000-0002-4296-0021
Krebs S. orcid.org/0000-0001-5112-9507
Solovieva A.D. orcid.org/0000-0003-2628-9554
Medugorac I. orcid.org/0000-0002-6891-3039
Zinovieva N.A. orcid.org/0000-0003-4017-6863

The authors declare no conflict of interests
Acknowledgements:
The equipment of the Center for Biological Resources and Bioengineering of Agricultural Animals (Ernst Federal Research Center for Animal Husbandry) was used.
Performed with the financial support of the Russian Science Foundation (RSF No. 21-66-00007). Sheep samples were obtained as part of the assignment of the Ministry of Science and Higher Education of the Russian Federation (No. 0445-2019-0024).
Received October 1, 2021

Abstract

Analysis of mitochondrial DNA (mtDNA) polymorphism is one of the most effective modern approaches to assess the genetic diversity of livestock species. The mtDNA sequencing is the most efficient approach for identifying mtDNA haplogroups in sheep (*Ovis aries*). Although this approach is widely used abroad, a systematic and comprehensive study of Russian sheep breeds with its aid has not yet been conducted. In this work, we analyzed the polymorphism of the complete sequence of the cytochrome b (*CytB*) gene in Russian sheep breeds of various origins. For the first time, we established the belonging of sheep from 25 Russian breeds to haplogroups and showed haplotype relationships between coarse wool, fine wool and semi-fine wool sheep breeds based on the analysis of polymorphism of the mitochondrial cytochrome b gene. The maternal variability of a wide range of local sheep breeds in comparison with transboundary breeds was assessed. In this research, we aimed to evaluate genetic diversity and to determine the haplotype variability and haplogroup belonging of Russian local sheep breeds based on the *CytB* gene sequences. The study was performed on 106 samples from 25 Russian sheep breeds in 2020-2021. Tissue samples (ear notches) were retrieved from the biological collection "Bank of genetic material of domestic and wild animal species and poultry" (registered by the Ministry of Education and Science of the Russian Federation No. 498808), which is established and maintained at the Ernst Federal Research Center for Animal Husbandry. The final study sample included nine fine-wool breeds, including Baikal (n = 3), Dagestan Mountain (n = 4), Groznensk (n = 5), Kulundin (n = 5), Manych Merino (n = 5), Salsky (n = 5), Soviet Merino (n = 3), Stavropol (n = 5) and Volgograd (n = 5); five semi-fine wool breeds, including Altai mountain(n = 5), Kuibyshev (n = 1), North Caucasian meat-wool (n = 5), Russian longhaired (n = 3) and Tsigai (n = 2); eleven coarse-wool breeds, including Romanov (n = 3), Andean black (n = 5), Buubei (n = 5), Karakul (n = 3), Karachaev (n = 5), Kuchugur (n = 3), Lezgin (n = 5), Tushin (n = 5), Tuva short-fat-tailed (n = 4), Edilbai (n = 5) and Kalmyk (n = 5). The complete sequences of the *CytB* gene of the studied sheep breeds were determined using the next generation sequencing (NGS) technology. To achieve this goal, three overlapping mtDNA fragments (overlapping region of more than 290 bp) with lengths of 6500, 5700, and 6700 bp were amplified. The ob-

tained polymerase chain reaction (PCR) products were used to prepare libraries, which were then sequenced by the method of paired terminal reads of 300 bp each with a MiSeq System Sequencer (Illumina, Inc., USA). The *CytB* gene sequence was recovered from the complete mtDNA sequence after alignment, which was performed using the MUSCLE algorithm in the MEGA 7.0.26 software. All studied breeds had high haplotype (HD = 0.400-1,000) and nucleotide diversity (π = 0.00058-0.00760). In total, we identified 82 haplotypes. Tuva short-fat-tailed sheep breed was represented by only one haplotype. The AMOVA results showed that genetic diversity was mainly determined by intrabreed differences (90.55 %). Four haplogroups including A, B, C and D were identified in the study sample. Such a haplogroup diversity might be explained by a wide geographical range of habitats of the studied animals. The most frequent haplogroups in Russian local sheep breeds were B (n = 64) and A (n = 34), which are typical for sheep of European and Asian origin respectively. Seven animals were assigned to haplogroup C, and haplogroup D was represented by one animal. The results contribute to a deeper understanding of the processes of migration and settlement of domestic sheep in Eurasia.

Keywords: domestic sheep, mitochondrial DNA, cytochrome b gene, haplogroups, haplotypes

Domestic sheep (*Ovis aries*) are one of the most economically significant livestock species, providing humans with food (meat and milk) and raw materials for light industry (wool, sheepskin, and astrakhan) [1]. Since domestication (between 11,000 and 10,500 BC), sheep have spread across all continents, except for Antarctica [2]. This has led to various local breeds with a unique composition of traits due to adaptation and artificial selection with the aim of producing livestock products [3].

Genetic diversity (variation in alleles and genotypes present in a population) reflects the size, history, ecology, and fitness of a population [4]. It plays an important role in ensuring the formation of traits that are responsible for the improvement, survival, and adaptation of a species [5]. Climate change, emerging diseases, scarcity of land and water resources, and changing market demands make the conservation and sustainable use of livestock genetic resources even more important [6]. The study of the genetic variability of the world gene pool of modern native sheep breeds makes it possible to comprehensively assess genetic diversity and indicators of selection, deepen knowledge about the breeds' origin and distribution, and determine the impact of human activity on these animals since domestication [7-9].

Single nucleotide polymorphisms (SNPs), although widely used in the study of the genomes of farm animals [10, 11], represent only one type of common genomic variation. Another effective approach for assessing genetic diversity that has not lost its relevance is the study of mitochondrial DNA (mtDNA) polymorphism [12, 13]. MtDNA demonstrates a high degree of polymorphism and is characterized by the absence of recombination. This makes it possible to study the genetic relationships between breeds and to track both ancient and relatively recent evolutionary events.

Phylogenetic studies have often focused on mitochondrial genes encoding ribosomal DNA (12S and 16S), but their use in broad taxonomic analysis is constrained by the predominance of insertions and deletions (indels); this greatly complicates the alignment of sequenced nucleotide sequences [14]. In this regard, 13 protein-coding genes, in which indels are rarely found due to a shift in the reading frame, are considered more suitable targets in the mitochondrial genome.

The cytochrome B (*Cytb*) gene has several advantages over other mtDNA genes. First, it has a wider range of phylogenetic signals than other mitochondrial genes. Second, nucleotides in the third codon position of *Cytb* show a high base substitution frequency, which is approximately three times higher than the rate of 12S or 16S rDNA, leading to accelerated molecular evolution [15]. Third, this gene evolves quite quickly, making it possible to distinguish

closely related species as well as phylogenetic groups within the same species [16, 17]. Therefore, mtDNA sequencing is the approach for identifying haplogroups. Although this approach is widely used [18-20], it has not been used to conduct a systematic and comprehensive study of Russian sheep breeds.

In 1996, Wood et al. [19] identified two haplogroups in domestic sheep from New Zealand. Then in 1998, after comparing the distribution of haplotypes in several breeds in Germany, Russia, and Kazakhstan, Hiendleder et al. [20] identified these haplogroups as Asian (haplogroup A) and European (haplogroup B). In 2005, based on the results of studies on local breeds from China and Turkey, Guo et al. [18] and Pedrosa et al. [21] expanded the composition of haplogroups to three generally recognized phylogenetic branches (with the inclusion of haplogroup C). Haplogroup C sequences were found at low frequency in sheep living in Portugal [22], suggesting gene flow from the Fertile Crescent to the Iberian Peninsula. Haplogroup C has also been shown to contain more genetic diversity than haplogroup A or B [21]; however, unlike haplogroup B, it does not correspond to any of the wild animals of the *Ovis* genus. Subsequently, in 2006, Tapio et al. [23] found a control region sequence in one Karachaev sheep that clustered separately from the three distinct clusters of domestic sheep mtDNA. This study provided evidence of the presence of a fourth maternal lineage, named haplogroup D. Lastly, in 2007, based on the analysis of polymorphism of the fragment of the control region and *Cytb* mtDNA in sheep, Meadows et al. [24] identified the fifth haplogroup, E.

Genetic analysis showed that haplogroups A and B are found in domestic sheep from all geographic regions (average combined frequency, 89%). Haplogroup A is mainly found in Asian populations [19, 25], whereas haplogroup B has a high frequency of occurrence in European and Asian populations. In contrast, haplogroup C is less common (mean frequency: 18%); only a small number of individuals have been identified in Asia (within the Fertile Crescent) and Europe (within the Caucasus and Iberian Peninsula) [23, 25, 26]. Haplogroups D and E have been identified more recently and are the least represented of the five lineages; sheep with these haplogroups have so far only been found in the Caucasus and Turkey [23, 24].

Through the use of mtDNA to determine the genetic diversity of sheep, insights into the history of sheep domestication and human-influenced global migration have been obtained [27]. In 2007, Pardeshi et al. [28] characterized the mtDNA diversity of three breeds of Indian sheep which all belonged to maternal line A. The Indian sheep network did not have a well-defined central haplotype, no haplotype exchange between populations was observed, and there was a strong breed structure. This haplotype structure of Indian sheep indicates that the history of these breeds was characterized by complete reproductive isolation and a very low frequency of crossing between populations. This is likely because Indian sheep farming is indeed based on maintaining cultural and traditional barriers that prevent genetic exchange between breeds [28].

In 2013, Zhao et al. [29] examined mtDNA variability in local sheep raised in seven regions of China. Phylogenetic analysis of mtDNA D-loop sequences from 16 indigenous Chinese sheep breeds confirmed the presence of three maternal haplogroups (A, B, and C) with high genetic diversity. Additionally, Lv et al. [27] identified two stages of migration in the history of the East Eurasian sheep. The authors concluded that the Mongolian Plateau region was a secondary center of settlement, acting as a "transport hub" in Eastern Eurasia. Sheep from the Middle Eastern center of domestication migrated through the Caucasus and Central Asia and arrived in northern and southwestern China

(haplogroups A, B, and C) and the Indian subcontinent (haplogroups B and C) [27].

The estimated time of divergence between the five main haplogroups occurred before domestication, as demonstrated by archaeological evidence [30]. For example, the time of divergence between the two most common lineages, A and B, was estimated to be 1.6-1.7 million years ago based on the *Cytb* sequences [20]. In addition, Pedrosa et al. [21] suggested that the divergence times of line C and lines A and B are approximately 0.42-0.76 and 0.45-0.75 Ma, respectively, based on analysis of *Cytb* sequences. However, a recent study [25] used 12 protein-coding genes to provide a different estimate of the divergence between lineages: 0.590±0.17 Ma between A and B, and 0.26±0.09 Ma between C and E. In 2020, Liu et al. [31] conducted a complete genome mtDNA sequencing study on Tibetan sheep and obtained similar results. This supports the existence of two maternal lines (haplogroups A and B) with high genetic diversity in 15 populations of Tibetan sheep in China. The ancestors of the maternal lines may have been mouflons (*O. gmelina*) and argali (*O. ammon*) [31]. Despite the wide coverage of mtDNA studies abroad, the Russian sheep breeds remain poorly understood. Sheep breeding has always been an important branch of animal husbandry in the Russian Federation because it provides the population with wool, which is in huge demand due to harsh climatic conditions. In the 1990s, sheep breeding in the Russian Federation fully met the domestic needs of the country [32], but by 2007 there was a sharp decrease in the number of sheep (by 65%) and the textile industry (by 85–90%) [33]. Many factors contributed to this, including a lack of demand for fine and crossbred wool, change of ownership, price disparity for industrial and agricultural products, an inundation of the domestic market with cheap imported goods made of wool, cotton, and leather, unpreparedness, and vulnerability of prices of the Russian commodities in the market [34]. Furthermore, the number of sheep breeding enterprises has decreased in Russia [35], and this has led to an economic decline in domestic sheep breeding. In 35 regions of the Russian Federation, 43 sheep breeds are bred, including 15 fine (34.9%), 12 semi-fine (27.9%), 2 semi-coarse (4.7%), and 14 coarse (32.5%) wool breeds [36].

Despite the problems with domestic sheep breeding, it has begun to recover. Currently, improving the potential meat productivity of raised breeds is considered promising for increasing the economic efficiency of the sheep breeding industry. This is due to a significant difference between the economic importance of wool (5% of the total income) and mutton (95%). Due to market reorientation, the share of wool breeds has decreased significantly from 90.0% in 1990 to 55.2% in 2020, whereas that of meat breeds have increased from 10.0 to 44.8% [37, 38]. These changes have had serious consequences. Some woolly breeds are on the verge of extinction. Most fine and semi-fine wool breeds were developed using native ewes as maternal forms, with sires of highly productive foreign breeds [39]. Local coarse wool breeds were created based on the genetic resources of native sheep and their history of origin, which has not yet been fully elucidated.

In this study, we analyzed the polymorphism in the complete sequence of *Cytb* in Russian breeds of sheep of various origins. For the first time, the haplogroups of sheep from 25 Russian breeds were established, and haplotype relationships between coarse, fine, and semi-fine wool breeds were determined. The characteristics of maternal variability in local sheep breeds were compared with those of transboundary breeds. Our goal was to determine the genetic diversity, haplotype variability, and haplogroup assignments of Russian local sheep breeds based on *Cytb* sequences.

Materials and methods. The study was performed on 25 Russian sheep breeds between 2020 and 2021. Tissue samples (ear notches) were obtained from the biocollection "Bank of genetic material of domestic and wild species of animals and birds" (registered by the Ministry of Education and Science of the Russian Federation No. 498808). It is established and maintained by the Ernst Federal Research Center for Animal Husbandry. The final dataset included nine fine wool breeds: Baikal fine-fleeced (BAKL, n = 3), Dagestan Mountain (DGMT, n = 4), Groznensk (GRZY, n = 5), Kulundin (KLND, n = 5), Manych Merino (MNCM, n = 5), Salsky (SLSK, n = 5), Soviet Merino (SVTM, n = 3), Stavropol (STVP, n = 5), and Volgograd (VLGD, n = 5); five semi-fine wool breeds: Altai Mountain (ALTM, n = 5), Kuibyshev (KBSV, n = 1), North Caucasian (NCCS, n = 5), Russian Longhaired (RSLH, n = 3), and Tsigai (TSIG, n = 2); and eleven coarse wool breeds: Romanov (RMNV, n = 3), Andean (ANDB, n = 5), Buubei (BUBI, n = 5), Karakul (KRKL, n = 3), Karachaev (KRCV, n = 5), Kuchugur (KHGR, n = 3), Lezgin (LZGN, n = 5), Tushin (TSHN, n = 5), Tuva short-fat-tailed (TUVA, n = 4), Edilbaev (EDLB, n = 5), and Kalmyk (KLMY, n = 5).

DNA was extracted using a DNA-Extran-2 kit (OOO Sintol, Russia) according to the manufacturer's recommendations. Quality control of the obtained DNA solutions was performed in two stages. In the first stage, the concentration was measured (DNA from 15 to 50 ng/μl was included) using a Qubit 4.0 fluorimeter (Invitrogen/Life Technologies, USA). In the second stage, the ratio of the degree of absorption OD_{260}/OD_{280} (DNA with a ratio ≥ 1.8 was included) was measured using a NanoDrop8000 spectrophotometer (Thermo Fisher Scientific, USA). Complete sequences of the *Cytb* gene of the sheep breeds were sequenced using next-generation sequencing technology. For this purpose, three overlapping mtDNA fragments (overlapping region > 290 bp), 6500, 5700, and 6700 bp long, were amplified using the following primer pairs: F1 5′-GTCCTTCGCCCTAATC-CTCTC-3′, R1 3′-AGGGTGCCGATATCTTTGTG-5′; F2 5′-ACCCAAAACTCTTCGTGCTC-3′, R2 3′-GGAAGTCAGAATGC-GATGGT-5′; and F3 5′-AC-ACCAAACCCACGCTTATC-3′, R3 3′-GG-GTGTTGATAGTGGGGCTA-5′. Reactions were performed at a final volume of 25 μl: 10 μl reaction buffer (2.5× HF Reaction buffer), 10.25 μl Milli-Q Water H_2O, 2.5 μl dNTPs, 1 μl primer mix, 0.25 μl SmartTaq HF-FuZZ DNA polymerase (Dialat, Russia), and 1 μl of DNA. After initial denaturation (2 min at 94 °C), amplification was performed on an Applied Biosystems SimpliAmp thermal cycler (Thermo Fisher Scientific, USA) using the following temperature-time regime: 30 s at 94 °C (1 cycle); 30 s at 61 °C, 6.5 min at 70 °C (10 cycles); 30 s at 94 °C, 30 s at 60 °C, 3.5 min at 70 °C (25 cycles); and the final stage for 10 min at 72 °C.

The obtained polymerase chain reaction products were purified using a Cleanup Standard kit for DNA purification from agarose gel and reaction mixtures (ZAO Evrogen, Russia) and used to prepare libraries, which were then sequenced using 300 bp paired-end sequencing on a MiSeq device (Illumina, Inc., USA). The *Cytb* sequence was reconstructed from the complete mtDNA sequence after alignment was performed using the MUSCLE algorithm [40] in MEGA 7.0.26 software [41]. A median-joining haplotype network [42] was constructed using PopART 1.7 software [43]. The best evolutionary models were determined in PartitionFinder 2 [44], using the adjusted Akaike information criterion [45]. The evolutionary models HKY and HKY + I were found to be optimal. An analysis of molecular variance (AMOVA] was performed using Arlequin 3.5.2.2 [46]. The Bayesian phylogenetic tree was constructed using MrBayes 3.2.7 [47] with subsequent visualization in FigTree 1.4.3 [48]. The *Cytb* sequence

of snow sheep (*O. nivicola*; GenBank accession number NC_039431.1) was used as the outgroup [49]. A Markov chain Monte Carlo search was performed using four chains with 10,000,000 steps, with trees sampled every 500 generations (the first 25% of the trees were discarded as burn-in). In the DnaSP 6.12.01 program [50], the following parameters of genetic diversity were calculated: the number of polymorphic sites (S), the average number of nucleotide differences (K), the number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π), and the standard error of the mean (±SEM).

Results. A total of 82 haplotypes were identified from 106 domestic sheep. All the individuals from the Tuva short-fat-tailed breed group had an identical haplotype. The highest haplotypic diversity (Hd = 1.000) was observed in the Baikal fine-fleeced, Kalmyk, Karakul, Lezgin, Russian Longhaired, Stavropol, Tsigai, Volgograd, and Manych Merino breeds (Table 1). The North Caucasian meat-wool and Tushin breeds showed the lowest haplotype diversity (Hd = 0.400). The lowest values of nucleotide diversity and smallest average number of nucleotide differences were recorded in the Soviet Merino breed (π = 0.00058, K = 0.667). The Baikal fine-fleeced breed was characterized by the highest values for these indicators (π = 0.00760, K = 8.667).

1. Indices of genetic diversity in populations of 25 Russian local breeds of domestic sheep (*Ovis aries*), based on the nucleotide sequence of the mitochondrial gene cytochrome B (Ernst Federal Research Center for Animal Husbandry, Moscow region, 2020–2021)

Population	*n*	S	K	H	Hd±SEM	π±SEM
ALTM	5	8	4.000	4	0.900±0.161	0.00351±0.00069
ANDB	5	5	2.600	4	0.900±0.161	0.00228±0.00049
BAKL	3	13	8.667	3	1.000±0.272	0.00760±0.00308
BUBI	5	13	5.800	3	0.800±0.164	0.00509±0.00208
DGMT	4	14	7.333	3	0.833±0.222	0.00643±0.00240
EDLB	5	6	2.800	4	0.900±0.161	0.00246±0.00064
GRZY	5	4	1.600	4	0.900±0.161	0.00140±0.00042
KBSV	3	2	1.333	2	0.667±0.314	0.00117±0.00055
KHGR	3	4	2.667	2	0.667±0.314	0.00234±0.00110
KLMY	5	8	3.600	5	1.000±0.126	0.00316±0.00065
KLND	5	5	2.000	4	0.900±0.161	0.00175±0.00051
KRCV	5	13	5.600	4	0.900±0.161	0.00491±0.00220
KRKL	3	6	4.000	3	1.000±0.272	0.00351±0.00141
LZGN	5	15	8.000	5	1.000±0.126	0.00702±0.00160
MNCM	5	7	2.800	5	1.000±0.126	0.00246±0.00051
NCCS	5	3	1.200	2	0.400±0.400	0.00105±0.00062
RMNV	3	2	1.333	2	0.667±0.314	0.00117±0.00055
RSLH	3	4	2.667	3	1.000±0.272	0.00234±0.00068
SLSK	5	6	2.400	4	0.900±0.161	0.00211±0.00065
STVP	5	18	7.800	5	1.000±0.126	0.00684±0.00212
SVTM	3	1	0.667	2	0.667±0.314	0.00058±0.00028
TSHN	5	2	0.800	2	0.400±0.237	0.00070±0.00042
TSIG	2	1	1.000	2	1.000±0.500	0.00088±0.00044
TUVA	4	0	0.000	1	0.000±0.000	0.00000±0.00000
VLGD	5	18	7.600	5	1.000±0.126	0.00667±0.00183

Note. *n* — number of samples, S — number of polymorphic sites, K — average number of nucleotide differences, H — number of haplotypes, Hd — haplotype diversity, π — nucleotide diversity. ALTM — Altai Mountain, ANDB — Andean, BAKL — Baikal fine-fleeced, BUBI — Buubei, DGMT — Dagestan Mountain, EDLB — Edilbaev, GRZY — Groznensk, KBSV — Kuibyshev, KHGR — Kuchugur, KLMY — Kalmyk, KLND — Kulundin, KRCV — Karachaev, KRKL — Karakul, LZGN — Lezgin, MNCM — Manych Merino, NCCS — North Caucasian, RMNV — Romanov, RSLH — Russian Longhaired, SLSK — Salsky, STVP — Stavropol, SVTM — Soviet Merino, TSHN — Tushin, TSIG — Tsigai, TUVA — Tuva short-fat-tailed, VLGD — Volgograd.

The coarse wool sheep breeds (Fig. 1, A) formed three clusters corresponding to haplogroups A, B, and C. These breeds were characterized by high genetic diversity. The exception was the Tuva short-fat-tailed breed, all the study individuals of which belonged to the same haplogroup A. Animals of the other breeds belonged to different haplogroups, which may indicate that these populations are of a mixed origin. The fine wool sheep breeds (Fig. 1, B) also had a

high haplotype diversity. As in the case of coarse wool sheep, animals of the same breed clustered into different haplogroups, with the exception of the Salsky breed. In contrast to Tuva short-fat-tailed sheep, sheep from the Salsky breed were characterized by higher nucleotide diversity and had different haplotypes within haplogroup B, which was the most numerous one among the fine wool breeds.

Sheep assigned to haplogroup C differed from those belonging to haplogroup A by nine nucleotide substitutions. Among the fine wool sheep, one individual of the Volgograd breed, which differed by eight nucleotide substitutions from haplogroups A and C, formed a separate cluster, haplogroup D.

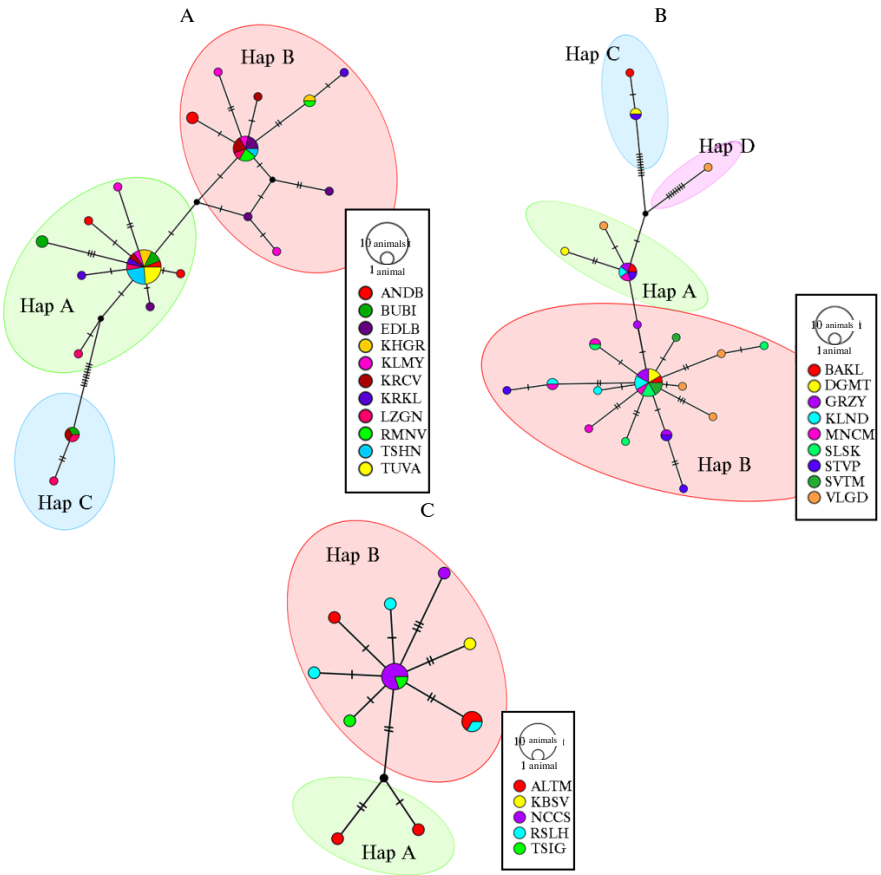


Fig. 1. Median-joining haplotype network displaying the relationships among haplotypes identified in 25 Russian local breeds of domestic sheep (*Ovis aries*), based on the mitochondrial gene Cytb (Ernst Federal Research Center for Animal Husbandry, Moscow region, 2020-2021).

A — median-joining haplotype network, constructed for coarse wool sheep breeds: ANDB — Andean (n = 5), BUBI — Buubei (n = 5), EDLB — Edilbaev (n = 5), KHGR — Kuchugur (n = 3), KLMY — Kalmyk (n = 5), KRCV — Karachaev (n = 5), KRKL — Karakul (n = 3), LZGN — Lezgin (n = 5), RMNV — Romanov (n = 3), TSHN — Tushin (n = 5), TUVA — Tuva short-fat-tailed (n = 4).

B — median-joining haplotype network, constructed for fine wool sheep breeds: BAKL — Baikal fine-fleeced (n = 3), DGMT — Dagestan Mountain (n = 4), GRZY — Groznensk (n = 5), KLND — Kulundin (n = 5), MNCM — Manych Merino (n = 5), SLSK — Salsky (n = 5), STVP — Stavropol (n = 5), SVTM — Soviet Merino (n = 3), VLGD — Volgograd (n = 5).

C — median-joining haplotype network, constructed for semi-fine wool sheep breeds: ALTM − Altai Mountain (n = 5), KBSV — Kuibyshev (n = 3), NCCS — North Caucasian (n = 5), RSLH — Russian Longhaired (n = 3), TSIG — Tsigai (n = 2).

Haplogroups: Hap A — haplogroup A, Hap B — haplogroup B, Hap C — haplogroup C, Hap D — haplogroup D. The diameter of each circle corresponds to the number of individuals belonging to a given haplotype. The number of transverse lines indicates the number of nucleotide substitutions. The black circles at network branching points indicate hypothetical haplotypes.

Most sheep of semi-fine wool breeds (Fig. 1, C) belonged to haplogroup B. Two animals of the Altai Mountain breed were clustered separately into haplogroup A.

Similar conclusions to those mentioned above were drawn based on the analysis of the Bayesian phylogenetic tree (Fig. 2). The largest number of animals was assigned to haplogroup B and two clusters, corresponding to haplogroups C and D, were separated from haplogroup A. The results of the AMOVA performed for the three groups of sheep (coarse, fine, and semi-fine wool breeds) confirmed the presence of genetic differentiation within the breeds, which corresponded to 90.55% of the variability (Table 2). The inter-breed difference was only 3.77%, and the genetic variation between groups was 5.68%.

2. The results of an analysis of molecular variance on populations of 25 Russian local breeds of domestic sheep (*Ovis aries*), based on the nucleotide sequence of the mitochondrial gene *Cytb* (n = 106, Ernst Federal Research Center for Animal Husbandry, Moscow, 2020–2021).

Source of variation	Degrees of freedom, d.f.	Sum of squares, SS	Variance components, VS	Percentage of variation, V%
Intergroup differences	2	12.052	0.11615	5.68
Interbreed differences within the group	22	47.906	0.7706	3.77
Intrabreed differences	81	150.033	1.85226	90.55
Total	105	209.991	2.04547	

Domestic sheep are a traditional and significant type of farm animal in Russia, providing for the needs of the population with food and raw materials for light industry [1]. However, previous studies that have been conducted on the genetic resources of Russian sheep populations are characterized to a greater extent by nuclear molecular genetic markers such as SNPs [51] and microsatellites [52]. In this regard, our study will serve as the basis for accumulating knowledge about maternal variability and genetic diversity based on mtDNA *Cytb* polymorphism in Russian sheep breeds.

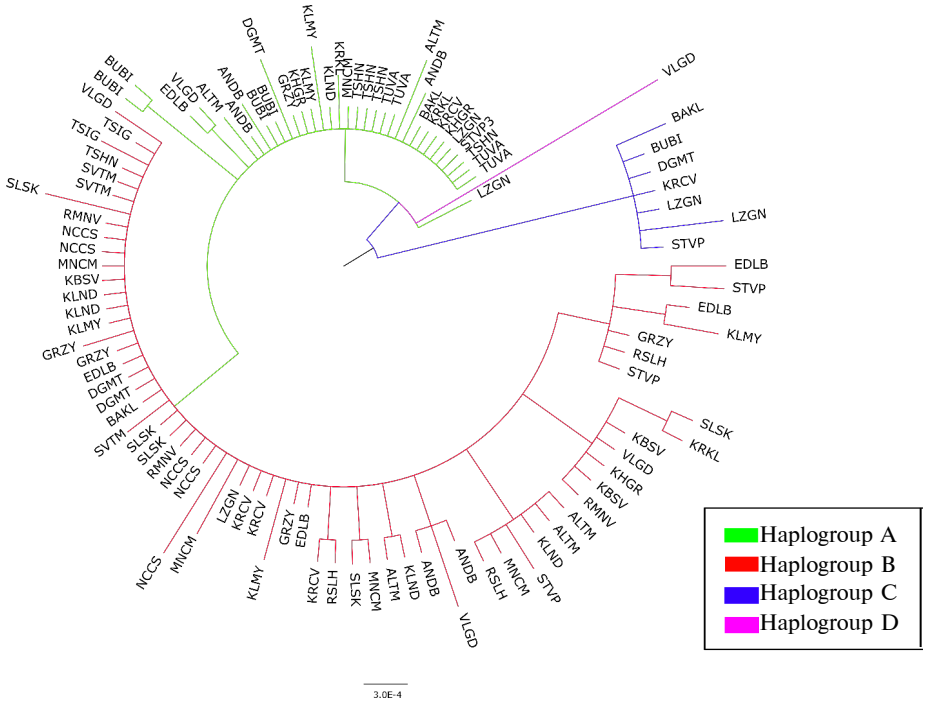


Fig. 2. Bayesian phylogenetic tree reflecting the genetic relationships of 25 Russian local breeds of

domestic sheep (*Ovis aries*) based on the nucleotide sequence of the mitochondrial gene *Cytb*: ALTM — Altai Mountain, ANDB — Andean, BAKL — Baikal fine-fleeced, BUBI — Buubei, DGMT — Dagestan Moun-tain, EDLB — Edilbaev, GRZY — Groznensk, KBSV — Kuibyshev, KHGR — Kuchugur, KLMY — Kalmyk, KLND — Kulundin, KRCV — Karachaev, KRKL — Karakul, LZGN — Lezgin, MNCM — Manych Merino, NCCS — North Cauca-sian, RMNV — Romanov, RSLH — Russian Longhaired, SLSK — Salsky, STVP — Stavropol, SVTM — Soviet Merino, TSHN — Tushin, TSIG — Tsigai, TUVA — Tuva short-fat-tailed, VLGD — Volgograd (Ernst Federal Research Center for Animal Husbandry, Moscow region, 2020–2021)

The haplotype diversity in Russian sheep populations (Hd = 0.400–1.000) was comparable to the values obtained in other studies on Tibetan (Hd = 0.464–1.000) [31] and Moroccan sheep (Hd = 0.963–0.996) [53]. Nucleotide diversity (π = 0.0000–0.00760) was slightly lower than that of Mexican (π = 0.00041–0.90000) [54] and Moroccan sheep (π = 0.01330–0.02260) [53], and close to the values obtained for Tibetan sheep (π = 0.00100–0.00600) [31]. Consequently, the genetic and nucleotide diversity of Russian sheep did not differ significantly from previously reported values, which supports the adequacy of our approach for calculating genetic indicators. According to the results of the AMOVA analysis, genetic diversity was mainly determined by intrabreed differences (90.55%). Similar results were obtained by Oliveira et al. [55], who reported that 91.54% of the genetic variation was due to intrabreed differences in Brazilian sheep raised in the state of Mato Grosso do Sul.

Four sheep haplogroups have been identified based on mtDNA nucleotide sequences: A, B, C, and D [18-21, 23]. Haplogroups B and A, which are typical for sheep of European and Asian origin, were found to be the most common among the Russian local sheep breeds. This result was expected and is consistent with the data obtained earlier by Wood et al. [19], Hiendleder et al. [20], and Meadows et al. [25]. Wood et al. [19] identified two haplogroups (A and B) in domestic sheep from New Zealand, with haplogroup A predominant in Asian populations. These haplogroups were characterized by Hiendleder et al. [20] as being of Asian (haplogroup A) and European (haplogroup B) origin because haplogroup B was prevalent among European breeds but was a minority in East Asia. Meadows et al. [25] obtained similar results, with haplogroups A and B being the most common (approximately 89%). Haplogroup A had a high frequency of occurrence (approximately 77%) in the Indian subcontinent, whereas in Europe, its frequency was less than 10%. In contrast, lineage B was mainly found in Europe, with the highest frequency (> 90%) in southwestern Europe. In our study, haplogroup C was also found in Russian local sheep breeds. Similar to earlier studies [22, 23, 26], haplogroup C was less common, and only a small number of individuals were identified in Asia and Europe. In addition, one animal from the Volgograd region clustered with haplogroup D. Tapio et al. [23] found haplogroup D in one animal of the Karachaev breed from the North Caucasus, indicating the presence of this maternal type in Russia.

Thus, our analysis of mtDNA *Cytb* polymorphism in domestic sheep showed that there is high genetic diversity in Russian sheep breeds. Four haplogroups (A, B, C, and D) were identified, which can be explained by the wide habitat of the study animals. Moreover, the diversity of the presented haplogroups, including the presence of an Asian and European phylogenetic root, could indicate that the processes of migration of domestic sheep in Eurasia, including the Russian Federation, took place in two directions.

REFERENCES

1. Chessa B., Pereira F., Arnaud F., Amorim A., Goyache F., Mainland I., Kao R.R., Pemberton J.M., Beraldi D., Stear M.J., Alberti A., Pittau M., Iannuzzi L., Banabazi M.H., Kazwala R.R., Zhang Y.P., Arranz J.J., Ali B.A., Wang Z., Uzun M., Dione M.M., Olsaker I., Holm

L.E., Saarma U., Ahmad S., Marzanov N., Eythorsdottir E., Holland M.J., Ajmone-Marsan P., Bruford M.W., Kantanen J., Spencer T.E., Palmarini M. Revealing the history of sheep domestication using retrovirus integrations. *Science*, 2009, 324(5926): 532-536 (doi: 10.1126/science.1170587).
2. 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).
3. Diamond J. Evolution, consequences and future of plant and animal domestication. *Nature*, 2002, 418(6898): 700-707 (doi: 10.1038/nature01019).
4. Sheriff O., Alemayehu K. Genetic diversity studies using microsatellite markers and their contribution in supporting sustainable sheep breeding programs: a review. *Cogent Food & Agriculture*, 2018, 4(1): 1459062 (doi: 10.1080/23311932.2018.1459062).
5. Zhong Y., Tang Z., Huang L., Wang D., Lu Z. Genetic diversity of *Procambarus clarkii* populations based on mitochondrial DNA and microsatellite markers in different areas of Guangxi, China. *Mitochondrial DNA. Part A*, 2020, 31(2): 48-56 (doi: 10.1080/24701394.2020.1721484).
6. FAO. *The state of food and agriculture: climate change, agriculture, and food security*. Food and Agriculture Organization of the United Nations, Rome, 2016.
7. 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(1): 194 (doi: 10.1186/s12864-015-1384-9).
8. Wang H., Zhang L., Cao J., Wu M., Ma X., Liu Z., Liu R., Zhao F., Wei C., Du L. Genome-wide specific selection in three domestic sheep breeds. *PLoS ONE*, 2015, 10(6): e0128688. (doi: 10.1371/journal.pone.0128688).
9. Fariello M.-I., Servin B., Tosser-Klopp G., Rupp R., Moreno C., International Sheep Genomics Consortium, San Cristobal M., Boitard S. Selection signatures in worldwide sheep populations. *PLoS ONE*, 2014, 9(8): e103813 (doi: 10.1371/journal.pone.0103813).
10. Deniskova T.E., Dotsev A.V., Fornara M.S., Sermyagin A.A., Reyer H., Wimmers K., Brem G., Zinov'eva N.A. The genomic architecture of the Russian population of Saanen goats in comparison with worldwide Saanen gene pool from five countries. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2020, 55(2): 285-294 (doi: 10.15389/agrobiology.2020.2.285eng).
11. Kostyunina O.V., Abdel'manova A.S., Martynova E.U., Zinov'eva N.A. Search for genomic regions carrying the lethal genetic variants in the Duroc pigs. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2020, 55(2): 275-284 (doi: 10.15389/agrobiology.2020.2.275eng).
12. Sulimova G.E., Stolpovskii Yu.A., Ruzina M.N., Zakharov-Gezekhus I.A. V sbornike: *Bioraznoobrazie i dinamika genofondov* [In: Biodiversity and gene pool dynamics]. Moscow, 2008: 211-214 (in Russ.).
13. Qiao G., Zhang H., Zhu S., Yuan C., Zhao H., Han M., Yue Y., Yang B. The complete mitochondrial genome sequence and phylogenetic analysis of Alpine Merino sheep (*Ovis aries*). *Mitochondrial DNA. Part B, Resources*, 2020, 5(1): 990-991 (doi: 10.1080/23802359.2020.1720536).
14. Doyle J.J., Gaut B.S. Evolution of genes and taxa: a primer. *Plant Molecular Biology*, 2000, 42(1): 1-23.
15. Knowlton N., Weigt L. New dates and new rates for divergence across the Isthmus of Panama. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 1998, 265: 2257-2263 (doi: 10.1098/rspb.1998.0568).
16. Cox A.J., Hebert P.D. Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. *Molecular Ecology*, 2001, 10(2): 371-386 (doi: 10.1046/j.1365-294x.2001.01188.x).
17. Wares J.P., Cunningham C.W. Phylogeography and historical ecology of the North Atlantic intertidal. *Evolution*, 2001, 55(12): 2455-2469 (doi: 10.1111/j.0014-3820.2001.tb00760.x).
18. Guo J., Du L.-X., Ma Y.-H., Guan W.-J., Li H.-B., Zhao Q.-J., Li X., Rao S.-Q. A novel maternal lineage revealed in sheep (*Ovis aries*). *Animal Genetics*, 2005, 36(4): 331-336 (doi: 10.1111/j.1365-2052.2005.01310.x).
19. Wood N.J., Phua S.H. Variation in the control region sequence of the sheep mitochondrial genome. *Animal Genetics*, 1996, 27(1): 25-33 (doi: 10.1111/j.1365-2052.1996.tb01173.x).
20. Hiendleder S., Mainz K., Plante Y., Lewalski H. Analysis of mitochondrial DNA indicates that domestic sheep are derived from two different ancestral maternal sources: no evidence for contributions from urial and argali sheep. *The Journal of Heredity*, 1998, 89(2): 113-120 (doi: 10.1093/jhered/89.2.113).
21. Pedrosa S., Uzun M., Arranz J.J., Gutiérrez-Gil B., San Primitivo F., Bayón Y. Evidence of three maternal lineages in Near Eastern sheep supporting multiple domestication events. *Proceedings of the Royal Society B: Biological Sciences*, 2005, 272(1577): 2211-2217 (doi: 10.1098/rspb.2005.3204).
22. Pereira F., Davis S.J., Pereira L., McEvoy B., Bradley D.G., Amorim A. Genetic signatures of a Mediterranean influence in Iberian Peninsula sheep husbandry. *Molecular Biology and Evolution*, 2006, 23(7): 1420-1426 (doi: 10.1093/molbev/msl007).
23. Tapio M., Marzanov N., Ozerov M., Cinkulov M., Gonzarenko G., Kiselyova T., Murawski

M., Viinalass H., Kantanen J. Sheep mitochondrial DNA variation in European, Caucasian, and Central Asian areas. *Molecular Biology and Evolution*, 2006, 23(9): 1776-1783 (doi: 10.1093/molbev/msl043).
24. Meadows J.R., Cemal I., Karaca O., Gootwine E., Kijas J.W. Five ovine mitochondrial lineages identified from sheep breeds of the near East. *Genetics*, 2007, 175(3): 1371-1379 (doi: 10.1534/genetics.106.068353).
25. Meadows J.R., Hiendleder S., Kijas J.W. Haplogroup relationships between domestic and wild sheep resolved using a mitogenome panel. *Heredity*, 2011, 106(4): 700-706 (doi: 10.1038/hdy.2010.122).
26. Meadows J.R., Li K., Kantanen J., Tapio M., Sipos W., Pardeshi V., Gupta V., Calvo J.H., Whan V., Norris B., Kijas J.W. Mitochondrial sequence reveals high levels of gene flow between breeds of domestic sheep from Asia and Europe. *The Journal of Heredity*, 2005, 96(5): 494-501 (doi: 10.1093/jhered/esi100).
27. 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).
28. Pardeshi V.C., Kadoo N.Y., Sainani M.N., Meadows J.R., Kijas J.W., Gupta V.S. Mitochondrial haplotypes reveal a strong genetic structure for three Indian sheep breeds. *Animal Genetics*, 2007, 38(5): 460-466 (doi: 10.1111/j.1365-2052.2007.01636.x).
29. Zhao E., Yu Q., Zhang N., Kong D., Zhao Y. Mitochondrial DNA diversity and the origin of Chinese indigenous sheep. *Tropical Animal Health and Production*, 2013, 45(8): 1715-1722 (doi: 10.1007/s11250-013-0420-5).
30. 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).
31. Liu J., Lu Z., Yuan C., Wang F., Yang B. Phylogeography and phylogenetic evolution in Tibetan sheep based on *MT-CYB* sequences. *Animals*, 2020, 10(7): 117 (doi: 10.3390/ani10071177).
32. Ul'yanov A.N., Kulikova A.Ya. *Ovtsy, kozy, sherstyanoe delo*, 2012, 1: 4-11 (in Russ.).
33. Aboneev V.V. *Dostizheniya nauki i tekhniki v APK*, 2008, 10: 37-39 (in Russ.).
34. Erokhin A.I., Karasev E.A., Erokhin S.A. *Ovtsy, kozy, sherstyanoe delo*, 2019, 3: 3-6 (in Russ.).
35. Aboneev V.V., Kolosov Yu.A. *Ovtsy, kozy, sherstyanoe delo*, 2020, 1: 43-46 (in Russ.).
36. Selionova M.I *Zhivotnovodstvo i kormoproizvodstvo*, 2019, 102(4): 272-276 (in Russ.).
37. Lescheva M., Ivolga A. Current state and perspectives of sheep breeding development in Russian modern economic condition. *Economics of Agriculture*, 2015, 2(62): 467-480.
38. Dunin I.M., Safina G.F., Chernov V.V., Grigoryan L.N., Khatataev S.A., Khmelevskaya G.N., Stepanova N.G., Pavlov M.B. V sbornike: *Ezhegodnik po plemennoi rabote v ovtsevodstve i kozovodstve v khozyaistvakh rossiiskoi federatsii (2019 god)* [In: Yearbook on breeding in sheep and goat on farms of the Russian Federation (2019)]. Moscow, 2020: 3-14 (in Russ.).
39. Ernst L.K., Dmitriev N.G., Paronyan I.A. *Geneticheskie resursy sel'skokhozyaistvennykh zhivotnykh v Rossii i sopredel'nykh stranakh* [Genetic resources of farm animals in Russia and neighboring countries]. St. Petersburg, 1994 (in Russ.).
40. Edgar R.C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 2004, 32(5): 1792-1797 (doi: 10.1093/nar/gkh340).
41. Kumar S., Stecher G., Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 2016, 33(7): 1870-1874 (doi: 10.1093/molbev/msw054).
42. Bandelt H.J., Forster P., Röhl A. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, 1999, 16(1): 37-48 (doi: 10.1093/oxfordjournals.molbev.a026036).
43. Leigh J.W., Bryant D. Popart: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 2015, 6(9): 1110-1116 (doi: 10.1111/2041-210X.12410).
44. Lanfear R., Frandsen P.B., Wright A.M., Senfeld T., Calcott B. PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*, 2017, 34(3): 772-773 (doi: 10.1093/molbev/msw260).
45. Akaike H. A new look at the statistical model identification. *IEEE Trans Auto Control*, 1974, 19(6): 716-723 (doi: 10.1109/TAC.1974.1100705).
46. Excoffier L., Lischer H.E. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 2010, 10(3): 564-567 (doi: 10.1111/j.1755-0998.2010.02847.x).
47. Ronquist F., Teslenko M., van der Mark P., Ayres D.L., Darling A., Hцhna S., Larget B., Liu L., Suchard M.A., Huelsenbeck J.P. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 2012, 61(3): 539-542 (doi: 10.1093/sysbio/sys029).

48. *Molecular evolution, phylogenetics and epidemiology*. Available: http://tree.bio.ed.ac.uk/software/figtree. Accessed: 30.07.2021.
49. Dotsev A.V., Kunz E., Shakhin A.V., Petrov S.N., Kostyunina O.V., Okhlopkov I.M., Deniskova T.E., Barbato M., Bagirov V.A., Medvedev D.G., Krebs S., Brem G., Medugorac I., Zinovieva N.A. The first complete mitochondrial genomes of snow sheep (*Ovis nivicola*) and thinhorn sheep (*Ovis dalli*) and their phylogenetic implications for the genus *Ovis*. *Mitochondrial DNA Part B*, 2019, 4(1): 1332-1333 (doi: 10.1080/23802359.2018.1535849).
50. Rozas J., Ferrer-Mata A., Sánchez-DelBarrio J.C., Guirao-Rico S., Librado P., Ramos-Onsins S.E., Sánchez-Gracia A. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution*, 2017, 34: 3299-3302 (doi: 10.1093/molbev/msx248).
51. Deniskova T.E., Abelmanova A.S., Dotsev A.V., Sambu-Khoo Ch.S., Reyer H., Selionova M.I., Fornara M.S., Wimmers K., Brem G., Zinovieva N.A. PSX-18 High-density genomic description of Russian native sheep breed of the Republic of Tyva. *Journal of Animal Science*, 2020, 98(4): 453-454 (doi: 10.1093/jas/skaa278.789).
52. Deniskova T.E., Selionova M.I., Gladyr' E.A., Dotsev A.V., Bobryshova G.T., Kostyunina O.V., Brem G., Zinov'eva N.A. Variability of microsatellites in sheep breeds raced in Russia. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2016, 51(6): 801-810 (doi: 10.15389/agrobiology.2016.6.801eng).
53. Kandoussi A., Boujenane I., Auger C., Serranito B., Germot A., Piro M., Maftah A., Badaoui B., Petit D. The origin of sheep settlement in Western Mediterranean. *Scientific Reports*, 2020, 10(1): 10225 (doi: 10.1038/s41598-020-67246-5).
54. Alonso R.A., Ulloa-Arvizu R., Gayosso-Vázquez A. Mitochondrial DNA sequence analysis of the Mexican Creole sheep (*Ovis aries*) reveals a narrow Iberian maternal origin. *Mitochondrial DNA. Part A*, 2017, 28(6): 793-800 (doi: 10.1080/24701394.2016.1192613).
55. Oliveira J.A., Egito A.A.D., Crispim B.D.A., Vargas Junior F.M., Seno L.O., Barufatti A. Importance of naturalized breeds as a base for the formation of exotic sheep (*Ovis aries*) breeds in tropical altitude regions. *Genetics and Molecular Biology*, 2020, 43(2): e20190054 (doi: 10.1590/1678-4685-GMB-2019-0054).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1148-1155
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Reproductive biotechnologies

UDC 636.2:591.391.1:57.085.23

doi: 10.15389/agrobiology.2021.6.1148eng
doi: 10.15389/agrobiology.2021.6.1148rus

THE EFFECT OF PROLACTIN ON THE QUALITY OF HEIFER OOCYTES RETRIEVED BY TRANSVAGINAL PUNCTURE OF FOLLICLES

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

Ernst Federal Science Center for Animal Husbandry, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail g_singina@mail.ru (✉ corresponding author), roman_chinarov@mail.ru, kristybatle@gmail.com, tima_voron2008@mail.ru
ORCID:
Singina G.N. orcid.org/0000-0003-0198-9757
Chinarov R.Yu. orcid.org/0000-0001-6511-5341
Lukanina V.A. orcid.org/0000-0003-4744-7873
Voroshbit T.A. orcid.org/0000-0002-2490-7489
The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Russian Foundation for Basic Research (project 20-316-90003) and the Ministry of Science and Higher Education of the Russian Federation
Received October 5, 2021

Abstract

In both dairy and beef cattle breeding, producing the larger number of the offspring from the best mothers to increase the degree of genetic progress through the generations is of particular interests. One of the attractive ways to resolve this problem is the development and implementation of the technology for obtaining embryos in vitro (in vitro embryo production, IVP) using oocytes derived from live animals by transvaginal puncture of follicles — Ovum-Pick-Up (OPU). The extracorporeal maturation of oocytes is an important element of this technology that may significantly affect its efficiency. In this paper, for the first time, we evaluated the advantage of the pituitary hormone prolactin (PRL) — a potential regulator of the quality of mammalian oocytes, during the maturation of OPU-oocytes. The effect of this hormone on the completion of nuclear maturation of the OPU-oocytes, as well as on the development and quality of IVP embryos, was studied. The mature Simmental heifers at the age from 19 to 25 months (n = 4) with a natural sexual cycle were used as the oocyte donors. Transvaginal aspiration of the follicles was performed every 4 days using the OPU system for cattle (Minitube, Germany). A total of 28 OPU sessions were carried out. The derived cumulus-oocyte complexes (COCs) were cultured in TS-199 medium supplemented with 10 % bovine fetal serum, 10 μg/ml of follicle-stimulating (FSH) and 10 μg/ml of luteinizing (LH) hormones in the absence (control) or presence of PRL. After 24 hours, the mature oocytes were subjected to fertilization to assess their developmental competence. Morphological analysis did not reveal the effect of culture conditions on the completion of nuclear maturation. The rate of mature oocytes was similar in both groups and was 82.8 and 88.9 %, respectively, in the control and the PRL groups. However, the oocyte cleavage rate after in vitro fertilization in the control group was lower comparing to the PRL group (69.7±2.4 % vs. 81.7±4.9 %, $p < 0.05$). A positive effect of the PRL on the development of mature oocytes to the blastocyst stage was observed. When COCs were cultured in the control medium, the yield of blastocysts was 11.0±1.8 %, while the adding of PRL into the IVM medium increased this indicator to 17.2±2.0 % ($p < 0.05$). However, we did not find significant differences among compared groups in the relation to the total number of nuclei in blastocysts. Thus, prolactin hormone in the maturation environment has a stimulating effect on the developmental competence of donor oocytes. The positive effect is observed at the stage of the first cleavage and maintained during the development of embryos to the blastocyst stage. Our data indicate the positive effect of prolactin on the quality of OPU-oocytes, that makes it reasonable the using this hormone at the maturation stage to increase the effectiveness of IVP technology.

Keywords: cattle, transvaginal aspiration of follicles, in vitro oocyte maturation, prolactin, embryonic development

Obtaining the largest number of calves from the best mothers for a more complete realization of their genetic potential in generations remains a challenge for both dairy and beef animal husbandry. The development and practical application

of in vitro embryo production (IVP) using eggs from live animals through transvaginal follicle puncture (ovum-pick-up, OPU) can address this urgent problem [1, 2]. It has been shown that OPU is the most flexible and reproducible method for obtaining embryos from living donors. Unlike multiple ovulation and embryo transfer, OPU does not interfere with the normal reproduction and production cycle of the donor. Any female between the ages of 6 months and the 3rd month of pregnancy and soon after calving (in 2-3 weeks) can be a suitable donor [2]. OPU has been now recognized as good alternative to the traditional in vivo embryo production program [3, 4] and is increasingly used commercially worldwide [5-7].

As is known, the efficiency of the IVP technology depends not only on the quality of the initial population of gametes isolated from the ovaries [8, 9], but also on the environmental impact that oocytes are exposed to in vitro [10, 11]. The maturation of oocytes is the most important stage of culture. By means of its modeling, it is possible to significantly increase both quantitative (the proportion of embryos at the blastocyst stage) and qualitative (the usefulness of blastocysts) indicators of the effectiveness of the IVP method [12]. The vast majority of modern research is focused primarily on the search for physiologically relevant substances (growth factors, hormones, steroids, fatty acids, amino acids, metabolites) that can specifically affect oocytes, increasing or maintaining their viability and developmental competence, as well as identifying mechanisms underlying such influence [13-16].

To date, it has been established that the pituitary hormone prolactin (PRL) affects the ovarian function of females and can positively modulate the maturation of oocytes and their ability for embryonic development [17-20]. Receptors for this hormone or its mRNA have been found in oocytes and associated cumulus cells of various mammalian species, including cows [20-23]. In in vitro conditions, the addition of PRL to the medium for maturation of post mortem bovine oocytes positively affects their nuclear maturation and quality and increases competence for further embryonic development [20, 24, 25]. In prolonging oocyte in vitro culture it has been shown that PRL inhibits destructive changes in the morphology of metaphase chromosomes and reduces the frequency of apoptotic degeneration of senescent oocytes of this species [26, 27]. In addition, prolactin increases the competence of mature oocytes for further embryonic development, which decreases with aging [27]. In general, prolactin is deemed a potential regulator of the female germ cells capable of increasing their quality under in vitro conditions.

In this work, we have for the first time revealed the positive effect of the pituitary hormone prolactin on the quality of donor OPU oocytes of cows during their maturation in vitro.

The aim of the work was to assess the effect of prolactin on the completion of nuclear maturation by oocytes obtained by transvaginal follicle puncture and on the development and quality of embryos after in vitro fertilization of donor oocytes.

Materials and methods. In all experiments, except for separately indicated cases, reagents from Sigma-Aldrich (USA) were used.

Oocytes were collected from mature heifers (*Bos taurus taurus*) of the Simmental breed aged from 19 to 25 months (n = 4) with a natural sexual cycle. Follicle puncture was performed every 4 days using an OPU system for cattle (Minitube, Germany), which included an SSD Pro Sound 2 ultrasound scanner, a convection sector probe, a vacuum pump, and a probe holder. Aspiration of all visible follicles was performed with a needle (1.2 mm in diameter and 75 mm long) connected with a silicone hose to a 50 ml vial. Phosphate buffered saline (PBS) supplemented with 10% bovine fetal serum (BFS), 18 IU/ml heparin, and 50 mg/ml gentamicin was used as aspiration fluid. Aspirates from each donor were

filtered individually, washed with PSB supplemented with 1% BFSPBS. Cumulus-oocyte complexes (COCs) were searched and evaluated under a stereomicroscope (Nikon, Japan). The isolated COCs were divided into those suitable for in vitro culture, including oocytes lacking cumulus cells, and those with obvious cytoplasmic abnormalities not suitable for in vitro culture. Selected COCs were incubated for 24 h to mature in TS-199 medium supplemented with 10% bovine fetal serum (BFS), 10 μg/ml follicle-stimulating hormone (FSH) and 10 μg/ml luteinizing hormone (LH) in the absence (control) or presence of PRL (50 ng /ml) (experiment).

Mature oocytes were fertilized to assess competence for embryonic development. COCs were washed once in BO-IVF fertilization medium (IVF Bioscience, UK) and placed in drops of the same medium 30 min before contact with spermatozoa.

Oocytes were fertilized using frozen-thawed semen from one Simmental bull. Straws with frozen sperm were thawed 1.5 hours before fertilization, and active spermatozoa were obtained by the swim-up method [28] using Sperm-TALP medium containing 1 mM sodium pyruvate, 6 mg/ml BSA [27]. The contents of the straws were layered with 220 μl in 1.8 ml tubes (Nunc, Denmark) containing 1 ml of Sperm-TALP medium and placed in an incubator (MCO-18AIC, Sanyo, Japan) for 50 min. At the end of the incubation, 750 μl of the upper layer was taken from the tubes, diluted with fresh medium and centrifuged (a centrifuge 3-30KS, Sigma, Germany) at 300 g for 7 min. The resulting sediment containing motile spermatozoa was introduced into the fertilization medium (BO-IVF) with previously transferred COCs to a final concentration of 1.5×10^6 spermatozoa per 1 ml.

COCs were maturated and fertilized in 4-well plates (Biomedical, Russia) in drops of 90 μl medium completely covered with light mineral oil.

After 16-18 h co-incubation with sperm, oocytes were carefully pipetted and washed in CR1aa medium [29] to remove cumulus cells and adhering spermatozoa. Simultaneously, morphology of isolated oocytes was assessed, the oocytes with target bodies (first or first and second) were counted and the percentage of maturation was determed. Putative zygotes (regardless of the presence or absence of polar bodies) were transferred to the CR1aa medium and cultured for 4.5 days. The developing embryos were placed in the same medium containing 5% BFS.

Embryos were developed in 4-well plates (Nunc, Denmark) in 90 μl of medium completely covered with light mineral oil. On day 2 after the fertilization of the oocytes, the morphological assessment of the fragmented zygotes was carried out; on day 7, the number of embryos that had developed to the blastocyst stage was determined. The evaluation was performed under an SMZ stereomicroscope (Nikon, Japan) at a magnification of ×40-60.

The oocyte maturation and fertilization, as well as the embryo culture occurred in a 5% CO_2 atmosphere at 38.5 °C and 90% humidity.

Embryos obtained on day 7 were fixed with 4% paraformaldehyde solution (60 min), permeabilized in 0.1% sodium citrate solution containing 0.5% Triton X-100 (30 min) and stained with DAPI to localize the nuclei (20 min). Embryos treated in this way were transferred to a glass slide and placed in Vectashield medium (Vector Laboratories, UK). Microphotography and evaluation of preparations were performed under an Axio Imager.M2 microscope (Carl Zeiss, Germany) using the ZEN 2 pro program (Carl Zeiss, Germany).

Statistical processing was performed by one-way analysis of variance using the SigmaStat program (Systat Software, Inc., USA). The data were expressed as means (M) and standard errors of the means (±SEM). The significance of differences between the compared mean values was assessed using Tukey's test.

Results. To date, significant progress has been made in the development of IVP technology in cattle using donor oocytes, however, the usefulness of embryos developed in vitro from OPU oocytes still remains significantly lower than those developed in vivo [5-8]. The identification of biologically relevant factors responsible for the regulation of oocyte quality during their in vitro maturation will contribute to solving this problem [12-13, 16].

Since PRL positively modulates post mortem maturation of bovine oocytes and their ability for embryonic development [20, 24, 25], it is likely that this hormone may similarly affect donor oocytes. In the present work, oocytes obtained by transvaginal follicle aspiration were cultured in the presence or absence of PRL (50 ng/ml), followed by fertilization in vitro and cultured to the blastocyst stage. The effect of PRL on the completion of nuclear maturation by OPU oocytes, as well as on the development and quality of IVP embryos, was evaluated.

A total of 360 follicles were aspirated from four Simmental heifers during 28 OPU sessions, of which 166 COCs were isolated. The number of oocytes isolated from individual donors (1 OPU session) averaged 5.9. COCs (Fig. 1, a) obtained in the OPU session, except for oocytes with obvious cytoplasmic abnormalities (total 140 COCs, 5.0 per 1 OPU session), were cultured in IVM medium until maturation was completed (see Fig. 1, b) without (control) or with PRL.

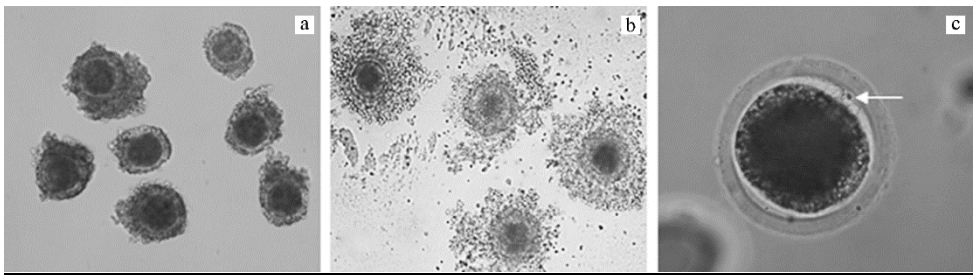


Fig. 1. Micrographs of oocytes of Simmental heifers collected by transvaginal follicle puncture: a — immature cumulus-oocyte complexes (magnification ×100), b — cumulus-oocyte complexes after 24 hours of maturation in vitro (magnification ×100), c — mature oocytes after the fertilization procedure in vitro (white arrow indicates polar bodies, magnification ×400) (microscope Eclipse Ti-U, Nikon, Japan).

Morphological analysis did not reveal the effect of prolactin on the completion of nuclear maturation. The proportion of mature oocytes as the ratio of oocytes with polar bodies (see Fig. 1, c) to the initial oocyte number determined after the IVF procedure when oocytes were separated from cumulus cells and spermatozoa, was high and did not differ significantly between the control and experimental groups (Table 1).

1. The competence of oocytes collected by transvaginal puncture of follicles of Simmental heifers and maturing in the presence of prolactin to embryonic development **after in vitro fertilization** (M±SEM)

Group	Oocytes, n	Mature oocytes, %	Cleaved oocytes, %	Oocytes developed to the blastocyst stage, %
Control	80	82.8±3.8	69.7±2.4	11.0±1.8
Experimental	60	88.9±3.9	81.7±4.9*	17.2±2.0*

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

The competence of mature oocytes to development after in vitro fertilization was assessed by their ability to enter the first cleavage division (Fig. 2, a) and reach the blastocyst stage (Fig. 2, b, Table 1). On day 2, the proportion of cleaved oocytes after in vitro fertilization in the control was lower than in the experiment ($p < 0.05$). A positive effect of the hormone on the development of

mature oocytes up to the blastocyst stage also occurred (see Table 1). In general, the number of blastocysts per OPU session in the experimental group was 1.5 times higher than in the control (Table 2).

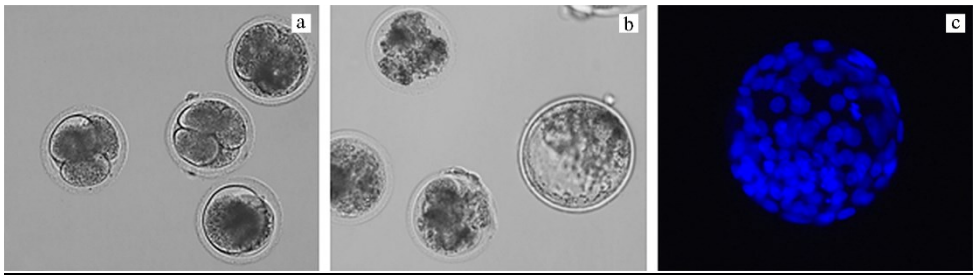


Fig. 2. Micrographs of embryos developed after in vitro fertilization of donor Simmental heifer oocytes collected by transvaginal follicle aspiration: a — cleaved oocytes (×200 magnification), b — embryos developed to the blastocyst stage (×100 magnification) (Eclipse Ti- U microscope, Nikon, Japan); c — staining of nuclei in the blastocyst with DAPI (blue color, cytological preparation, magnification ×400; microscope Axio Imager.M2, Carl Zeiss, Germany).

Prolactin did not significantly change the quality of the IVP embryos, which was assessed by the number of nuclei on day 7 after fertilization (see Fig. 2, c), however, this parameter tended to increase upon maturation of OPU oocytes in the presence of PRL (see Table 2).

2. Efficiency of IVP (in vitro embryo production) **technology and quality of IVP embryos from oocytes collected by transvaginal aspiration of follicles of Simmental heifers and matured in the presence of prolactin (*M*±SEM)**

Group	OPU sessions, *n*	Embryos at the blastocyst stage, *n*	Blastocysts per OPU session, *n*	Nuclei per blastocyst, *n*
Control	16	9	0.59±0.11	67.3±3.0
Experimental	12	10	0.86±0.09	78.6±5.2

N o t e. OPU — ovum-pick-up. For a description of the groups, see the Materials and methods section.

The stimulatory effect of PRL during IVM on the development of fertilized oocytes up to the blastocyst stage was previously reported for rabbit and mouse [30, 19]. A similar effect of PRL was also observed in the co-culture of post mortem bovine COCs with granulosa cells [25] and in the presence of gonadotropic hormones [20]. In the latter case, the addition of prolactin to the COCs culture medium containing FSH and LH led to an increase in the yield of embryos from the total number of oocytes fertilized in vitro, a 2-fold increase in the yield of blastocysts, and an increase in the average number of nuclei per blastocyst. In our study, the addition of PRL to the INM medium with FSH and LH, although it had a similar effect on the oocyte ability to develop in vitro, did not provide such a significant yield of blastocysts and a statistically significant change in the number of their nuclear material.

As is known, cumulus cells are involved in maintaining normal maturation and fertilization of mammalian oocytes [31]. In addition, the presence of cumulus cells ensures the positive effect of PRL on the embryonic development of post mortem oocytes described above [20]. Post mortem oocytes, unlike donor oocytes, are carefully selected prior to in vitro culture by morphological features, in particular, by the presence of a compact multilayer cumulus. In this work, we used for culture not only morphologically normal COCs but also oocytes partially enclosed by cumulus cells, as well as practically devoid of cumulus cells. Selection criteria may have influenced the nature of the revealed positive effect of the studied hormone.

Thus, the pituitary hormone prolactin added to the maturation medium stimulates the competence of donor bovine oocytes collected by transvaginal

follicle puncture to further embryonic development. The beneficial effect occurs at the first cleavage division and persists during the development of embryos up to the blastocyst stage. This indicates a positive influence of the hormone on the oocyte quality and the possibility to use prolactin to increase the effectiveness of the IVP technology at the stage of extracorporeal oocyte maturation.

REFERENCES

1. Boni R. Ovum pick-up in cattle: a 25 years retrospective analysis. *Animal Reproduction*, 2012, 9(3): 362-369.
2. Qi M., Yao Y., Ma H., Wang J., Zhao X., Liu L., Tang X., Zhang L., Zhang S., Sun F. Transvaginal ultrasoundguided ovum pick-up (OPU) in cattle. *Journal of Biomimetics Biomaterials and Tissue Engineering*, 2013, 18: 118.
3. Sanches B.V., Zangirolamo A.F., Seneda M.M. Intensive use of IVF by large-scale dairy programs. *Animal Reproduction*, 2019, 16(3): 394-401 (doi: 10.21451/1984-3143-AR2019-0058).
4. Viana J. 2019 Statistics of embryo production and transfer in domestic farm animals. *Embryo Technology Newsletter*, 2020, 38(4): 7-26.
5. Sirard M.A. 40 years of bovine IVF in the new genomic selection context. *Reproduction*, 2018, 156(1): R1-R7 (doi: 10.1530/REP-18-0008).
6. van Wagtendonk-de Leeuw A.M. Ovum pick up and in vitro production in the bovine after use in several generations: a 2005 status. *Theriogenology*, 2006, 65(5): 914-925 (doi: 10.1016/j.theriogenology.2005.09.007).
7. Baldassarre H., Bordignon V. Laparoscopic ovum pick-up for in vitro embryo production from dairy bovine and buffalo calves. *Animal Reproduction*, 2018, 15(3): 191-196 (doi: 10.21451/1984-3143-AR2018-0057).
8. Aguila L., Treulen F., Therrien J., Felmer R., Valdivia M., Smith L.C. Oocyte selection for in vitro embryo production in bovine species: noninvasive approaches for new challenges of oocyte competence. *Animals*, 2020, 10(12): 2196 (doi: 10.3390/ani10122196).
9. Saini N., Singh M.K., Shah S.M., Singh K.P., Kaushik R., Manik R.S., Singla S.K., Palta P., Chauhan M.S. Developmental competence of different quality bovine oocytes retrieved through ovum pick-up following in vitro maturation and fertilization. *Animal*, 2015, 9(12): 1979-85 (doi: 10.1017/S1751731115001226).
10. Stroebech L., Mazzoni G., Pedersen H.S., Freude K.K., Kadarmideen H.N., Callesen H., Hyttel P. In vitro production of bovine embryos: revisiting oocyte development and application of systems biology. *Animal Reproduction*, 2015, 12(3): 465-472.
11. Gilchrist R.B., Thompson J.G. Oocyte maturation: emerging concepts and technologies to improve developmental potential in vitro. *Theriogenology*, 2007, 67(1): 6-15 (doi: 10.1016/j.theriogenology.2006.09.027).
12. Blanco M.R., Demyda S., Moreno Millán M., Genero E. Developmental competence of in vivo and in vitro matured oocytes: a review. *Biotechnology and Molecular Biology Reviews*, 2011, 6(7): 155-165 (doi: 10.5897/BMBR2011.0015).
13. Lonergan P., Fair T., Forde N., Rizos D. Embryo development in dairy cattle. *Theriogenology*, 2016, 86(1): 270-277 (doi: 10.1016/j.theriogenology.2016.04.040).
14. Lonergan P., Fair T. Maturation of oocytes in vitro. *Annual Review of Animal Biosciences*, 2016, 4: 255-268 (doi: 10.1146/annurev-animal-022114-110822).
15. 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(12): 737-756 (doi: 10.3923/ajava.2016.737.756).
16. Ferré L.B., Kjelland M.E., Strøbech L.B., Hyttel P., Mermillod P., Ross P.J. Review: Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. *Animal*, 2020, 14(5): 991-1004 (doi: 10.1017/S1751731119002775).
17. Wise T., Suss U., Stranzinger G., Wuthrich K., Maurer R.R. Cumulus and oocyte maturation and in vitro and in vivo fertilization of oocytes in relation to follicular steroids, prolactin, and glycosaminoglycans throughout the estrous period in superovulated heifers with a normal LH surge, no detectable LH surge, and progestin inhibition of LH surge. *Domestic Animal Endocrinology*, 1994, 11(1): 59-86 (doi: 10.1016/0739-7240(94)90036-1).
18. Jinno M., Katsumata Y., Hoshiai T., Nakamura Y., Matsumoto K., Yoshimura Y. A therapeutic role of prolactin supplementation in ovarian stimulation for in vitro fertilization: the bromocriptine-rebound method. *The Journal of Clinical Endocrinology and Metabolism*, 1997, 82(11): 3603-3611 (doi: 10.1210/jcem.82.11.4349).
19. Bole-Feysot C., Goffin V., Edery M., Binart N., Kelly P.A. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocrine Reviews*, 1998, 19(3): 225-268 (doi: 10.1210/edrv.19.3.0334).

20. Lebedeva I.Y., Singina G.N., Volkova N.A., Vejlsted M., Zinovieva N.A., Schmidt M. Prolactin affects bovine oocytes through direct and cumulus-mediated pathways. *Theriogenology*, 2014, 82(8): 1154-1164.e1 (doi: 10.1016/j.theriogenology.2014.08.005).
21. Picazo R.A., García Ruiz J.P., Santiago Moreno J., González de Bulnes A., Muñoz J., Silván G., Lorenzo P.L., Illera J.C. Cellular localization and changes in expression of prolactin receptor isoforms in sheep ovary throughout the estrous cycle. *Reproduction*, 2004, 128(5): 545-553 (doi: 10.1530/rep.1.00343).
22. Nakamura E., Otsuka F., Inagaki K., Miyoshi T., Yamanaka R., Tsukamoto N., Suzuki J., Ogura T., Makino H. A novel antagonistic effect of the bone morphogenetic protein system on prolactin actions in regulating steroidogenesis by granulosa cells. *Endocrinology*, 2010, 151(11): 5506-5518 (doi: 10.1210/en.2010-0265).
23. Kiapekou E., Loutradis D., Patsoula E., Koussidis G.A., Minas V., Bletsa R., Antsaklis A., Michalas S., Makrigiannakis A. Prolactin receptor mRNA expression in oocytes and preimplantation mouse embryos. *Reproductive BioMedicine Online*, 2005, 10(3): 339–346 (doi: 10.1016/s1472-6483(10)61793-2).
24. Kuzmina T.I., Lebedeva I.Y., Torner H., Alm H., Denisenko V.Y. Effects of prolactin on intracellular stored calcium in the course of bovine oocyte maturation in vitro. *Theriogenology*, 1999, 51(7): 1363-1374 (doi: 10.1016/S0093-691X(99)00080-1).
25. Kuz'mina T.I., Lebedeva I.Yu., Torner Kh., Al'm Kh. *Ontogenez*, 2001, 32(2): 140-147 (in Russ.).
26. Lebedeva I.Y., Singina G.N., Lopukhov A.B., Shedova E.N., Zinovieva N.A. Prolactin and growth hormone affect metaphase II chromosomes in aging oocytes via cumulus cells using similar signaling pathways. *Frontiers in Genetics*, 2015, 6: 274 (doi: 10.3389/fgene.2015.00274).
27. Singina G.N., Shedova E.N., Lopukhov A.V., Mityashova O.S., Lebedeva I.Y. Delaying effects of prolactin and growth hormone on aging processes in bovine oocytes matured in vitro. *Pharmaceuticals*, 2021, 14(7): 684 (doi: 10.3390/ph14070684).
28. Parrish J.J., Susko-Parrish J.L., Leibfried-Rutledge M.L., Critser E.S., Eyestone W.H., First N.L. Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology*, 1986, 25(4): 591-600 (doi: 10.1016/0093-691X(86)90143-3).
29. Rosenkrans C.F. Jr., First N.L. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. *Journal of Animal Science*, 1994, 72(2): 434-437 (doi: 10.2527/1994.722434x).
30. Yoshimura Y., Hosoi Y., Iritani A., Nakamura Y., Atlas S.J., Wallach E.E. Developmental potential of rabbit oocytes matured in vitro: the possible contribution of prolactin. *Biology of Reproduction*, 1989, 41(1): 26-33 (doi: 10.1095/BIOLREPROD41.1.26).
31. Tanghe S., Van Soom A., Nauwynck H., Coryn M., de Kruif A. Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Molecular Reproduction and Development*, 2002, 61(3): 414-424 (doi: 10.1002/mrd.10102).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1156-1171
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Feed additives

UDC 636.4:636.084:591.1

doi: 10.15389/agrobiology.2021.6.1156eng
doi: 10.15389/agrobiology.2021.6.1156rus

THE EFFECT OF DIHYDROQUERCETIN ON THE GROWTH AND USE OF FEED BY PIGS (*Sus scrofa domesticus* Erxleben, 1777) UNDER MODERATE HEAT STRESS

R.V. NEKRASOV[1] ✉, M.G. CHABAEV[1], E.Yu. TSIS[1], N.V. BOGOLYUBOVA[1], A.A. SEMENOVA[1, 2]

[1]*Ernst Federal Research Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail nek_roman@mail.ru (✉ corresponding author), chabaev.m.g-1@mail.ru, tsis-elen@yandex.ru, 652202@mail.ru;
[2]*Gorbatov Federal Center for Food Systems RAS,* 26, ul. Talalikhina, Moscow, 109316 Russia, e-mail semmm@mail.ru
ORCID:
Nekrasov R.V. orcid.org/0000-0003-4242-2239
Chabaev M.G. orcid.org/0000-0003-1889-6063
Tsis E.Yu. orcid.org/0000-0003-1988-1189
Bogolyubova N.V. orcid.org/0000-0002-0520-7022
Semenova A.A. orcid.org/0000-0002-4372-6448
The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation, project No. 19-16-00068
Received July 15, 2021

Abstract

Crossbred pigs are characterized by nervous instability, limited thermoregulation, and susceptibility to stress. Climate stress causes behavioral, physiological, functional, productive changes in farm animals. The aim of the research was to assess the influence of a moderate climatic stress factor (an increase in ambient temperature) on feeding, the digestibility of nutrients and productivity of intensively growing young pigs fed with dihydroquercetin (DHQ) during different periods of rearing and fattening (the physiological yard of the Ernst Federal Research Center for Animal Husbandry, 2020). For groups of crossbred boars F_2 (Large White × Landrace) × Duroc, $N = 36$) were subjected to moderate heat stress (4-6 °C above the optimum). Control animals (group 1, $n = 9$) fed a basal diet (BD), group 2 ($n = 9$) received BD + adaptogen dihydroquercetin (DHQ) during the rearing period, group 3 ($n = 9$) during the rearing and fattening, and group 4 ($n = 9$) during periods of technological stress (7 days after transportation, after transferring to other feeds, and before slaughter). The adaptogen we used as dietary supplement was Ecostimul-2 (LLC Ametis, Russia; 45 mg/kg of feed, or 32 mg DHQ/kg of feed). Moderate heat stress during feeding period (weeks 12-15 of the experiment) led to a significant increase in air concentration of ammonia up to 16.7 mg/m^3, hydrogen sulfide up to 1.67 mg/m^3, and carbon dioxide up to 0.14 mg/m^3. The blood cortisol level was 291.60 nmol/l in control group 1 (or 23.0 % above the upper value of reference limits of 41-237 nmol/l), 299.89 nmol/l in group 4, and 210 nmol/l ($p > 0.05$) in groups 2 and 3. At slaughter, the cortisol level was the highest in the control animals (284.77 nmol/l) while feeding DHQ in groups 3 and 4 decrease it to 234-253 nmol/l. Adverse external stimuli increased the mortality in the control to 11 % vs. 0 % in other groups. The animals were weighed weekly, and the average daily weight gain was assessed for each of the periods as compared to control with regard to environmental factors (microclimate parameters) and technology elements (change of feed, vaccination, etc.). During the growing period, the weight gain in all groups with DHQ were 1.5-1.7 % greater than in control group 1 (week 1, group 3, $p < 0.05$) that indicates better adaptation after transportation. Our study showed a significant increase in the average daily weight gain in certain periods of co-action of moderate heat stress and other stress factors, e.g., during vaccination (week 8, vaccination against classic swine fever, group 2 at $0.05 < p \le 0.1$; groups 3 and 4 at $p < 0.05$). Over the experiment (growing and fattening periods), the largest average daily weight gain was in group 4 which received 32 mg/kg DHQ during technological stress, the difference with the control was 13.6 % ($p > 0.05$). In group 3 (32 mg/kg DHQ during the final fattening), there was a trend towards an increase in gross growth (by 6.2 %, $0.05 < p \le 0.1$) compared to control. The balance test during the final fattening revealed a tendency to higher digestibility of dry matter in groups 3 and 4 (by 1.31 and 0.93 %, respectively; $0.05 < p \le 0.1$). In the groups received DHQ, the nitrogen excretion with urine was lower (by 21.20, 14.47, and 21.91 g in groups 2, 3, and 4, respectively) compared to control group 1 ($p = 0.18$-0.37). Thus, dietary DHQ contributed to the retention and more efficient use of nitrogen by growing young pigs. With DHQ, excretion of calcium

in the feces was also lower (by 3.48 g, $p < 0.05$; 1.68 g, $p > 0.05$; 2.87 g, $p = 0.06$) while its deposition in the body of growing young pigs was higher (by 3.52 g, $p < 0.05$; 1.62 g, $p > 0.05$; 2.85 g, $p = 0.06$) in groups 2, 3, and 4, respectively. Calcium utilization was 9.82 % higher ($p < 0.05$) in the animals of groups 2, 3, and 4. Thus, the control animals were more susceptible to the heat stress and had worse growth parameters, nutrient utilization, and higher mortality. Dietary DHQ applied during pig growing and fattening improves adaptive abilities of animals resulting in their better growth and productive performance.

Keywords: adaptogen, dihydroquercetin, stress, young pigs, productivity, average daily live weight gain, digestibility

To increase the efficiency of pig raising, it is necessary to ensure the optimal microclimate in the premises (temperature and humidity, the concentration of harmful gases, air exchange). A proper microclimate positively influences the physiological state of animals. Conversely, an uncontrolled microclimate or underestimation of the effect of stressors of various strengths and degrees of impact on the animals weakens their resistance, which leads to the emergence and development of diseases of various etiologies, impairs productivity [1], reproductive ability, and causes a number of other undesirable consequences [2], including the decline in pork quality [3].

The physical state and chemical composition of the air environment are fickle factors and are subject to large fluctuations. The animal organism can adapt to these changes, but only up to certain limits. In particular, in order to maintain normal vitality, animals must expend a certain amount of nutrients to generate heat, which is necessary for metabolism [4]. The more the body spends energy materials to adapt to environmental conditions, the less nutrients will be used to ensure productivity [5].

The air environment, which determines the state of the microclimate of closed livestock buildings, affects heat exchange, gas exchange, physical and chemical properties of blood, body and skin temperature, and other indicators [6]. The body reacts to any impact of the environment with a multilevel physiological and biochemical reaction, which causes the development of stress and then, as a consequence, adaptation. The damaging effect of the consequences of stress is due to an excessive increase in the adaptive lipotropic effect, which increases the activity of phospholipases and the intensity of free-radical lipid oxidation through catecholamines and protein kinases. Stress effects lead to the restructuring of metabolism and some physiological functions, which initially increases the stability of the animal organism [7]. However, prolonged exposure to stress depletes the internal defense systems, which ultimately affects the health of animals, their resistance to diseases, productivity and safety [8]. The ambient temperature has a significant impact on the physiological changes in the body and the productivity of pigs, while the effect of air humidity on these indicators is less pronounced. In studies on animals kept under conditions of complex exposure to environmental factors, significant violations of immune reactivity were revealed. It was established [9] that in 60-kilogram pigs the upper critical temperatures for such important physiological reactions as respiratory rate, heat production, rectal temperature were within the range of 21.3-22.4 °C, 22.9-25.5 °C and 24.6-27.1 °C, respectively, depending on the change in relative air humidity from 50 to 80%.

Physiological balance under microclimatic stressors is maintained as long as the action of external stimuli does not exceed the adaptive capacity of the body [10, 11]. The consequences of the manifestation of climatic stress and its duration depend largely on the composition of the diet, the system of housing and watering, the density of animals in the pig breeding complex, microclimate conditions - relative humidity, air velocity and its composition [12].

The trend towards intensification of animal husbandry is likely to continue, and the problem of heat stress is likely to be exacerbated by global warming and climate change. The development of methods for preventing and eliminating the negative consequences of stress, in particular heat stress, is undoubtedly an important tool for increasing the productivity of animal husbandry [1, 13]. During the construction of large pig-breeding complexes, there will be a need for more precise control of all factors affecting production efficiency. It is necessary to identify and study the possibilities to level the consequences of abiotic stresses, including through the use of feed adaptogens. In this regard, a promising solution may be the use of natural and synthetic bioactive substances with antioxidant properties, which reduce the effect of stressors on homeostasis by stabilizing free radical oxidation and increasing the adaptive properties of the body [14, 15]. It has been reported that the use of dihydroquercetin (DHQ) helps to reduce lipid peroxidation, reducing the negative effects on the body of pigs of the effects of transport and feed stress [16, 17].

In the present work, we have shown that the feeding of natural bioflavonoid dihydroquercetin as an additional prophylactic feed component can favorably affect the safety and productivity of intensively growing pigs, contributing to their better adaptation to feeding and housing conditions.

The purpose of this study was to assess the effect of the adaptogen dihydroquercetin, fed to pigs at different periods of growing and fattening, on productivity and nutrient use under conditions of moderately pronounced heat stress with accompanying technological stresses (transportation, switching to another feed recipe, vaccination and slaughter).

Materials and methods. Physiological studies were carried out on 36 hybrid boletus (*Sus scrofa domesticus*) F_2 [(large white × Landrace) × Duroc] with a live weight at the beginning of the experiment of 17.20-17.43 kg at the age of 58 days (physiological yard of the Ernst FRC VIZh, 2020). The duration of the experiment was 120 days.

Young hogs purchased from Verkhnyaya Khava (Voronezh Province) were delivered by special vehicles for animal transport (transportation at a distance of 500 km) in compliance with transportation standards and the necessary veterinary and sanitary control. After delivery, the animals were divided into four groups, which were kept under the same conditions.

In a comparative study, groups 2, 3 and 4 (n = 9 each) received DHQ in addition to the diet (Ecostimul-2 preparation, Ametis JSC, Russia; dosage 45 mg/kg of feed, or 32 mg DHQ/kg feed as per active substance), in group 2 (n = 9) only during the growing period (DHQd), in group 3 (n = 9) during growing and fattening (DHQd+o), in group 4 (n = 9) during feed and technological stresses (DHQtech) (7 days after transportation, 7 days before and 7 days after switching to another type of compound feed, and also not less than 7 days before slaughter). Group 1 (n = 9) fed the basal diet without DHQ was used as a control.

The fattening pigsty for 48 posts complied with the standards for keeping animals (GOST 12.1.005 and MR for technological design) during growing and fattening. Feeding occurred 2 times a day from group feeders with dividers for individual feeding (1.5×2 m pens, 1×1.5 m rubber mat; 3 pigs per pen during growing to slaughter, 0.8 m^2 per head with an actual area of 3 m^2). Teat drinkers were located in the corner of the machine directly in front of the manure removal channel, the animals had constant access to water; dry compound feeds were moistened directly during distribution. The basal components of the diet were SK-4 (during growing), SK-5 (during the 1st fattening period) and SK-6 (during the

final fattening) (the manufacturer of the compound feed is Agrovitex LLC, Russia). The feeds were balanced in terms of nutritional substances and energy according to modern norms and the recommended feeding regimen [18].

In the premises, as per veterinary and sanitary requirements, cleaning with the removal of manure was performed twice a day. To control the mode of the simulated environment, the temperature and relative humidity of the air in the pigsty were measured (at 16:00) using a stationary electronic weather station. Using infrared lamps in each machine, in the warm period when the outdoor air temperature was above 10 °C, the indoor temperature was increased by 5 °C relative to the calculated summer outdoor temperature (up to 26-28 °C the most) to simulate stress conditions.

The temperature-humidity index (THI) was calculated for the entire period of the experiment based on the records of the electronic weather station [19]:

$$THI = (0.8 \times t) + [(\varphi/100) \times (t - 14.4)] + 46.4,$$

where t is the dry bulb temperature, °C; φ is relative air humidity, %.

The volume fraction of methane (%vol.f.), mass concentration (mg/m^3) of carbon dioxide, ammonia, hydrogen sulfide and methane in the air of the working area was measured (a multicomponent gas analyzer MAG-6, MAG-6 P-K, EXIS JSC, Russia).

Animals were individually weighed (a REUS-300 electronic balance, OOO Tenzosila, Russia) before the start of the experiment and every 7 days until its completion. Based on the weighing and assessment of feed consumption, gross, average daily weight gains and feed costs per unit of gain were calculated.

In animals from all groups ($N = 20$, $n = 5$), blood was taken from the jugular vein at the end of rearing, in the middle of fattening period and before the end of the experiment. Blood concentration of cortisol was measured by the enzyme immunoassay method (an automatic microplate photometer Immunochem-2100, High Technology, Inc., USA; reagent kits X-3964 Cortisol-IFA-BEST, Vector-Best, Russia; sensitivity 5 nmol/l, measurement range 0-1200 nmol/l).

To determine the digestibility of nutrients in the diet and to study the metabolism of nitrogen and minerals in intensively growing young pigs at the end of the final fattening period, a balance experiment was carried out ($N = 12$, groups of $n = 3$) as per common standard methods [20, 21]. All animals ($N = 12$) during the balance experiment were kept in special individual cages to record feed consumption and the amount of excrements. Recording was carried out for 5 days, after which average samples were taken for chemical analysis using standard methods.

Statistical analysis was performed using the STATISTICA package, version 10.0 (StatSoft, Inc., USA). Quantitative data are presented as the arithmetic mean (M) and standard error of the mean (±SEM). Identification of the relationship of the studied factor with the indicators of nutrient digestibility, nitrogen retention, blood parameters was performed on a sample of animals using one-way analysis of variance (ANOVA) with Dunnett's test. Differences from control were statistically significant at $p < 0.05$ and were considered a trend at $p \geq 0.05$ and $p \leq 0.1$.

Results. Table 1 shows the experiment scheme. Table 2 shows the composition of diets over the experiment.

The environmental conditions directly affect the vital activity, substrate and energy metabolism of animals. The temperature is one of the main influencing factors. The optimum temperature for pigs of different sex and age groups is not the same, 27 °C for sucking pigs, 25 °C for piglets with live weight from 15 to 25 kg, 22 °C from 25 to 45 kg, 20 °C from 45 to 85 kg, and 17 °C from 85 to 120 kg. Any deviation from the optimal parameters activates the thermoregulation system, and the greater the deviation, the more the animal is exposed to stress with high energy costs to maintain a constant body temperature [18].

1. Experiment scheme to assess the effect of dihydroquercetin (DHQ) on pigs (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] adaptation to simulated heat stress (physiological yard of Ernst FRC VIZH, Moscow Province, May-September 2020)

Group	*n*	Diet	DHQ feeding scheme
1(control)	9	BD	
2	9	BD + DHQ	During growing (DHQd)
3	9	BD + DHQ	During growing and fattening (DHQd+o)
4	9	OBD + DHQ	During feed and technological stresses (ДКВtech)

N o t e. BD — a basal diet (Table 2), DHQ (Ecostimul-2 preparation, Ametis JSC, Russia) was fed at a dosage of 45 mg/kg feed (32 mg DHQ active substance/kg feed). The dosage of the supplements was preliminarily estimated [2, 5, 12, 30].

2. The nutritional value of compound feed (at natural humidity) **in assessment of the dihydroquercetin (DHQ) effects on pigs (*Sus scrofa domesticus*) F_2 [(large white × Landrace) × Duroc] adaptation to simulated heat stress** (physiological yard of Ernst FRC VIZH, Moscow Province, May-September 2020)

Parameter	Units	Compound feed (OOO Agrovitex, Russia)		
		SK-4	SK-5	SK-6
Exchange energy	MJ/kg feed	11.85	11.65	11.47
Moisture	%	12.00	13.50	14.00
Crude protein	%	18.50	17.20	12.20
Crude fat	%	1.86	2.35	2.60
Crude fiber	%	4.24	5.72	5.04
Lysine	%	1.13	1.00	0.75
Methionine + cystine	%	0.65	0.62	0.46
Threonine	%	0.70	0.64	0.50
Tryptophan	%	0.22	0.20	0.16
Calcium	%	0.85	0.75	0.60
Phosphorus	%	0.56	0.55	0.48
Salt (NaCl)	%	0.54	0.53	0.50

In our research, the temperature regime varied from 22.1 to 29.6 °C (or from 71.8 to 85.3 °F). Thus, there was an excess of the temperature optimum of 18-20 °C by an average of 4-6 °C during most of the experiment. Relative air humidity varied on average from 65 to 85% and generally corresponded to zoohygienic standards (60-85%) [22] (Fig. 1).

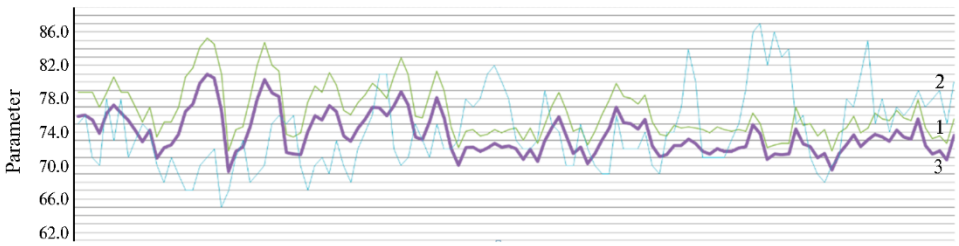


Рис. 1. Temperature (°F, 1)**, relative air humidity** (%, 3) **and temperature-humidity index** (THI, 2) **in the experiment on assessing the dihydroquercetin effects on pigs (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] adaptation to simulated thermal stress** (physiological yard of Ernst FRC VIZH, Moscow Province, May-September 2020).

Based on measurements of the relative humidity and temperature of the room where the animals were kept, a temperature-humidity index (THI) was calculated, confirming that the animals were under moderate stress. The THI values were 72.0-77.3 units (Table 3). Along with a moderate increase in temperature, the room air was saturated with CO_2 to 0.14 mg/m^3, remaining within the permissible concentration (< 0.2 mg/m^3). During the experiment, the content of ammonia and hydrogen sulfide also remained within the normal range (up to 20 and 10 mg/m^3, respectively).

The dynamics of blood cortisol level (Table 4) showed that the animals were exposed to stress factors during the experiment. In some periods, the level of cortisol often exceeded the physiological norm for pigs (41-237 nmol/l) [23].

3. Microclimate parameters during assessing the dihydroquercetin effect on pigs (*Sus scrofa domesticus*) F2 [(Large White × Landrace) × Duroc] adaptation to simulated thermal stress (*n* = 6, *M*±SEM, physiological yard of Ernst FRC VIZH, Moscow Province, May-September 2020)

Week	Parameter							
	φ, %	t, °C	t, °F	THI	NH_3, mg/m^3	H_2S, mg/m^3	CH_4, %	CO_2, %
1	73.2±1.37	21.8±0.28	78.8±0.51	75.8±0.50	4.3±1.1	0	0	0.06±0.01
2	71.6±1.37	22.2±0.45	76.5±0.82	73.7±0.77	3.8±0.8	0	0	0.06±0.01
3	68.1±0.81	25.2±0.88	81.2±1.59	77.3±1.36	5.8±1.1	0.05±0.01	0	0.07±0.01
4	66.7±1.49	24.1±0.84	78.1±1.51	74.7±1.32	5.5±1.0	0.06±0.01	0	0.06±0.01
5	67.5±0.88	24.3±0.40	77.4±0.72	74.5±0.63	6.8±1.2	0.08±0.01	0	0.11±0.02
6	66.0±1.85	25.2±0.51	78.3±0.91	75.1±0.67	6.5±1.2	0	0	0.09±0.02
7	73.5±0.97	25.9±0.73	79.6±1.32	76.7±1.12	5.8±1.1	0.05±0.01	0	0.06±0.01
8	70.5±1.22	24.2±0.08	76.5±0.14	73.8±0.12	6.6±1.2	0.08±0.01	0	0.07±0.01
9	77.3±1.18	22.7±0.51	74.1±0.92	72.2±0.80	5.5±1.1	0	0	0.08±0.01
10	72.7±1.18	23.3±0.58	75.4±1.04	72.9±0.93	4.9±1.3	0	0	0.08±0.01
11	69.8±2.09	23.6±0.07	75.6±0.12	72.8±0.25	7.0±1.3	0	0	0.07±0.01
12	70.9±0.76	24.2±0.49	76.4±0.88	73.5±0.77	16.7±2.0	0	0	0.13±0.04
13	74.4±2.09	22.5±0.07	74.5±0.12	72.3±0.25	12.8±1.8	0.38±0.05	0.01±0.005	0.09±0.02
14	77.6±2.57	22.9±0.28	74.3±0.50	72.5±0.57	15.8±2.2	1.67±0.21	0.02±0.005	0.11±0.02
15	77.4±2.73	22.6±0.38	74.0±0.68	72.0±0.49	13.3±1.8	1.42±0.15	0.02±0.005	0.14±0.03
16	73.1±2.56	22.3±0.28	74.1±0. 50	72.0±0.56	9.6±1.5	0	0	0.11±0.02
17	76.2±0.70	21.5±0.21	76.1±0.37	73.8±0.37	5.5±1.0	0	0	0.11±0.02

4. Blood cortisol levels in pigs (*Sus scrofa domesticus*) F2 [(Large White × Landrace) × Duroc] under simulated heat stress, as influenced by the dihydroquercetin (DHQ) **additive** (physiological yard of Ernst FRC VIZH, Moscow Province, May-September 2020)

Time frames	Group			
	1 (control)	2 (DHQ_d)	3 (DHQ_d +o)	4 (DHQ_{tech})
Final growing period	291.60±42.68	210.81±18.46	210.26±33.65	299.89±52.35
Transition to final fattening	147.58±27.50	140.83±13.16	93.53±16.19*	133.74±16.64
Before slaughter	284.77±86.81	275.59±86.41	234.36±61.23	253.20±46.39

N o t e. For a description of the groups, see the Materials and methods section. DHQ was fed during the growing period (DHQd), during growing and fattening (DHQd+o), and during feed and technological stresses (DHQtech).
* Differences from control are statistically significant at $p < 0.05$

5. Weight gain and feed consumption in pigs (*Sus scrofa domesticus*) F2 [(Large White × Landrace) × Duroc] under simulated heat stress, as influenced by the dihydroquercetin (DHQ) **additive** (*n* = 9, *M*±SEM, physiological yard of Ernst FRC VIZH, Moscow Province, May-September 2020)

Parameter	Group			
	1 (control)	2 (DHQ_d)	3 (DHQ_d +o)	4 (DHQ_{tech})
Over growing period				
n	9	9	9	9
Days	34	34	34	34
Live weight at the beginning of the experiment kg	17.20±0.63	17.40±0.51	17.31±0.52	17.43±0.65
Live weight at the end of the period, kg	38.83±0.89	39.34±1.09	39.31±0.81	39.39±1.26
Gross gain, kg	21.63±0.46	21.94±0.82	22.00±0.63	21.96±0.80
Daily gain, g	636.18±13.39	645.42±24.01	647.06±18.57	645.75±23.39
Over the 1st fattening period				
n	8	9	9	9
Days	49	49	49	49
Live weight at the end of 1st fattening period, kg	84.32±1.67[a]	84.47±1.93	84.56±1.26	84.38±2.75
Gross gain, kg	45.09±0.99[a]	45.12±1.30	45.24±1.18	44.99±1.85
Daily gain, g	920.18±20.11[a]	920.86±26.54	923.36±24.08	918.14±37.73
Over the 2nd fattening period a				
n	8	9	9	9

Continued Table 5

Days on average	37	37	37	37
Live weight at the end of fattening:				
total, kg	121.33±1.59	122.50±1.90	123.86±1.48	123.22±2.28
to control, %	100.0	101.0	102.1	101.6
Gross gain, kg	37.01±0.92	38.03±0.69	39.30±0.93^{+}	38.84±0.74
Daily gain, g	1000.30±24.93	1027.93±18.58	1062.16±25.01^{+}	1049.85±19.94
Over the whole experiment				
n	8	9	9	9
Days	120	120	120	120
Gross gain, kg	103.82±1.44	105.10±1.76	106.54±1.59	105.79±1.90
Daily gain, g	865.19±12.02	875.83±14.63	887.87±13.23	881.57±15.81
Feed consumption over the whole experiment				
Total, kg	320.8	312.1	312.1	312.1
Feed per 1 kg weigh gain:	3.09	2.97	2.93	2.95
total;, kg				
o control, %	100	96.1	94.8	95.5

Note. For a description of the groups, see the Materials and methods section. DHQ was fed during the growing period (DHQd), during growing and fattening (DHQd+o), and during feed and technological stresses (DHQtech); [a] — the value is calculated without estimates for one pig died in the middle of the period, 07/28/2020); + means trend of differences from control at $0.05 < p \leq 0.1$.

We revealed differences in the dynamics of live weight in animals during the experiment (Table 5, Fig. 2).

Thus, growing animals fed DHQ differed from the control in the average daily weight gain by 1.5-1.7% ($p > 0.05$). According to the results of the 1st fattening period, the animals showed identical parameters of live weight gain, but one animal dropped out of the control group (because of paralysis of the heart muscle due to a moderately pronounced heat stress), for the rest ($n = 8$) growth parameters were the same as in animals of the experimental groups. In the 2nd fattening period, the animals showed similar growth parameters ($p < 0.05$), but one more animal dropped out of the control group a few days before slaughter (the consequences of stress, the hind limbs were stretched, forced slaughter was carried out). In group 3, there was a tendency ($p = 0.01$) to better weight gains (1062.2 vs. 1000.3 g in the control group), which indicated a positive effect of feeding DHQ during the final fattening period. We have established the fact of greater susceptibility to stress among the animals of the control group compared to those treated with DHQ. Losses due to the disposal of animals in the control group influenced the cost of feed for the 1st fattening period (2.9-3.5% less in the experimental groups), for the 2nd fattening period (2.7-5% and 8% less in the experimental groups) and in general for the experiment (3.9-5.2% less in groups 2-4).

When compared, there was a tendency ($0.05 < p$ 0.1) to increase the digestibility of dry matter by 1.31% in animals treated with DHQ during the fattening period, by 0.93% in those receiving DHQ during technological stress (Table 6). The digestibility of crude fiber increased in group 3 (DHQd+o) by 3.23%. In groups 3 and 4, there was a tendency ($0.05 < p \leq 0.1$) to increase the digestibility of the feed dry matter. Changes in feed digestibility were accompanied by a reduced excretion of nitrogen in the urine (in group 2 by 21.20 g, in group 3 by 14.47 g, and in group 4 by 21.91 g) compared to control group ($p > 0.05$) (Table 7).

DHQ in the diet of pigs from groups 2, 3 and 4 contributed to less calcium excretion with faeces (by 3.48 at $p < 0.05$; 1.68 at $p > 0.05$; 2.87 g at $p = 0.06$. respectively) and its increased deposition in the body (by 3.52 at $p < 0.05$, 1.62 at $p > 0.05$, and 2.85 g at $p = 0.06$) compared to control. The proportion of used Ca from that received with food in groups fed DHQ was higher by 9.82% ($p < 0.05$), 4.52 and 7.94% ($0.05 < p \leq 0.1$), respectively. In animals from groups 3 and 4, the deposition of phosphorus was somewhat lower than in the control, but the decrease was not statistically significant.

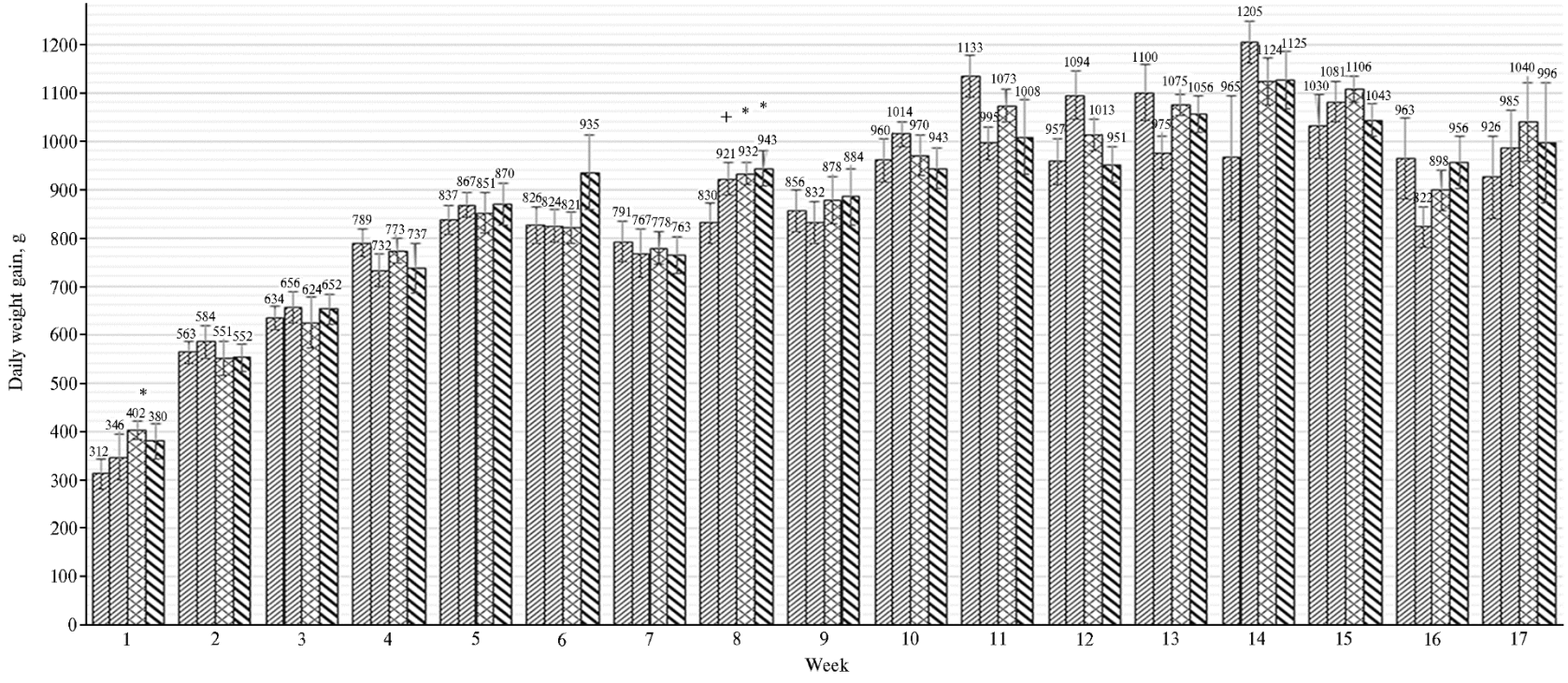


Fig. 2. Dynamics of average daily weight gain (AGA) **in pigs (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] under simulated heat stress, as influenced by the dihydroquercetin** (DHQ) **additive:** weekly from left to right) group 1 (control), groups 2, 3, and 4. For a description of the groups, see the Materials and methods section ($n = 9$, $M \pm$SEM, physiological yard of Ernst FRC VIZH, Moscow Province, May-September 2020)).

* Differences from control are statistically significant at $p < 0.05$; + means trend at $0.05 < p \leq 0.1$.

6. Digestibility of nutrients in pigs (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] under simulated heat stress, as influenced by the dihydroquercetin (DHQ) **additive** (n = 3, M±SEM, physiological yard of Ernst FRC VIZH, Moscow Province, May-September 2020)

Nutrient	Group			
	1 (control)	2 (DHQ_d)	3 (DHQ_d +o)	4 (DHQ_{tech})
Dry matter	73.89±0.36	75.66±0.89	75.20±0.42$^+$	74.82±0.20$^+$
Organic matter Органическое вещество	76.71±0.59	77.51±0.77	77.13±0.39	76.71±0.24
Crude protein	76.45±1.08	77.36±1.83	76.53±1.03	75.61±0.54
Crude fat	59.32±6.63	65.25±0.26	66.81±3.94	60.88±7.42
Crude fiber	40.26±2.47	40.44±1.47	43.49±2.88	40.89±2.10
Nitrogen-free extractives	81.63±0.13	82.24±0.49	81.53±0.37	81.76±0.30

N o t e. For a description of the groups, see the Materials and methods section. DHQ was fed during the growing period (DHQd), during growing and fattening (DHQd+o), and during feed and technological stresses (DHQtech); $^+$ means trend at $0.05 < p \le 0.1$.

7. Nitrogen, calcium, and phosphorus utilization in pigs (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] under simulated heat stress, as influenced by the dihydroquercetin (DHQ) **additive** (n = 3, M±SEM, physiological yard of Ernst FRC VIZH, Moscow Province, May-September 2020)

Parameter	Group			
	1 (control)	2 (DHQ_d)	3 (DHQ_d +o)	4 (DHQ_{tech})
N i t r o g e n b a l a n c e				
Input with feed, g	112.82	112.82	112.82	112.82
Output with faeces, g	27.79±0.38	25.54±2.06	26.48±1.17	27.52±0.61
Digested, g	85.03±0.31	87.28±1.68	86.35±0.95	85.30±0.50
Output with urine, g	60.37±11.48	39.17±8.78	45.90±8.51	38.46±7.32
Deposited in the body:				
total, g	24.66±11.77	48.11±7.23	40.45±9.64	46.84±7.28
of the input, %	21.86±10.44	42.64±6.41	35.85±8.54	41.51±6.45
of digested, %	28.91±13.69	55.46±9.20	46.57±10.60	54.92±8.61
C a l c i u m b a l a n c e				
Input with feed, g	35.83	35.83	35.83	35.83
Output with faeces, g	20.31±0.77	16.83±0.45*	18.63±1.33	17.44±0.77$^+$
Output with urine, g	0.31±0.04	0.27±0.06	0.38±0.04	0.34±0.04
Deposited in the body	15.20±0.78	18.72±0.45*	16.82±1.34	18.05±0.77$^+$
Utilized, % of the input	42.43±2.19	52.25±1.26*	46.95±3.73	50.37±2.16$^+$
P h o s p h o r u s b a l a n c e				
Input with feed, g	22.53	22.53	22.53	22.53
Output with faeces, g	10.23±0.09	9.48±0.57	9.85±0.18	10.10±0.15
Output with urine, g	3.12±0.39	2.89±0.65	4.40±0.37$^+$	4.01±0.70
Deposited in the body	9.17±0.40	10.16±0.54	8.28±0.54	8.42±0.73
Utilized, % of the input	40.70±1.79	45.09±2.38	36.74±2.39	37.38±3.25

N o t e. For a description of the groups, see the Materials and methods section. DHQ was fed during the growing period (DHQd), during growing and fattening (DHQd+o), and during feed and technological stresses (DHQtech). * Различия с контролем статистически значимы при $p < 0{,}05$; $^+$ means тенденция при $0{,}05 < p \le 0{,}1$.

Thus, pigs normally performed physiological functions when being in a neutral thermal zone. It also depends on animal age, body weight and the effective perceived temperature, which, in turn, is influenced by air movement, bedding, humidity and temperature of the walls and floor. Pigs do not sweat and have a relatively small lung capacity. Because of these physiological limitations and the relatively thick layer of subcutaneous fat, pigs are more susceptible to heat stress. Pigs with a live weight of 25, 50 and 75 kg respond differently to an increase in ambient temperature from 14 to 35 °C. The average daily live weight gain of 75 kg pigs begins to decrease at the temperature exceeding 23 °C, of 25 kg pigs at the temperature above 27°°C [1].

The concentration of ammonia, hydrogen sulfide and carbon dioxide varied during the experiment depending on the temperature and humidity conditions of the premice. When modeling moderately pronounced heat stress during the fattening period (12-15 weeks of the experiment), with an increase in the animal

live weight, the concentration of ammonia increased to 16.7 mg/m^3, hydrogen sulfide up to 1.67 mg/m^3, and carbon dioxide up to 0.14 mg/m^3 (see Table 3). We believe that these values indicate the complex negative impact of the simulated heat stress on animals. The change in the microclimate of the premise together with current technological manipulations to a certain extent influence the physiological processes, reducing the adaptive capabilities of some individuals. As a result, one animal from the control group failed to acclimatize and died. Thus, the mortality of livestock was 11% in the control group vs. 0% in the rest animals.

In growing, individuals fed DHQ had greater average daily weight gains compared to control, especially during the 1st week ($p < 0.05$, group 3). In our opinion, it indicates better adaptation after distant (500 km) transportation together with simulated moderate heat stress (see Table 5, Fig. 2). This followed from the blood cortisol level which in the control exceeded the upper limit of the norm. In animals that received DHQ only during technological and feed stresses (group 4), the cortisol concentration was comparable to control while in groups 2 and 3 it corresponded to the norm. This indicates a positive role of DHQ additives. The effect of DHQ similar to that found during growing was noted in group 3 during the 1st fattening period when cortisol levels decreased to 93.5 vs. 147.6 nmol/l in the control ($p < 0.05$). Dietary DHQ led to an increase in the average daily weight gain in groups 2, 3 and 4 groups during technological stress, together with moderate simulated thermal stress. It occurs during growing (1st week, group 3, $p < 0.05$) and at vaccination against classical swine fever in the beginning of fattening (8th week, group 2, $0.05 < p \leq 0.1$; groups 3 and 4, $p < 0.05$) (see Fig. 2). Note that constantly fed dietary DHQ (group 3) ensured the live weight gain which did not decrease (as compared to the control) throughout the entire experiment. When the supplement was stopped (group 2) or periodically fed (group 4, 11th and 16th weeks of the experiment), the gains were lower than in the control.

The blood cortisol level before slaughter was the highest in the control animals (20.2% above the physiological norm). In animals that received DHQ only during growing, it also exceeded the norm. Feeding with DHQ during the entire growth and at some subsequent periods stabilized this parameter (234-253 nmol/l), indicating a positive effect of DHQ on stress resistance of animals. The best result was provided by the constant DHQ input during growth and fattening.

As is known, in response to heat stress, an animal tries to lower its body temperature by increasing sweating, respiration rate and salivation [18]. These reactions energy-consuming and, accordingly, part of the feed energy used under optimal conditions to ensure live weight gain is redirected to thermoregulation. Digestion also transforms chemical energy in feeds into thermal energy in the body, and animals change their feeding behavior [24]. Some researchers believe [25] that the primary response to heat stress in different animal species is to reduce food intake (metabolic heat reduction strategy).

It is difficult to quantify the effects of climatic stress on animal productivity compared to a normal temperature regime [1, 26, 27]. Stress-induced reduction in feed intake creates the prerequisites for a decrease in the productivity of growing animals. In recent years, the negative consequences of thermal stress in pigs have become more obvious, probably due to increased susceptability of these animals to heat as a result of genetic selection for heat-producing traits [24]. The negative effect of heat stress on productivity is primarily explained by a decrease in feed intake, although the experimental results of recent years contradict this conclusion. In our experiments, no suppression of feed intake occurred with an increase in ambient temperature. As long as the animal consumes enough food (including dry

matter and metabolizable energy) to provide growth, development and physiological response to heat stress, stress does not lead to negative consequences, but at certain periods, the body needs additional support (increased feed energy, administration of adaptogenes, etc.). It is assumed that heat stress directly and indirectly affects the physiological processes that determine the health and productivity of animals. In our experiment, we found a trend towards an increase in dry matter digestibility, by 1.31% in animals fed DHQ during fattening and by 0.93% when subjected to technological stress ($p < 0.1$). In our opinion, it is explained by DHQ-mediated activation of metabolism due to the antioxidant properties of the adaptogene, by optimization of energy consumption with a moderate increase in ambient temperature, and alteration of metabolic priorities through direct or indirect mechanisms. At large pig breeding complexes, it was found [28] that about 5% of the nitrogen used as feed is emitted as NH_3 and another 1% with wastewater. Our data, including those obtained previously [5, 12], suggest that in growing pigs, feeding dietary DHQ may promote better utilization and deposition of nitrogen due to less excretion in the urine. Further studies of the influence of DHQ on the nitrogen compound redistribution in heat-stressed pigs will confirm or refute our assumption. In intensive pig breeding, the DHQ used constantly or in courses under stresses can increase pig adaptability and prevent the undesirable effects of technological, feed, transport and heat stresses. Improving the health of animals increases their livability and stabilizes daily weight gain under stress.

Suray and Fisinina [13] showed that immediately after the temperature impact on chickens, their growth slows down but then compensatory growth follows which contributes to a higher final live weight in broilers compared to birds not subjected to thermal training. That is, short-term stressful situations train animals, causing them to have a physiological response to stress. Long-term chronic stress, even of a moderate strength, worsens the growth performance and leads to premature retirement of the livestock, which was also confirmed by our findings. Dietary adaptogenes can neutralize the negative effects of stress and improves adaptive reactivity of animals [29, 30]. Previously, we have shown that dietary DHQ prevents negative effect of simulated technological stress on metabolism, including lipid peroxidation, stimulates anabolic processes, positively affectes the clinical health and nonspecific resistance of animals. In pigs fed DHQ, the level of cortisol, a hormone involved in the development of stress reactions was the lowest (134 nmol/l during growth and 215 nmol/l at the final fattening) [30], which additionally confirms our data of 2020. There are several aspects of the leveling effect of dietary adaptogens under various stresses. Phytogenic feed additives with a high content of antioxidants may reduce oxidative stress in pigs caused, among other reasons, by thermal exposure of pigs [31]. Feeding gamma-aminobutyric acid provides better performance due to improved functions of the nervous system and increased stress resistance of piglets [32]. Research has shown that targeted bioactives reduce adrenaline production and increase growth hormone production in piglets [32], resulting in consuming more feed and wasting less time and energy on aggressive behavior and associated stress. Immunoprophylaxis of various stresses with immunotropic drugs can reduce the age of the first insemination in gilts and increase their fertility [33, 34]. Normotimics and adaptogens may accelerate animal growth and improve the quality of meat products [35, 36].

Thus, a reduced heat generation and proper intake of dietary nutrients help to mitigate the effects of various stresses in pigs. We suggest an integrated approach to prevent negative effect of heat stress in pigs. It includes breeding animals for heat tolerance; the use of special anti-stress feed additives (with

thermal exposure, changes in the feeding regime, transportation and other stress factors); proper ventilation and air cooling regime in the premise. Timely forecasting of heat and other stresses and adequate preventive measures will help to avoid negative consequences of stresses for young animals and economic losses under intensive pig farming.

REFERENCES

1. Pearce S.C., Gabler N.K., Ross J.W., Escobar J., Patience J.F., Rhoads R.P., Baumgard L.H. The effects of heat stress and plane of nutrition on metabolism in growing pigs. *Journal of Animal Science*, 2013, 91: 2108-2118 (doi: 10.2527/jas.2012-5738).
2. Semenova A.A., Kuznetsova T.G., Nasonova V.V., Nekrasov R.V., Bogolyubova N.V., Tsis E.Yu. Use of antioxidants as adaptogens fed to pigs (*Sus scrofa domesticus* Erxleben, 1777) (meta-analysis). *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2020, 55(6): 1107-1125 (doi: 10.15389/agrobiology.2020.6.1107eng).
3. Zhang M., Dunshea F.R., Warner R.D., DiGiacomo K., Osei-Amponsah R., Chauhan S.S. Impacts of heat stress on meat quality and strategies for amelioration: a review. *International Journal of Biometeorology*, 2020, 64(9): 1613-1628 (doi: 10.1007/s00484-020-01929-6).
4. Yahav S., Mcmurthy J.P. Thermotolerance acquisition in broiler chickens by temperature conditioning early in life — The effect of timing and ambient temperature. *Poultry Science*, 2001, 80: 1662-1666 (doi: 10.1093/ps/80.12.1662).
5. Nekrasov R.V., Chabaev M.G., Bogolyubova N.V., Tsis E.Yu., Rykov R.A., Semenova A.A. *Agrarnaya nauka*, 2019, 10: 49-54 (doi: 10.32634/0869-8155-2019-332-9-49-54) (in Russ.).
6. Niyas P.A.A., Chaidanya K., Shaji S., Sejian V., Bhatta R., Bagath M., Rao G.S.L.H.V.P., Kurien E.K., Girish V. Adaptation of Livestock to Environmental Challenges. *J. Vet. Sci. Med. Diagn.*, 2015, 4: 3 (doi: 10.4172/2325-9590.1000162).
7. Kolesnikova L.R. *Vestnik Smolenskoi gosudarstvennoi meditsinskoi akademii*, 2018, 17(4): 30-36 (in Russ.).
8. Bogolyubova N.V., Chabaev M.G., Fomichev Yu.P., Tsis E.Yu., Semenova A.A., Nekrasov R.V. Ways to reduce adverse effects of stress in pigs using nutritional factors. *Ukrainian Journal of Ecology*, 2019, 9(2): 239-245 (doi: 10.15421/2019_70).
9. Huynh T.T., Aarnink A.J., Verstegen M.W., Gerrits W.J., Heetkamp M.J., Kemp B., Canh T.T. Effects of increasing temperatures on physiological changes in pigs at different relative humidities. *Journal of Animal Science*, 2005, 83(6): 1385-1396 (doi: 10.2527/2005.8361385x).
10. Kavtarashvili A.Sh. *RatsVetInform*, 2011, 7(119): 9-11 (in Russ.).
11. Baumgard L.H., Rhoads R.P. Effects of heat stress on postabsorptive metabolism and energetics. *Annual Review of Animal Biosciences*, 2013, 1: 311-337 (doi: 10.1146/annurev-animal-031412-103644).
12. Chabaev M.G., Tsis E.Yu., Mishurov A.V., Alyaudinov Yu.A., Semenova A.A. *Svinovodstvo*, 2020, 5: 19-23 (doi: 10.37925/0039-713X-2020-5-19-23) (in Russ.).
13. Surai P., Fisinin V.I. The modern anti-stress technologies in poultry: from antioxidants to vitagenes. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2012, 4: 3-13 (doi: 10.15389/agrobiology.2012.4.3eng).
14. Carpenter R., O'Grady M.N., O'Callaghan Y.C., O'Brien N.M., Kerry J.P. Evaluation of the antioxidant potential of grape seed and bearberry extracts in raw and cooked pork. *Meat Science*, 2007, 76(4): 604-610 (doi: 10.1016/j.meatsci.2007.01.02).
15. Sekretar S., Schmidt S., Vajdak M., Zahradnikova L., Annus J. Antioxidative and antimicrobial effects of some natural extracts in lard. *Czech Journal of Food Sciences*, 2018, 22: 215-218 (doi: 10.17221/10664-CJFS).
16. Costa L.G., Garrick J.M., Roqии P.J., Pellacani C. Mechanisms of neuroprotection by quercetin: counteracting oxidative stress and more. *Oxidative Medicine and Cellular Longevity*, 2016, 2016: ID 2986796 (doi: 10.1155/2016/2986796).
17. Zou Y., Wei H. K., Xiang Q.-H., Wang J., Zhou Y.-F., Peng J. Protective effect of quercetin on pig intestinal integrity after transport stress is associated with regulation oxidative status and inflammation. *Journal of Veterinary Medical Science*, 2016, 78(9): 1487-1494 (doi: 10.1292/jvms.16-0090).
18. Nekrasov R.V., Golovin A.V., Makhaev E.A., Anikin A.S., Pervov N.G., Strekozov N.I., Mysik A.T., Duborezov V.M., Chabaev M.G., Fomichev Yu.P., Gusev I.V. *Normy potrebnostei molochnogo skota i svinei v pitatel'nykh veshchestvakh* /Pod redaktsiei R.V. Nekrasova, A.V. Golovina, E.A. Makhaeva [Nutrient requirements for dairy cattle and pigs. R.V. Nekrasov, A.V. Golovin, E.A. Makhaev (eds.)]. Moscow, 2018 (in Russ.).
19. Mader T.L., Kreikemeier W.M. Effects of growth promoting agent and seasons on blood metabolites and body temperature in heifers. *Journal of Animal Science*, 2006, 84(4): 1030-1037 (doi:

10.2527/2006.8441030x).
20. Tomme M.F. *Metodika opredeleniya perevarimosti kormov i ratsionov* [Method for determining the digestibility of feed and rations]. Moscow, 1969 (in Russ.).
21. Ovsyannikov A.I. *Osnovy opytnogo dela v zhivotnovodstve* [Experienced livestock fundamentals]. Moscow, 1976 (in Russ.).
22. Khramtsov V.V., Tabakov G.P. *Zoogigiena s osnovami veterinarii i sanitarii* [Zoo hygiene with the basics of veterinary medicine and sanitation]. Moscow, 2004 (in Russ.).
23. Kondrakhin I.P., Arkhipov A.V., Levchenko V.I., Talanov G.A., Frolova L.A., Novikov V.E. *Metody veterinarnoi klinicheskoi laboratornoi diagnostiki: spravochnik* /Pod redaktsiei V.N. Saitanidi [Methods of veterinary clinical laboratory diagnostics: reference book. V.N. Saitanidi (ed.)]. Moscow, 2004 (in Russ.).
24. Renaudeau D., Anais C., Tel L., Gourdine J.L., Effect of temperature on thermal acclimation in growing pigs estimated using a nonlinear function. *Journal of Animal Science*, 2010, 88(11): 3715-3724 (doi: 10.2527/jas.2009-2169).
25. Mayorga J., Renaudeau D., Ramirez B., Ross J., Baumgard L. Heat stress adaptations in pigs. *Animal Frontiers*, 2018, 9(1): 54-61 (doi: 10.1093/af/vfy035).
26. Baumgard L.H., Rhoads R.P. Effects of heat stress on postabsorptive metabolism and energetics. *Annual Review of Animal Biosciences*, 2013, 1: 311-337 (doi: 10.1146/annurev-animal-031412-103644).
27. Ross J.W., Hale B.J., Gabler N.K., Rhoads R.P., Keating A.F., Baumgard L.H. Physiological consequences of heat stress in pigs. *Animal Production Science*, 2015, 55: 1381-1390 (doi: 10.1071/AN15267).
28. Ross J.W., Hale B.J., Seibert J.T., Romoser M.R., Adur M.K., Keating A.F., Baumgard L.H. Physiological mechanisms through which heat stress compromises reproduction in pigs. *Molecular Reproduction Development*, 2017, 84(9): 934-945 (doi: 10.1002/mrd.22859).
29. Harper L.A., Sharpe R.R., Simmons J.D. Ammonia emissions from swine houses in the Southeastern United States. *Journal of Environmental Quality*, 2004, 33(2): 449-457 (doi: 10.2134/jeq2004.4490).
30. Nekrasov R.V., Chabaev M.G., Tsis E.Yu., Bogolybova N.V., Mishurov A.V., Rykov R.A. Effect of feed antioxidants on behavior and stress resistance of fattening pigs. *Journal of Animal Science*, 2020, 98(Suppl_4): 364-365 (doi: 10.1093/jas/skaa278.640).
31. Fomichev Yu.P., Bogolyubova N.V., Nekrasov R.V., Chabaev M.G., Rykov R.A., Semenova A.A. Physiological and biochemical effects of two feed antioxidants in modeling technological stress in pigs (*Sus scrofa domesticus* Erxleben, 1777). *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2020, 55(4): 750-769 (doi: 10.15389/agrobiology.2020.4.750eng).
32. Jycsák I., Tossenberger J., Végvári G., Sudár G., Varga-Visi E., Tyth T. How is the effect of phytogenic feed supplementation tested in heat stressed pigs? Methodological and sampling considerations. *Agriculture*, 2020, 10: 257 (doi: 10.3390/agriculture10070257).
33. Kuznetsov A.S. *Svinovodstvo*, 2019, 6: 24-26 (in Russ.).
34. Semenov V.G., Uspeshnyi A.V., Gladkikh L.P., Nikitin D.A., Tikhonov A.S., Mikhailova R.V. *Uchenye zapiski Kazanskoi gosudarstvennoi akademii veterinarnoi meditsiny im. N.E. Baumana*, 2020, 243(3): 233-236 (doi: 10.31588/2413-4201-1883-243-3-233-237) (in Russ.).
35. Uspeshnyi A.V., Gladkikh L.P., Semenov V.G., Nikitin D.A. *Vestnik CHuvashskoi gosudarstvennoi sel'skokhozyaistvennoi akademii*, 2020, 1(12): 77-81 (doi: 10.17022/hspc-fn13) (in Russ.).
36. Zou Y., Xiang Q., Wang J., Wei H., Peng J. Effects of oregano essential oil or quercetin supplementation on bodyweight loss, carcass characteristics, meat quality and antioxidant status in finishing pigs under transport stress. *Livestock Science*, 2016, 192: 33-38 (doi: 10.1016/j.livsci.2016.08.005).
37. Ostrenko K.S., Lemeshevsky V.O., Ovcharova A.N., Galochkina V.P., Sofronova O.V. Effect of adaptogens on the quality of pig meat. *Ukrainian Journal of Ecology*, 2020, 10(1): 344-348 (doi: 10.15421/2020_54).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1172-1182
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 664.872

doi: 10.15389/agrobiology.2021.6.1172eng
doi: 10.15389/agrobiology.2021.6.1172rus

A TECHNOLOGY FOR OBTAINING A PROTEIN CONCENTRATE FROM YEAST BIOMASS OF *Kluyveromyces marxianus* Van der Walt (1965)

I.A. FOMENKO, I.A. DEGTYAREV, L.A. IVANOVA, N.G. MASHENTSEVA ✉

Moscow State University of Food Production, 11, Volokolamskoe sh., Moscow, 125080 Russia, e-mail iv.fomenko@mail.ru, Ivand152@yandex.ru, biotech@mgupp.ru, natali-mng@yandex.ru (✉ corresponding author)
ORCID:
Fomenko I.A. orcid.org/0000-0003-0081-6336
Degtyarev I.A. orcid.org/0000-0002-3842-1391
Ivanova L.A. orcid.org/0000-0003-1993-8333
Mashentseva N.G. orcid.org/0000-0002-9287-0585
The authors declare no conflict of interests
Received July 8, 2021

Abstract

Lack of protein in the diet leads to a violation of nitrogen metabolism. Experts estimate that the feed protein market will exceed US $ 200 billion by 2024. In Russia, the deficit of fodder proteins is more than 1 million tons. Traditional protein sources cannot meet the daily need for food and feed proteins because of economic and social reasons, so there is a growing interest in alternative protein sources. This communication presents the results of obtaining a protein concentrate based on the biomass of thermotolerant yeast *K. marxianus* grown on a waste of oilseed production that is little used in bioconversion - sunflower husk. Yeast of this type increases the digestibility of feed, is used in the food industry for the fermentation of soy milk, soft cheese and as a flavor enhancer. The aim of the study was to develop a technology for obtaining a protein concentrate from the yeast biomass of *K. marxianus* and to substantiate the feasibility of its use as a feed and food additive. The *K. marxianus* Y-4570 strain was selected as a result of screening on sunflower husk fermentolysate as the most productive in terms of biomass accumulation (up to 30 g/l) and crude protein (59.29±2.96 %). Using a multicyclic semi-continuous method, yeast was cultured in a laboratory fermenter on a saline medium with sunflower husk fermentolysate to obtain protein biomass. Technological parameters were determined to obtain the protein concentrate containing at least 60 % of the true protein, no more than 2 % of lipids and no more than 2 % of nucleic acids. The biomass was defatted with 60 % ethyl alcohol with a hydromodule of 1:2.5 at 60 °C for 1 hour. The residual lipid content was 1.94±0.09 %. Denucleinization was performed by activating the cell's own endonucleases at 40-60 °C. Nucleic acids were removed at a 50 °C for 1 hour with a hydromodule 1:7. The residual content of nucleic acids was 1.97±0.10 %. The final product contains 65.94±3.14 % of true protein, which meets the requirements for protein concentrates. Analysis of the amino acid profile of the protein concentrate showed that the content of almost all essential amino acids exceeds that in the original yeast biomass, with the exception of glycine, leucine and histidine. A relative increase in the content of amino acids occurs due to the removed lipids, nucleic acids, the loss of moisture and the concentration of substances of the original biomass with drying. Protein concentrate based on the biomass of the yeast *K. marxianus* Y-4570 is intended for use as a feed and food additive in order to enrich products with essential amino acids.

Keywords: protein concentrate, protein, lipids, nucleic acids, *Kluyveromyces marxianus*, yeast, denuclearization, degreasing

In intensive animal farming, because of restrictions on the use of feed antibiotics, protein preparations are recently attracting increasing attention to improve animal health and accelerate the growth [1]. *Basidiomycetes* can be a source of complete protein. However, fungi grown in natural conditions are capable of accumulating toxic heavy metals [2, 3], therefore, under industrial conditions, champignons and oyster mushrooms are most often grown on artificial soils. Mushrooms are only 16.47-36.96% protein, which is their main disadvantage [4], although this figure for edible mushrooms such as champignons and oyster mushrooms is approximately 2 times higher than for vegetable crops [5]. Algae are rich in protein (on average up to 60-65% of dry matter) [6] and contain biolactive

substances that have a beneficial effect on humans and animal health (vitamins, minerals, antioxidants) [7]. However, algae, like champignons and oyster mushrooms, are able to accumulate heavy metals from the environment, so algae cannot be considered as a proper replacement for traditional protein sources either [8]. Among protein-oil crops, soybean is the leader in yield. However, discussions about the dangers of soybeans for human do not stop: its use is associated with the occurrence of cancer and the appearance of allergic reactions [9, 10].

The range of yeast feed preparations is quite wide. Dietary yeast primarily enrich feed with essential amino acids, in particular lysine [1, 11]. Yeast feed additives may be obtained from potato processing waste [12] and from the fermentation of cheese whey with the yeast *Kluyveromyces fragilis* [13]. Some yeasts have therapeutic and prophylactic effects on humans and animals [14]. Adding *Saccharomyces cerevisiae* protein preparations into feed increased the number of villi in the intestine and stimulated animal productivity [15-18]. The use of *Kluyveromyces marxianus* as a feed additive in fish farming provides a 40% replacement of the protein in expensive fishmeal without loss in salmon growth rates [19]. Yeast is a promising biologically active feed and food additive [20]. They accumulate up to 60% protein in dry mass, contain B vitamins [21, 22], some species, in particular *Saccharomyces cerevisiae*, serve as a rich source of ergosterol [23].

In microbial synthesis for protein production, cheap substrates [24, 25], in particular, cheese whey [13] and lignocellulose-containing waste and products of processing the cellulose-containing raw materials [26] are the indisputable advantages. Growing yeast biomass on chicken manure allows utilization of poultry waste that is toxic to the environment [27]. Microbiological bioconversion of agricultural waste into protein products reduces the negative impact on the environment [28].

Along with biologically valuable protein and vitamins, yeasts synthesize organic acids, polyhydric alcohols, and enzymes [29]. However, it is necessary to control the content of lipids and nucleic acids in yeast preparations [3]. Lipids cause unpleasant taste, odor, as they enter into oxidation reactions, and nucleic acids contain nitrogen, which accumulates in the form of urates, causing urolithiasis [30].

Kluyveromyces marxianus is an ascomycete yeast with pronounced thermotolerant properties used in the biotechnological production of enzymes, in particular inulinase, β-galactosidase and pectinase [31]. *K. marxianus* is also used in agriculture and the food industry, including for the production of ethanol, aromatics, and as starter cultures [32-35]. A number of data confirm the safety of the *Kluyveromyces* yeast for human and animal health [36, 37].

The main reasons hindering the industrial production of microbial protein using yeast fungi are the high cost of technologies due to expensive equipment and significant energy consumption during fermentation. For baker yeast *Saccharomyces cerevisiae* and *Candida*, *Cryptococcus*, and *Torulopsis* yeasts used for microbiological synthesis, the optimum growth temperature is 28-32 °C, while in *K. marxianus* it is 34-40 °C [1]. Yeast cultures are heat generating. Therefore, it is profitable to cool the medium in the bioreactor to a higher temperature, reducing the coolant consumption. In addition, from the point of view of waste bioconversion and cost reduction, it is important to expand the raw material base of such production. This report presents the results of obtaining a protein concentrate based on the biomass of thermotolerant yeast *K. marxianus* grown on sunflower husks, which are little used in bioconversion. The strain selected during screening on a nutrient medium containing enzymatic lysate of sunflower husk accumulates 59.29±2.96% of crude protein, the biomass yield reaches 30 g/l.

The study aimed to develop a technology for production of a protein concentrate from the *K. marxianus* yeast biomass and to substantiate the feasibility of its use as a feed and food additive.

Materials and methods. Sunflower husks were ground to a particle size of 30-100 μm (a rotor beater mill Retsch SR 200, RETSCH GmbH, Germany) controlled with a particle analyzer HELOS (H3908) & RODOS/L, R5 (Sympatec GmbH, Germany). The crushed particles were delignified by suspending in a 4% NaOH solution at a hydromodulus of 1:8.5 followed by incubation at 125±1 °C for 2 h; the extractant was separated by centrifugation. The resulting wet sediment of delignified husks was suspended in water and subjected to enzymatic hydrolysis for 24 h at 50±1 °C, pH 5.0±0.1. We used the enzyme preparation RovabioMax AP (Adisseo France S.A.S., France); cellulolytic activity 1900 units CA/g as per GOST R 55293-2012, xylanase activity 23500 units XA/g as per GOST R 55302-2012, the dosage of the preparation was determined at cellulase activity of 35 ClA/g raw material). After 24 h, the suspension was centrifuged, and the enzyme lysate was used as a substrate.

The yeast biomass of the *Kluyveromyces marxianus* Y-4570 strain (obtained from the collection of the NBC VKPM NRC Kurchatov Institute—GosNIIgenetika, Moscow) was produced using deep culture in a medium containing 0.50% $NH_4H_2PO_4$, 0.10% $MgSO_4$, 0.06% KH_2PO_4, 0.20% yeast extract, and up to 100% sunflower husk enzymatic lysate (8% DM).

Yeast was cultured in a multicyclic semi-continuous way in a laboratory fermenter MD-300 (L.E. MARUBISHI, Japan), aeration 1 v/(v · min) (air volume to nutrient medium volume), 38±1 °C, pH 5.0 (25% aqueous ammonia solution served as a titrant; alkalization of the medium indicated the need to add fresh enzyme lysate). A yeast suspension (4%) obtained in flasks on a liquid Sabouraud medium (5×10^6 cells/ml, counted with a Goryaev chamber) was used as an inoculum. After 10-day culture, the yeast biomass was separated at 5000 rpm for 15 min in a laboratory centrifuge (MLWT23D, OOO Medtehnika-Servis, Ukraine).

The amount of dry matter in the samples were determined gravimetrically by drying samples to constant weight at 105°°C. Crude protein in the biomass was measured according to Kjeldahl [38] (an automatic LK-500 distillation system. ZAO Laboratory Equipment and Devices, Russia). The analyzed sample was mineralized, ammonia was distilled off for 10 min in a Parnas-Wagner apparatus (PJSC Khimlaborpribor, Russia). The excess acid from the receiving flask was titrated with 0.1 N sodium hydroxide solution. The true protein was measured by the Barnstein method. The precipitate was filtered, washed, and the amount of nitrogen was determined by the Kjeldahl method [48]. Lipids were determined according to Folch [39] by distillation in a device for distilling liquids (NPO Laborkomplekt, Russia), and then drying to constant weight in a ShS-80-01 SPU drying cabinet (OAO Smolensk SKTB SPU, Russia). Nucleic acids were measured by Spirin method (an SSP-715 spectrophotometer, ZAO Spectroscopic Systems, Russia) [40].

The amino acid composition was analyzed by capillary electrophoresis (Kapel-105M system, Lumex LLC, Russia; the M-04-38-2009 methodology amended No. 1 of February 1, 2010, in accordance with the manufacturer's recommendations).

For defatting, the yeast biomass was extracted with ethyl alcohol (food alcohol Lux from grain raw materials; 40, 60 and 70%) in the ratio of biomass:ethanol 1:1.5; 1:2.0, and 1:2.5 in three doses of 20 min each. An appropriate amount of alcohol was added successively every 20 min, for a total of 60 min, at 50, 60 and 70 °C (a water bath TW-2.02, Elmi, Latvia). The partially defatted biomass was dried in an oven to constant weight. The amount of extracted lipids was

determined by the difference between the initial dry yeast biomass and the defatted biomass.

Nucleic acid degradation in yeast biomass occurred due to own enzyme activation at 50-70 °C. The incubation in a water bath took from 30 min to 1.5 h with hydromodules of 1:3, 1:5 and 1:7.

Statistical processing of quantitative data was performed using the STATISTICA 23.0 software package (StatSoft, Inc., USA). All measurements were performed in 3 replicates. Results are presented as weighted arithmetic mean (*WAM*) with standard deviation (±SD). Statistical significance was calculated using the non-parametric Mann-Whitney U-test and Kruskal-Wallis H-test. The critical significance level of the null hypothesis (p) was equal to 0.05.

Results. In the sunflower husk enzymatic lysate used to grow *K. marxianus* Y-4570 was 7.0-8.0% dry matter, 3.0-3.5% reducing substances (according to Bertrand), 69.65±3.48% glucose, 16.08±0.80% cellobioses, and 14.27±0.71% higher sugars.

The resulting *K. marxianus* Y-457 biomass was 59.29±2.96% crude protein, 13.45±0.67% lipids, and 8.85±0.44% nucleic acids. The residual amount of lipids and nucleic acids in protein preparations, which worsens their safety and provokes the formation of stones, should not exceed 2.00% [30].

The fundamental difference between our method and similar methods is the use of edible ethyl alcohol (95%) to remove lipids. For this purpose, the Folch method is most commonly used, extracting lipids with a mixture of chloroform:methanol (2:1 v/v) [41]. For food protein, it is advisable to use ethyl alcohol without chloroform. The amount of extracted lipids did not differ much compared to the Bligh-Dyer method, where chloroform is used for extraction in addition to ethyl alcohol, 33.04±0.16% without chloroform vs. 33.18±0.24% with chloroform according to the Bligh-Dyer method [42].

With an increase in the concentration of ethanol, the amount of extracted lipids increased, reaching a maximum for 70% ethanol (Table 1). However, it is preferable to use 60% ethanol, since 40% ethanol insufficiently removed lipids while the use of 70% alcohol increases costs, despite the fact that the amount of extracted lipids differs only by 1-2%. Ethanol 60% (1:2.5 v/v) removes up to 80% of lipids. In addition, sonication pre-treatment ensures the removal of a large amount of lipids [43].

1. Lipids (of initial content, %) **extracted from yeast *Kluyveromyces marxianus* biomass under various conditions** (n = 27, *WAM*±SD, lab test)

Temperature and ethanol concentration	Hydromodul		
	1:1.5	1:2.0	1:1.5
50 °C:			
40 %	72.41 ± 3.62^{Aa}	74.18 ± 3.70^{Ab}	75.02 ± 3.75^{Ac}
60 %	74.24 ± 3.71^{Bb}	75.24 ± 3.76^{Bc}	77.70 ± 3.88^{Bd}
70 %	75.12 ± 3.75^{Cc}	76.30 ± 3.81^{Cd}	78.52 ± 3.92^{Cf}
60 °C:			
40 %	75.46 ± 3.77^{Ca}	76.74 ± 3.83^{Cb}	82.90 ± 4.14^{Dd}
60 %	76.18 ± 3.80^{Cb}	78.10 ± 3.90^{Dc}	85.57 ± 4.27^{Ee}
70 %	77.12 ± 3.85^{Dc}	81.32 ± 4.06^{Ed}	86.46 ± 4.32^{Ee}
70 °C:			
40 %	78.54 ± 3.92^{Da}	82.15 ± 4.10^{Ec}	87.17 ± 4.35^{Fe}
60 %	79.60 ± 3.90^{Db}	83.47 ± 4.17^{Fd}	87.89 ± 4.39^{Fe}
70 %	81.04 ± 4.05^{Ec}	84.30 ± 4.21^{Fd}	89.55 ± 4.47^{Gf}

$^{A-G}$ Differences between the values in the column are statistically significant and differ at $p < 0.05$.
$^{a-f}$ Differences between the columns are statistically significant and differ at $p < 0.05$.

As the temperature increased, the amount of extracted lipids increased (see Table 1). The largest amount of lipids was remove at 70 °C (but this temperature regime is economically unfavorable), the smallest amount at 50 °C, therefore, a

temperature of 60 °C was chosen.

After choosing the concentration of ethanol and temperature, the ratio of ethanol:biomass was determined to extract the largest amount of lipids. Ethyl alcohol was used at ratios to biomass of 1:1.5; 1:2.0 and 1:2.5. With 60% ethanol and 60 °C, the optimal ethanol:biomass ratio was 1:2.5 (see Table 1), resulting in 85.57±4.27% extracted lipids. For 1:1.5 and 1:2.0 ratios, less lipids were removed, and their residual amount was more than 2.0%. In a similar study, the following parameters were proposed, the biomass:ethanol ratio 1:40, 135 °C and P = 1.5 MPa [44]. In this case, the disadvantages are the high consumption of the extractant, the high temperature which negatively affects the amino acid composition of the protein concentrate, and the use of excess pressure which leads to additional costs.

Thus, based on the data obtained, we propose the ratio of yeast biomass:60% ethanol of 1:2.5 at a temperature of 60 °C as technological parameters for lipid extraction. These treatments reduce the residual lipid content to 1.94±0.09%. One of the works reported on the preparation of a baker's yeast protein concentrate in which the amount of residual lipids after extraction was 6.47% [45].

The extraction temperature has the greatest influence on the amount of extracted nucleic acids (factor load 0.700).

With yeast biomass:water ratio of 1:7, the samples were kept at 40, 50 and 60 °C in a water bath for 0.5; 1 and 1.5 h for nucleic acid degradation. Less amount of nucleic acids was removed at 40 °C compared to 50 and 60 °C (see Table 2). It can be concluded that with an increase in temperature to a certain level, the activity of the yeast's own enzymes will increase, i.e., at 40 °C it is lower than at 50 °C) while at 60 °C the enzymes inactivation occurs. At 70 °C, nucleic acid residual was approximately 3.00%, but the amount of lysine also reduced. At 50 °C, nucleic acids would account for approximately 2.50%, while lysine loss would not be so significant [46].

2. Nucleic acids (of initial content, %) **extracted from yeast *Kluyveromyces marxianus* biomass under various conditions** (n = 27, *WAM*±SD, lab test)

Temperature and extraction time	Hydromodul		
	1:3	1:5	1:3
40 °C:			
0.5 h	56,32±2,81^{Aa}	58,75±2,93^{Ab}	59,60±2,98^{Ab}
1.0 h	58,60±2,93^{Bb}	60,12±3,00^{Bd}	64,02±3,20^{Be}
1.5 h	62,75±3,13^{Cc}	65,43±3,27^{Ce}	66,05±3,30^{Ce}
50 °C:			
0.5 h	67,89±3,39^{Da}	68,98±3,44^{Db}	70,25±3,51^{Dc}
1.0 h	69,34±3,46^{Eb}	71,30±3,56^{Ed}	77,74±3,88^{Ee}
1.5 h	70,02±3,50^{Ec}	72,46±3,62^{Ed}	79,88±3,99^{Ef}
60 °C:			
0.5 h	66,16±3,30^{Da}	67,18±3,35^{Db}	68,07±3,40^{Cc}
1.0 h	67,25±3,36^{Db}	68,93±3,44^{Dc}	69,74±3,48^{Dd}
1.5 h	68,73±3,43^{Ec}	69,80±3,49^{Dd}	70,10±3,50^{Df}

$^{A-D}$ Differences between the values in the column are statistically significant and differ at $p < 0.05$.
$^{a-f}$ Differences between the columns are statistically significant and differ at $p < 0.05$.

The final step was to determine the optimal time for nucleic acid degradation. In our experiment, it was 1.0 h (see Table 2). At 50 °C for 1 h and hydromodule of 1:7, up to 77.74±3.88% nucleic acids were removed. Thus, these parameters were selected for nucleic acid degradation.

Pacheco et al. [47] developed a technology for the production of a protein concentrate from baker's yeast with a true protein content of about 75.0% on average, for which salts (sodium perchlorate and sodium trimetaphosphate) were used. In this regard, we note that there are reports of a negative effect of sodium perchlorate on thyroid function [48]. In our work, ethyl alcohol and native yeast endonucleases were used to obtain the concentrate. In a similar study,

the biochemical composition of the *K. marxianus* and *S. cerevisiae* autolysates was compared [49]. The *K. marxianus* biomass contains a large amount of nucleic acids (approximately 10%) and 56% of true protein vs. approximately 9% and 57% for *S. cerevisiae* [30]. In our work (Table 3), the *K. marxianus* initial biomass was 13.45±0.67% lipids and 8.85±0.44% nucleic acids vs. 1.94±0.09% and 1.97±0.10%, respectively, for the produced protein concentrate.

3. Biochemical composition (of dry matter, %) **of initial yeast *Kluyveromyces marxianus* biomass and the protein concentrate after extraction of lipids and nucleic acids** (n = 8, *WAM*±SD, lab test)

Parameter	Biomass	Protein concentrate
Crude protein	59.29 ± 2.96^{Aa}	71.65 ± 3.43^{Bb}
True protein	54.60 ± 2.73^{Aa}	65.94 ± 3.14^{Cc}
Lipids	13.45 ± 0.67^{Da}	1.94 ± 0.09^{Fd}
Nucleic acids	8.85 ± 0.44^{Ea}	1.97 ± 0.10^{Fd}

$^{A\text{-}F}$ Differences between the values in the column are statistically significant and differ at $p < 0.05$.
$^{a\text{-}d}$ Differences between the columns are statistically significant and differ at $p < 0.05$.

The yeast biomass protein of *K. marxianus* was lower in the contents of lysine, threonine, and sulfur-containing amino acids (Table 4). However, the resulting protein concentrate was almost completely balanced in all essential amino acids, except for sulfur-containing amino acids, phenylalanine and tyrosine. Processing under selected technological parameters significantly increased the content of lysine, threonine, serine, arginine, proline, glutamine and aspartic amino acids, however, as compared to the initial biomass, the amount of leucine, histidine and glycine decreased. The relative increase in the content of amino acids occurred due to a decrease in the content of lipids, nucleic acids and the removal of moisture during drying. Compared to the initial biomass, the lysine level increased by 1.75%, threonine by 0.50%, serine by 0.62%, arginine by 1.02%, proline by 2.66%, aspartic acid by 1.93%, and glutamic acid by 1.76%. The amount of glycine decreased by 2.14%, leucine by 1.22%, and histidine by 0.16%, since heating destroys these amino acids.

4. Amino acid composition (g/100 g protein) **of initial yeast *Kluyveromyces marxianus* biomass and the protein concentrate after extraction of lipids and nucleic acids as compared to the FAO ideal protein** (*WAM*±SD, lab test)

Amino acid	Yeast biomass	Protein concentrate	"Ideal" protein
Phenylalanine + tyrosine	5.77±0.28	5.20±0.26	6.0
Leucine	6.70±0.33	5.48±0.27	5.9
Lysine	3.75±0.18	5.50±0.27	5.5
Valine	4.36±0.21	4.86±0.24	4.9
Isoleucine	4.28±0.22	4.35±0.21	4.0
Threonine	1.87±0.09	2.31±0.11	3.3
Tryptophan	1.23±0.06	1.34±0.06	1.0
Glutamic acid	2.55±0.12	4.31±0.21	–
Arginine	3.46±0.17	4.48±0.22	–
Glycine	4.59±0.23	2.45±0.12	–
Aspartic acid	3.54±0.17	5.47±0.27	–
Methionine + cysteine	2.03±0.10	2.20±0.11	3.5
Proline	2.61±0.13	5.27±0.26	–
Histidine	1.82±0.09	1.66±0.08	1.5
Alanine	4.32±0.21	5.53±0.27	–
Serine	0.41±0.02	1.03±0.05	–

N o t e. Dashes mean that these amino acids are not determined for the "ideal" protein.

Lysine is the main limiting amino acid in pig feed. In the protein concentrate we obtained its amount is 5.50±0.27%. This is enough to meet the daily needs of farm animals. For birds, the limiting amino acids are cysteine and methionine the amount of which in the protein concentrate (2.2±0.11%) can also cover daily requirement. One study used a yeast protein concentrate to feed *Cyprinidae* fish [50] and showed that this concentrate could replace up to 50% of

expensive fishmeal in carp diets without any negative effects on fish health and growth. In addition, the amino acid composition of the yeast biomass of *K. marxianus* and the protein concentrate is comparable in composition to the ideal FAO/WHO protein [51, 52] (see Table 4).

The main advantage of our technology is the use of thermotolerant yeast *K. marxianus*, which can be cultured at higher temperatures (34-40 °C). In addition, to date, most studies have focused on the physiology and metabolism rather than on practical applications of *K. marxianus* [32]. Baker's yeast is most commonly culture at approximately 32 °C [53]. Yeast culture generates a large amount of heat, so it is necessary to cool the medium in the bioreactor. *K. marxianus* yeast requires less cooling than baker's yeast, which reduces the amount of coolant.

Using the developed technology, we have obtained a prototype protein concentrate. It is a paste-like mass after drying in an oven at 103 °C to a 5.56% residual moisture content. Characteristics of the prototype are as follows:

Parameter	Regulatory document	Показатель
Crude protein	GOST 20083-74	71.65 %
True protein	GOST 20083-74	65.94 %
Appearance	GOST P 54731-2011	Homogeneous dry fine powder
Color	GOST P 54731-2011	Light beige or light brown
Flavor	GOST P 54731-2011	Yeast-specific, without off-flavours
Taste	GOST P 54731-2011	Yeast-specific, without extraneous aftertaste
Moisture	GOST P 54731-2011	5.56 %
Microbiological indicators	Technical regulation of the Customs Union 021/2011	Matched
Heavy metal content	Technical regulation of the Customs Union 021/2011	Matched

Thus, the sunflower oil production generates a large amount of wastes. We have developed the biotechnology for manufacturing a protein-rich product from the yeast *Kluyveromyces marxianus* Y-4570 biomass with the use of sunflower husk enzymatic lysate as the culture medium. The technology includes nucleic acid degradation and defattening. Lipid removal includes extraction with 60% ethanol for 1.0 h at 60 °C and a hydromodule of 1:2.5 (the residual amount of lipids does not exceed 2%). Nucleic acid degradation occurs during 1.0 h due to yeast's own endonucleases at 50 °C and a hydromodulus of 1:7 (the residual amount of nucleic acids also does not exceed 2%). The resultant protein concentrate contains at least 65% of true protein. The increase in the content of amino acids in the protein concentrate occurs due to a decrease in the content of lipids and nucleic acids. The amino acid composition of yeast biomass and protein concentrate is comparable to that of an ideal FAO/WHO protein. The introduction of a concentrate from the yeast biomass of *K. marxianus* Y-4570 into food products will enrich them with protein with a high content of essential amino acids and improve the organoleptic qualities. Protein concentrate can also be used in animal husbandry.

REFERENCES

1. Shurson G.C. Yeast and yeast derivatives in feed additives and ingredients: Sources, characteristics, animal responses, and quantification methods. *Animal Feed Science and Technology*, 2018, 235: 60-76 (doi: 10.1016/j.anifeedsci.2017.11.010).
2. Cocchi L., Vescovi L., Petrini L.E., Petrini O. Heavy metals in edible mushrooms in Italy. *Food Chemistry*, 2006, 98(2): 277-284 (doi: 10.1016/j.foodchem.2005.05.068).
3. Demirba A. Accumulation of heavy metals in some edible mushrooms from Turkey. *Food Chemistry*, 2000, 68(4): 415−-419 (doi: 10.5772/52771).
4. Bach F., Helm C.V., Bellettini M.B., Maciel G.M., Haminiuk C.W.I. Edible mushrooms: a potential source of essential amino acids, glucans and minerals. *International Journal of Food Science & Technology*, 2017, 52(11): 2382-2392 (doi: 10.1111/ijfs.13522).
5. Hamzah R.U., Jigam A.A., Makun H.A., Egwim E.C. Antioxidant properties of selected African vegetables, fruits and mushrooms: a review. In: *Mycotoxin and food safety in developing countries*. H. Makun. IntechOpen, London, 2013: 203-250 (doi: 10.5772/52771).
6. Becker E.W. Micro-algae as a source of protein. *Biotechnology Advances*, 2007, 25(2): 207-210

(doi: 10.1016/j.biotechadv.2006.11.002).
7. Wells M.L., Potin P., Craigie J.S., Raven J.A., Merchant S.S., Helliwell K.E., Brawley S.H. Algae as nutritional and functional food sources: revisiting our understanding. *Journal of Applied Phycology*, 2017, 29(2): 949-982 (doi: 10.1007/s10811-016-0974).
8. Arulkumar A., Nigariga P., Paramasivam S., Rajaram R. Metals accumulation in edible marine algae collected from Thondi coast of Palk Bay, Southeastern India. *Chemosphere*, 2019, 221: 856-862 (doi: 10.1016/j.chemosphere.2019.01.007).
9. Fallon S., Enig M. Soy alert-tragedy and hype. *Nexus Magazine*, 2000, 7(3): 17-23.
10. Savage J.H., Kaeding A.J., Matsui E.C., Wood R.A. The natural history of soy allergy. *Journal of Allergy and Clinical Immunology*, 2010, 125(3): 683-686 (doi: 10.1016/j.jaci.2009.12.994).
11. Wang W., Li Z., Lv Z., Zhang B., Lv H., Guo Y. Effects of *Kluyveromyces marxianus* supplementation on immune responses, intestinal structure and microbiota in broiler chickens. *PLoS ONE*, 2017, 12(7): e0180884 (doi: 10.1371/journal.pone.0180884).
12. Gélinas P., Barrette J. Protein enrichment of potato processing waste through yeast fermentation. *Bioresource Technology,* 2007, 98(5): 1138-1143 (doi: 10.1016/j.biortech.2006.04.021).
13. Ghaly A.E., Kamal M.A. Submerged yeast fermentation of acid cheese whey for protein production and pollution potential reduction. *Water Research*, 2004, 38(3): 631-644 (doi: 10.1016/j.watres.2003.10.019).
14. Palma M.L., Zamith-Miranda D., Martins F.S., Bozza F.A., Nimrichter L., Montero-Lomeli M., Douradinha B. Probiotic Saccharomyces cerevisiae strains as biotherapeutic tools: is there room for improvement. *Applied Microbiology and Biotechnology*, 2015, 99(16): 6563-6570 (doi: 10.1007/s00253-015-6776-x).
15. Haldar S., Ghosh T.K., Bedford M.R. Effects of yeast (*Saccharomyces cerevisiae*) and yeast protein concentrate on production performance of broiler chickens exposed to heat stress and challenged with *Salmonella enteritidis*. *Animal Feed Science and Technology,* 2011, 168(1-2): 61-71 (doi: 10.1016/j.anifeedsci.2011.03.007).
16. Moallem U., Lehrer H., Livshitz L., Zachut M., Yakoby S. The effects of live yeast supplementation to dairy cows during the hot season on production, feed efficiency, and digestibility. *Journal of Dairy Science*, 2009, 92(1): 343-351 (doi: 10.3168/jds.2007-0839).
17. Liu S., Shah A.M., Yuan M., Kang K., Wang Z., Wang L., Xue B., Zou H., Zhang X., Yu P., Wang H., Tian G., Peng Q. Effects of dry yeast supplementation on growth performance, rumen fermentation characteristics, slaughter performance and microbial communities in beef cattle. *Animal Biotechnology*, 2021: 1-11 (doi: 10.1080/10495398.2021.1878204). Epub ahead of print.
18. Mogensen G., Salminen S., Crittenden R., Bianchi Salvadori B., Zink R. Inventory of microorganisms with a documented history of use in food. *Bulletin-International Dairy Federation*, 2002, 377: 10-19 (doi: 10.1016/j.ijfoodmicro.2011.12.030).
19. Øverland M., Karlsson A., Mydland L.T., Romarheim O.H., Skrede A. Evaluation of *Candida utilis*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* yeasts as protein sources in diets for Atlantic salmon (*Salmo salar*). *Aquaculture*, 2013, 402: 1-7 (doi: 10.1016/j.aquaculture.2013.03.016).
20. Rakowska R., Sadowska A., Dybkowska E., Swiderski F. Spent yeast as natural source of functional food additives. *Roczniki Państwowego Zakładu Higieny*, 2017, 68(2): 115-121.
21. Pérez-Torrado R., Gamero E., Gymez-Pastor R., Garre E., Aranda A., Matallana E. Yeast biomass, an optimised product with myriad applications in the food industry. *Trends in Food Science & Technology*, 2015, 46(2): 167-175 (doi: 10.1016/j.tifs.2015.10.008).
22. Ferreira I., Pinho O., Vieira E., Tavarela J.G. Brewer's *Saccharomyces* yeast biomass: characteristics and potential applications. *Trends in Food Science & Technology*, 2010, 21(2): 77-84 (doi: 10.1016/j.tifs.2009.10.008).
23. Hu Z., He B., Ma L., Sun Y., Niu Y., Zeng B. Recent advances in ergosterol biosynthesis and regulation mechanisms in Saccharomyces cerevisiae. *Indian Journal of Microbiology*, 2017, 57(3): 270-277 (doi: 10.1007/s12088-017-0657-1).
24. Gervasi T., Pellizzeri V., Calabrese G., Di Bella G., Cicero N., Dugo G. Production of single cell protein (SCP) from food and agricultural waste by using *Saccharomyces cerevisiae*. *Natural Product Research*, 2018, 32(6): 648-653 (doi: 10.1080/14786419.2017.1332617).
25. Jin Y.S., Cate J.H. Metabolic engineering of yeast for lignocellulosic biofuel production. *Current Opinion in Chemical Biology*, 2017, 41: 99-106 (doi: 10.1016/j.cbpa.2017.10.025).
26. Kistaubayeva A., Savitskaya I., Shokataeva D., Zhabakova A., Kuli Z. Utilization of agricultural waste by yeast-bacterial conversion of cellulose-containing substrates to protein feed products. *Eurasian Journal of Ecology*, 2017, 51(2): 34-43 (doi: 10.26577/EJE-2017-2-765).
27. Yan Z., Liu X., Yuan Y., Liao Y., Li X. Deodorization study of the swine manure with two yeast strains. *Biotechnology and Bioprocess Engineering*, 2013, 18(1): 135-143 (doi: 10.1007/s12257-012-0313-x).
28. Matassa S., Boon N., Pikaar I., Verstraete W. Microbial protein: future sustainable food supply route with low environmental footprint. *Microbial Biotechnology*, 2016, 9(5): 568-575 (doi: 10.1111/1751-7915.12369).
29. Halász A., Lásztity R. *Use of yeast biomass in food production*. CRC Press, 1991 (doi: 10.1201/9780203734551).
30. Ivanova I.S. *Razrabotka tekhnologii biologicheski aktivnoi dobavki k pishche v vide belkovo-*

uglevodnogo kontsentrata iz biomassy khlebopekarnykh drozhzhei. Kandidatskaya dissertatsiya [Development of technology for bioactive food additive in the form of protein-carbohydrate concentrate from baker's yeast biomass. PhD Thesis]. Moscow, 2003 (in Russ.).
31. Lane M.M., Burke N., Karreman R., Wolfe K.H., O'Byrne C.P., Morrissey J.P. Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*. *Antonie Van Leeuwenhoek*, 2011, 100(4): 507-519 (doi: 10.1007/s10482-011-9606-x).
32. Fonseca G.G., Heinzle E., Wittmann C., Gombert A.K. The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Applied Microbiology and Biotechnology*, 2008, 79(3): 339-354 (doi: 10.1007/s00253-008-1458-6).
33. Karim A., Gerliani N., Aıder M. *Kluyveromyces marxianus*: an emerging yeast cell factory or applications in food and biotechnology. *International Journal of Food Microbiology*, 2020, 333: 108818 (doi: 10.1016/j.ijfoodmicro.2020.108818).
34. Varela J.A., Gethins L., Stanton C., Ross P., Morrissey J.P. Applications of *Kluyveromyces marxianus* in biotechnology. In: *Yeast diversity in human welfar*e. Springer, Singapore, 2017: 439-453 (doi: 10.1007/978-981-10-2621-8_17).
35. Lee M.H., Lin J.J., Lin Y.J., Chang J.J., Ke H.M., Fan W.L., Li W.H. Genome-wide prediction of CRISPR/Cas9 targets in *Kluyveromyces marxianus* and its application to obtain a stable haploid strain. *Scientific Reports*, 2018, 8(1): 1-10 (doi: 10.1038/s41598-018-25366-z).
36. Fadda M.E., Mossa V., Deplano M., Pisano M.B., Cosentino S. In vitro screening of *Kluyveromyces* strains isolated from Fiore Sardo cheese for potential use as probiotics. *Food Science and Technology*, 2017, 75: 100-106 (doi: 10.1016/j.lwt.2016.08.020).
37. EFSA Panel on Biological Hazards (BIOHAZ). Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update). *EFSA Journal*, 2013, 11(11): 3449 (doi: 10.2903/j.efsa.2013.3449).
38. Weigand E., Kirchgessner M. Protein and energy value of vinasse for pigs. *Animal Feed Science and Technology*, 1980, 5(3): 221
39. Folch J., Lees M., Sloane-Stanley G.H. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, 1957, 226: 497-509 (doi: 10.1016/S0021-9258(18)64849-5).
40. Karklinya V.A., Birska I.A., Limarenko Y.A. Quantitative determination of nucleic acids in *Salmonidae* milt by various methods. *Chem. Nat. Compd.*, 25, 1989: 109-112 (doi: 10.1007/BF00596713).
41. Schneiter R., Daum G. Extraction of yeast lipids. *Methods Mol. Biol.*, 2006, 313: 41-45 (doi: 10.1385/1-59259-958-3:041).
42. Yang F., Xiang W., Sun X., Wu H., Li T., Long L. A novel lipid extraction method from wet microalga *Picochlorum* sp. at room temperature. *Marine Drugs*, 2014, 12(3): 1258-1270 (doi: 10.3390/md12031258).
43. Kot A.M., Gientka I., Bzducha-Wrybel A., Błażejak S., Kurcz A. Comparison of simple and rapid cell wall disruption methods for improving lipid extraction from yeast cells. *Journal of Microbiological Methods*, 2020, 176: 105999 (doi: 10.1016/j.mimet.2020.105999).
44. Chen M., Chen X., Liu T., Zhang W. Subcritical ethanol extraction of lipid from wet microalgae paste of *Nannochloropsis* sp. *Journal of Biobased Materials and Bioenergy*, 2011, 5(3): 385-389 (doi: 10.1166/jbmb.2011.1157).
45. Caballero–Córdoba G.M., Sgarbieri V.C. Nutritional and toxicological evaluation of yeast (*Saccharomyces cerevisiae*) biomass and a yeast protein concentrate. *Journal of the Science of Food and Agriculture*, 2000, 80(3): 341-351 (doi: 10.1002/10970010(200002)80:3%3C341::AIDJSFA533%3E3.0.CO;2-M).
46. Hedenskog G., Ebbinghaus L. Reduction of the nucleic acid content of single - cell protein concentrates. *Biotechnology and Bioengineering*, 1972, 14(3): 447-457 (doi: 10.1002/bit.260140313).
47. Pacheco M.T.B., Caballero-Cordoba G.M., Sgarbieri V.C. Composition and nutritive value of yeast biomass and yeast protein concentrates. *Journal of Nutritional Science and Vitaminology*, 1997, 43(6): 601-612 (doi: 10.3177/jnsv.43.601).
48. Leung A.M., Pearce E.N., Braverman L.E. Perchlorate, iodine and the thyroid. *Best Practice & Research Clinical Endocrinology & Metabolism*, 2010, 24(1), 133-141 (doi: 10.1016/j.beem.2009.08.009).
49. Lukondeh T., Ashbolt N.J., Rogers P.L. Evaluation of *Kluyveromyces marxianus* as a source of yeast autolysates. *Journal of Industrial Microbiology and Biotechnology*, 2003, 30(1): 52-56 (doi: 10.1007/s10295-002-0008-y).
50. Omar S.S., Merrifield D.L., Kühlwein H., Williams P.E., Davies S.J. Biofuel derived yeast protein concentrate (YPC) as a novel feed ingredient in carp diets. *Aquaculture*, 2012, 330: 54-62 (doi: 10.1016/j.aquaculture.2011.12.004).
51. Andrade J., Pereira C.G., de Almeida J.C. Jr., Viana C.C.R., Neves de Oliveira L.N., Fonseca da Silva P.H., Bell, M.J.V., de Carvalho dos Anjos V. FTIR-ATR determination of protein content to evaluate whey protein concentrate adulteration. *LWT*, 2019, 99: 166-172 (doi: 10.1016/j.lwt.2018.09.079).
52. Consultation F.E. *Dietary protein quality evaluation in human nutrition. FAO Food and Nutrition Paper, 92.* Rome, 2013.
53. Salvady Z., Arroyo-López F.N., Guillamyn J.M., Salazar G., Querol A., Barrio E. Temperature adaptation markedly determines evolution within the genus *Saccharomyces*. *Applied and Environmental Microbiology*, 2011, 77(7): 2292-2302 (doi: 10.1128/aem.01861-10).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1183-1198
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

Veterinary diagnostics

UDC 636.2:619:637.04

doi: 10.15389/agrobiology.2021.6.1183eng
doi: 10.15389/agrobiology.2021.6.1183rus

DIFFERENTIAL SOMATIC CELL COUNT IN MILK AS CRITERIA FOR ASSESSING COWS' UDDER HEALTH IN RELATION WITH MILK PRODUCTION AND COMPONENTS

A.A. SERMYAGIN[1] ✉, I.A. LASHNEVA[1], A.A. KOSITSIN[1], L.P. IGNATIEVA[1], O.A. ARTEMIEVA[1], J. SÖLKNER[2], N.A. ZINOVIEVA[1]

[1]*Ernst Federal Research Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail alex_sermyagin85@mail.ru (✉ corresponding author), lashnevaira@gmail.com, ksicins@gmail.com, ignatieva-lp@mail.ru, vijmikrob@mail.ru, n_zinovieva@mail.ru);
[2]*Universität für Bodenkultur,* 1180 Wien, Österreich, Gregor-Mendel-Straße 33, e-mail johann.soelkner@boku.ac.at
ORCID:
Sermyagin A.A. orcid.org/0000-0002-1799-6014
Lashneva I.A. orcid.org/0000-0003-4276-8782
Kositsin A.A. orcid.org/0000-0001-8484-4902
Ignatieva L.P. orcid.org/0000-0003-2625-6912
Artemieva O.A. orcid.org/0000-0001-7706-4182
Sölkner J. orcid.org/0000-0002-1517-5829
Zinovieva N.A. orcid.org/0000-0003-4017-6863

The authors declare no conflict of interests
Acknowledgements:
We express our gratitude to the company Foss Electric LLC (Russia branch, Moscow) for the information support provided in the implementation of these studies, with personal thanks to the Head of the Dairy Farming and Feed Division Irina Elizarova and the service engineer Denis Karlin.
Supported financially by the Russian Foundation for Basic Research in the framework of scientific project No. 20-316-90050 for the analysis of somatic cell count in cows' milk and by the Russian Science Foundation project No. 21-76-20046 for studying extended milk component composition based on infrared spectrometry
Received September 30, 2021

Abstract

The somatic cell count in cow's milk is used to control the inflammatory infection process and to assess the likelihood of subclinical and clinical mastitis. In the article, within the framework of experimental design of observations in a dairy cattle herd, the possibility in the cows' mammary gland monitoring status, based on the total somatic cell count determination and proportion for lymphocytes and polymorphonuclear neutrophils (PMN) in raw milk is shown for the first time in Russia. The obtained results confirm the relationship between somatic cell count (SCC) and daily milk yield for lactating animals. The aim of this work is to assess the relationship between the number of somatic cells in milk and their differentiation by species with milk production, milk component traits, and the risk of progressing subclinical and clinical mastitis in Holsteinized Black-and-White cows. The work was carried out from June 2020 to May 2021 (an experimental herd of Holsteinized Black-and-White cattle, PZ Ladozhsky — a branch of the Ernst Federal Research Center for Animal Husbandry, Krasnodar Territory). The total sample in a data set was 313 animals; the number of milk lactation records was 1931. The analysis of milk components was carried out using an automatic analyzer CombiFoss 7 DC (FOSS, Denmark) based on express methods of infrared spectroscopy and flow cytometry. The following milk traits were studied: daily milk yield, percentage of fat, protein, casein, lactose, dry matter, dry skimmed milk residue, traces of acetone and beta-hydroxybutyrate (BHB), freezing point and acidity, fatty acids (FA), SCC, DSCC (fraction of lymphocytes and PMN in the total amount of cells). In order to indirectly assess the mammary gland condition of cows, animals in the herd were conditionally divided into four groups: A — SCC $\leq$ 200 thousand cells per ml, DSCC $\leq$ 70 %; B — SCC $\leq$ 200 thousand cells per ml, DSCC > 70 %; C — SCC > 200 thousand cells per ml, DSCC > 70 %; D — SCC > 200 thousand cells per ml, DSCC $\leq$ 70 %. Also, the following animal gradation was applied regardless of the probability of mastitis: two groups with DSCC $\leq$ 70 and DSCC > 70 %; four subgroups with SCC $\leq$ 200, 201-500, 501-1000 and $\geq$ 1001 thousand cells per ml. We used logarithmic (normalized) SCC scores according to G.R. Wiggans et al. (1987) approach. The individual economic value of the daily milk yield of cows was determined. For assessing effects of environmental factors and their elimination on daily milk component traits, the equation of generalized linear models (GLM) was used. Estimates of phenotypic means for milk features were obtained by the GLM-equation using the least squares method. The pairwise comparison between means was performed using Tukey's test. Principal component analysis (PCA) was used to study the variability of milk composition

depending on their formation in the animal organism in order to determine the most significant parameters that determine the productivity of dairy cows. Healthy individuals and animals with suspected mastitis (predisposed to the onset of infection) (groups A and B) had desirable features of milk production, the daily milk yield was 25.7-27.7 kg, the average economic efficiency of milk production was 714-744 rubles per day per cow. Cows assigned to groups C (subclinical or clinical forms of active mastitis) and revealed as D (chronic mastitis) had the milk component traits superior to other groups with a relatively lower daily milk yield. Animals with high SCC values as well as with a chronic form of mastitis were most susceptible to metabolic disorders or ketosis, regardless of DSCC. An increase in the fat percentage in milk by 0.18-0.37 % ($p \leq 0.001$) for animals with SCC $\geq$ 1001 thousand cells per ml led to rise the share of saturated FA by 1.1-1.4 percentage points (p.p.), palmitic FA — by 0.4-1.2 p.p., medium-chain FA — by 1.0-1.4 p.p. An increase in the normalized scores of SCC by one point (limits from 1 to 10) led to a decrease in the daily milk yield by 0.6 kg, lactose percentage — by 0.062 p.p. and an increase in fat and protein by 0.090 and 0.055 p.p., respectively. Analysis of the main components revealed clear clusters for the protein and fat milk fractions, urea and fatty acids, acetone and BHB, freezing point and pH values, SCC and DSCC. A separate group included the daily milk yield and lactose percentage (together with ketone bodies) traits not related to other milk composition traits, thereby indicating the independent nature of the variability of these features. Further study of the relationship between the milk components synthesis in mammary gland and animal physiological status will make it possible to clarify the direction of selection in dairy cattle and define the genetic determination of milk production traits.

Keywords: milk, cow, somatic cell count, milk yield, fat, protein, fatty acids, acetone, BHB, mastitis, ketosis

The total somatic cell count (SCC) in the milk of dairy cows is crucial for detecting various mastitis, monitoring the inflammatory process, predicting subclinical and clinical mastitis, and assessing the health status of animals and milk quality. A more detailed analysis of the milk composition helps to develop ways to improve milk quality and milk yield [1, 2].

Despite of considerable progress in cow udder health over the past 40 years, mastitis continues to cause enormous economic damage to dairy cattle throughout the world. This is a complex pathology influenced by the environment, feeding, keeping conditions and genetic factors. In addition, pathogens that cause mastitis are constantly mutating, which requires adjustment of treatment regimens. Now dairy cattle are more productive than 20-30 years ago, which changes the type of herd management and breeding approaches [3].

Since the milk somatic cells are mostly lymphocytes, macrophages and polymorphonuclear neutrophils (PMN), for a more accurate characterization of the udder state and the prognosis of the occurrence of mastitis, in addition to evaluation of total SCC, it is advisable to differentiate somatic cells by their type [4-6]. Three major populations of cells found in milk play a key role in the mammary gland inflammation [7, 8]. Lymphocytes regulate the induction and suppression of immune responses. Macrophages recognize pathogens and initiate an immune response to invasion, resulting in a massive influx of PMN. Macrophages engulf bacteria, cellular debris, accumulated milk components and participate in tissue repairing. PMNs also protect the udder from bacterial entry in the event of mastitis [9].

In disease, the total number of SCCs and the composition of cells involved in the immune response usually change. In milk of healthy animals, the counts of somatic cells, mainly macrophages and lymphocytes, are low [10-13]. With any infection, the SCC increases significantly, and PMNs become predominant [14].

Flow cytometry coupled with infrared spectroscopy is a relatively inexpensive rapid method to assess SCCs and milk composition compared to fluorescence microscopy and arbitration methods [15, 16]. The method allows an accuracy of 0.839 to differentiate somatic cell types, 0.994 to detect SCCs, 0.820 to detect beta-hydroxymalic acid, and 0.800-0.950 for other milk components.

Many practitioners deem the number of somatic cells in milk to be insignificant for breeding, since the state of the mammary gland is more influenced by

the environment (including bacterial microbiota, especially staphylococci) than by genetic factors. However, population monitoring can contribute to breeding the most resistant and genetically adapted individuals. In Russia, in cows with SCC from 201 to 500 thousands per milliliter, milk production over lactation was 274 kg, or 4%, lower compared to animals with SCC not exceeding 200 thousands per milliliter. For SCC from 501 to 1000 thousands per milliliter, productivity was 348 kg (5%) lower, for SCC above 1001 thousand cells per ml408 kg (5.9%) lower. Note that the mass fraction of milk protein was 0.19% higher in animals with SCC of more than 1000 thousands per milliliter, which indirectly indicates physico-chemical changes in milk [17, 18].

At present, dairy cattle breeding for higher milk protein content is relevant because of general deficiency of protein in the human diet with constantly growing milk consumption. Also important is the traditional breeding for milk fat content to meet the market needs for butter, sour cream, cream, soft cheeses. However, without understanding the nature of the onset and course of the inflammation in the mammary gland, it is difficult to obtain high-quality products. It is obvious that an increase in productivity creates an additional physiological burden on the cow's body, which leads to metabolic stress, a decrease in resistance and a change in the composition of milk.

All these stimulate interest in search for putative biomarkers of the physiological state and productivity traits of dairy cows. Various types of somatic cells in milk can be such biomarkers.

For the first time, our observations in a herd of dairy cattle revealed that the total SCC in milk and the proportions of lymphocytes and PMN in SCC might be indicative of the state of cows' mammary gland. Our finding confirmed the relationship between the SCC score and daily milk production of lactating cows.

This work aimed to reveal the relationship of somatic cell counts (total and by species) with milk yielding, milk composition and the likelihood of occurrence of subclinical and clinical mastitis in Holsteinized Black-and-White cows.

Materials and methods. The work was carried out from June 2020 to May 2021 in an experimental herd of Holsteinized Black-and-White cows (PZ Ladoga — a branch of the Ernst FRC VIZH, Krasnodar Territory). The total number of measurements of daily milk indicators on 334 cows was 2023. After quality control for normal distribution and extreme values (outliers) for the main parameters, i.e., the mass fractions of fat (MFF), protein (MFP), lactose (MFL) and dry matter (DM), the sample size was 313 animals, with 1931 measurements in total. Control milking, individual sampling and conservation of milk samples using Microtabs tablets (USA) were carried out three times a day, in the morning (5.00-7.00), in the afternoon (12.00-13.30), and in the evening (18.00-20.00).

Milk component assay was performed (an automatic analyzer CombiFoss 7 DC, FOSS, Denmark; the analyzer consists of a MilkoScan for near infrared spectroscopy coupled with Fossomatic 7 DC for flow cytometry). All indicators were recorded automatically, the data were uploaded to the Microsoft Excel program for each sample. Before starting milk sample analysis, the readings for a standard milk sample and a synthetic medium containing somatic cells were recorded.

Each milk sample was analyzed individually; the values obtained were reduced to average daily values. The following parameters were determined: daily milk yield (DMY), MFF, MFP, mass fraction of casein (MFC), MFL, DM, dry skimmed milk residue (DSMR), traces of acetone and beta-hydroxybutyrate (BHB), freezing point (FP) and acidity (pH), fatty acids (FA) — $C_{14:0}$ (myristic), $C_{16:0}$ (palmitic), $C_{18:0}$ (stearic), $C_{18:1}$ (oleic), long-, medium- and short-chain fatty acids (LCFA, MCFA , SCFA), mono- and polyunsaturated FAs (MUFAs, PUFAs), saturated FAs (SFAs), transisomers (TFA), SCC, DSCC (differential

somatic cell count, DSCC as the proportion of lymphocytes and PMN in the total amount of cells). The device cannot record the proportion of macrophages, so it was calculated as the difference between the SCC taken as 100% and the DSCC percentage.

To indirectly assess the mammary gland state, all cows were conditionally grouped according to Schwarz [26] in our modification. Group A was healthy individuals (SCC ≤ 200 thousands per milliliter, DSCC ≤ 70%); group B was individuals with suspected mastitis (SCC ≤ 200 thousands per milliliter, DCC > 70%); C was individuals with subclinical/clinical mastitis, SCC > 200 thousands per milliliter, DSCC > 70%); D was individuals with chronic (persistent) mastitis (SCC > 200 thousands per milliliter, DSCC ≤ 70%). In addition, regardless of the likelihood of mastitis, animals were grouped by DSCC scores (two groups with DSCC ≤ 70 and > 70%) and by SCC scores (four subgroups with SCC ≤ 200, 201-500, 501-1000 and ≥ 1001 thousands per milliliter) (eight subgroups in total for the DSCC and SCC combination).

We used logarithmic (normalized) SCC estimates (SCCE) according to Wiggans [19]. The best animals in terms of SCC in milk corresponded to 1 point, the worst to 10 points, with a one-point step SCC:

$$SCCE = \log_2(SCC/100) + 3. \quad (1)$$

The individual estimated milk value (EMV) in phenotypic terms was determined based on the milk price (60% for MFP, 40% for MFF expressed for the basal contents of 3.0% protein and 3.4% fat) in terms of the physical mass of the chilled raw material. The economic value was specified by increasing and decreasing coefficients for raw material for processing which depend on the SCC value by (1.1 for SCC < 250 thousands per milliliter, 1.0 for SCC 250-400 thousands per milliliter, 0.9 for SCC = 400-1000 thousands per milliliter, and 0.5 for SCC > 1000 thousands per milliliter).

To assess the effect of environmental factors and their elimination on the daily indicators, the equation of generalized linear models (GLM) implemented in the STATISTICA 10 program [20] was used:

$$y_{ikm} = ML_i + DM_k + a_1 DIM + DSCC\_SCC_m + e_{ikm}, \quad [2]$$

where y is the estimated parameter for group m, month i, and milkmaid k; ML is the month of the productivity estimation (i = 10); DM is the fixed effect of the milkmaid (k = 6); DIM is a continuous effect of lactation days from calving (a_1 is regression coefficient); DSCC_SCC is the fixed effect of the group the animal is assigned to with regard to SCC and DSCC scores (m = 8); e is residual variance of the model.

Based on the GLM equation using the least squares method (Least-Squares, LS), estimates of phenotypic means for milk performance were obtained. Pairwise comparisons between means were made using Tukey's test. The principal component analysis (PCA) method was used for spatial visualization and analysis of the variability of milk composition depending on the individual characteristics of its formation to establish the most significant parameters that determine the dairy productivity. Descriptive statistics were calculated using the STATISTICA 10 program (StatSoft, Inc., USA) and Microsoft Excel 2013. The tables show the means (M), their standard errors (±SEM), and coefficients of variation (Cv, %). The differences were considered statistically significant at $p \leq 0.05$.

Results. The milk composition estimates provided by infrared spectroscopy or flow cytometry assay, serve as a kind of biomarker to control the productive traits through breeding and to manage animal health.

DSCC in milk from cows in our experiments ranged from 0 (no blood

cells detected) to 93.8%. I.e., the proportion of macrophages at the maximum DSCC was the smallest, which could indicate an active inflammatory process in the mammary gland.

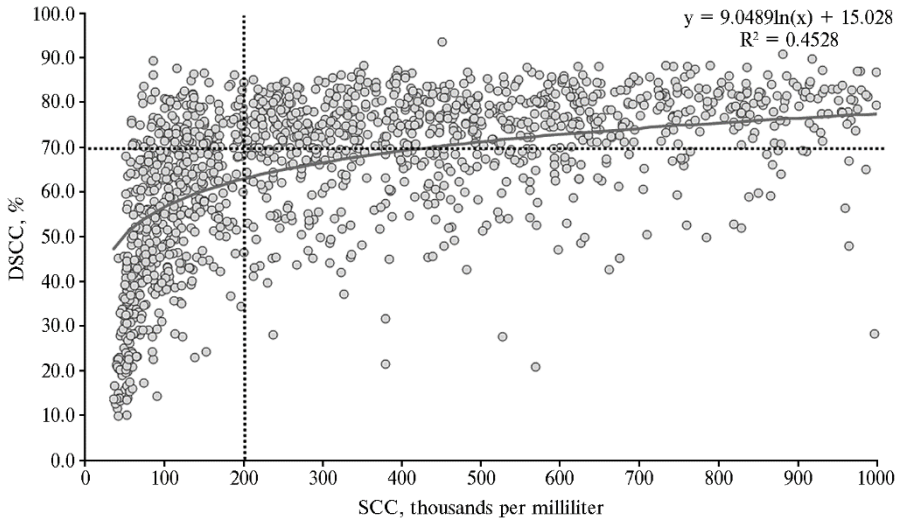


Fig. 1. Somatic cell differentiation (DSCC, lymphocytes and polymorphonuclear neutrophils) **in milk of Holsteinized Black-and-White cows (individual daily values) depending on somatic cell counts** (SCC) (PZ Ladoga — a branch of the Ernst FRC VIZH, Krasnodar Territory, 2020-2021). Dotted lines indicate the threshold values for DSCC and SCC.

Figure 1 shows the distribution of DSCC depending on the total number of somatic cell counts (with threshold values of 70% for DSCC and 200 thousand cells per mlSCC). With an increase in the SCC in milk, the DSCC index increased in a logarithmic function ($R^2 = 0.453$). The percentage of records with the lower threshold for SCC, regardless of the DSCC score, was 35.9%, with 13.4 and 50.7% excess for SCC < 70% and > 70%, respectively. These results draw to the conclusion that approximately two thirds of the herd have a subclinical pattern of mammary dysfunction, which often occur in high-yielding herds whose diet is high in concentrated feed. For 13.4% of animals, deviations in the functional properties of the udder were chronic.

The average daily milk yield in the studied animals was 25.4±0.2 kg with a mass fraction of fat and protein of 3.61±0.02 and 3.20±0.01%, respectively (Table 1). In our opinion, the low milk fat content is associated with the climatic conditions of the breeding area and the diet intended for high milk production. Values of metabolic biomarkers (ketone bodies or traces of acetone and BHB in milk) were within the physiological norm, the 0.30-0.35 and 0.15 mmol/l, respectively. In 1.1% of cases, the appearance of clinical (new-calving cows, from day 9 to day 78 after calving) and subclinical (second half of lactation, from day 167 to day 598 after calving) forms of ketosis occurred. Similar results for acetone and BHB in milk were obtained in the herds of the Black-and-White and Holstein breeds in the Tyumen region [21]. The relationship between the amount of acetone and BHB was linear ($R^2 = 0.631$). The concentration of urea in milk exceeded the optimal values (15.1-30.0 mg/100 ml) and amounted to 38.7 mg/100 ml. Earlier work on a dairy cattle population in the Moscow Province showed that an increase in the urea content in milk to 35.0-37.6 mg/100 ml led to a 0.27-0.55% decrease in MFF [22].

1. The impact of generalized linear model (GLM) **factors on milk productivity and composition of Holsteinized Black-and-White cows** (n = 1931, PZ Ladoga — a branch of the Ernst FRC VIZH, Krasnodar Territory, 2020-2021)

Parameter	M±SEM	Cv, %	Factor				R^2
			DL	MM	ML	DSCC/SCC	
Daily milk yield, kg	25.4±0.2	28.0	***	***	***	***	0.240
MFF, %	3.61±0.02	26.0	***	t	***	***	0.273
MFP, %	3.20±0.01	13.8	***	nr	***	***	0.315
MFC, %	2.69±0.01	13.8	***	nr	***	***	0.310
MFL, %	4.82±0.01	4.5	***	nr	**	***	0.285
Dry matter, %	12.72±0.028	9.5	***	nr	***	***	0.268
DSMR, %	9.08±0.01	5.3	***	nr	***	***	0.195
Acetone, mmol/l	0.055±0.002	160.1	***	***	***	**	0.171
BHB, mmol/l	0.013±0.001	326.4	*	***	***	***	0.256
Urea, mg/100 ml	38.7±0.1	16.8	nr	***	***	t	0.514
$C_{14:0}$, g/100 g	0.336±0.002	25.9	***	t	***	***	0.196
$C_{16:0}$, g/100 g	0.887±0.005	26.2	***	t	***	***	0.182
$C_{18:0}$, g/100 g	0.336±0.003	34.9	nr	nr	***	***	0.200
$C_{18:1}$, g/100 g	1.156±0.007	27.5	***	t	***	***	0.252
LCFA, g/100 g	1.426±0.010	30.7	**	*	***	**	0.263
MCFA, g/100 g	1.358±0.008	26.4	***	*	***	***	0.207
SCFA, g/100 g	0.479±0.003	31.0	***	nr	***	***	0.304
MUFAs, g/100 g	1.076±0.007	27.4	***	t	***	***	0.211
PUFAs, g/100 g	0.131±0.001	22.9	***	***	***	***	0.325
SFAs, g/100 g	2.373±0.015	27.7	***	t	***	***	0.275
SCC, thousand cells per ml	832±31	166.1	nr	nr	nr	***	0.526
DSCC, %	63.5±0.6	39.8	nr	t	***	***	0.685

N o t e. DL — lactation days, MM — milkmaid, ML — lactation month, DSCC/SCC — proportion of lymphocytes + polymorphonuclear neutrophils (DSCC) in total somatic cell counts (SCC) (8 subgroups), R^2 — determination coefficient; MFF — mass fraction of fat, MFP — mass fraction of protein, MFC — mass fraction of casein, MFL — mass fraction of lactose, DSMR — dry skimmed milk residue, BHB — beta-hydroxybutyrate, fatty acids $C_{14:0}$ — myristic, $C_{16:0}$ — palmitic, $C_{18:0}$ — stearic, $C_{18:1}$ — oleic, LCFA — long-chain fatty acids, MCFA — medium-chain fatty acids, SCFA — short-chain fatty acids, MUFAs — monounsaturated FAs, PUFAs — polyunsaturated FAs, SFAs — saturated FAs.
$^t p \le 0.1$, * $p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$; nr — unreliable.

The milk fatty acid distribution was as follows: by saturation of the carbon chain 66.3% SFA, 30.1% MUFA, 3.7% PUFA; by the length of the carbon chain 39.8% LCFA, 37.9% MCFA, SCFA 13.4%, trans-isomers of fatty acids 2.7%; by the number of carbon atoms in the FA chain 32.3% oleic ($C_{18:1}$), 24.8% palmitic ($C_{16:0}$), 9.4% myristic ($C_{14:0}$) and stearic ($C_{18:0}$) each. The proportions of fatty acids in the population of Holsteinized cattle in the Moscow region was similar [23], however, the proportion of the milk fat fraction was higher (4.46%), while SFA accounted for 78.2%, MUFA for 21.8%, PUFA for 0.1% [23].

The detected milk SCC was consistent with an increased incidence of subclinical and clinical forms of mammary gland diseases in the herd, as well as persistent chronic mastitis. The coefficient of phenotypic variability was the highest for SCC (166.1%), acetone content (160.1%) and BHB (326.4%), which may indicate a significant influence of paratypical conditions (keeping technology, management, diet of animals). The minimum variability occurred for MDL (4.5%), DSMR (5.3%) and DM (9.5%). The variability of the main indicators of milk production ranged from 13.8% for protein to 26.0% for fat (including 22.9-34.9% FA) and 28.0% for daily milk yield. According to the model equation for a number of fixed and regression factors, the day of lactation and the stage of lactation had no effect on the of milk urea concentration, the content of stearic FA, SCC and DSCC.

The milkmaid factor turned out to be the least significant of all those considered and was not statistically significant for the content of protein, casein, lactose, dry matter and DSMR, stearic FA, short-chain FA and SCC. The milkmaid factor slightly affected ($p \le 0.1$) the MFF, the content of myristic and palmitic

FAs, MUFAs, SFAs and DCSCs In all likelihood, the effect of milking on the composition of cows' milk was eliminated by other more significant factors.

The month of lactation had a highly significant significance ($p \leq 0.01$-0.001) for all the studied parameters, except for SCC. The influence of factors from eight DSCC/SCC subgroups on the variability of milk parameters turned out to be highly significant ($p \leq 0.01$-0.001), with the exception of the concentration of urea ($p \leq 0.1$). The coefficient of determination of the model expectedly showed higher values for SCC ($R^2 = 0.526$), DSCC ($R^2 = 0.685$) and for urea ($R^2 = 0.514$). In other cases, the reliability of the model ranged from $R^2 = 0.171$ for acetone to $R^2 = 0.325$ for polyunsaturated fatty acids.

Table 2 submits the distribution of the least squares estimates for the trait of milk productivity, depending on the predicted state of the mammary gland, with the gradations of groups A, B, C and D. The highest daily milk yield (27.7 kg, $p \leq 0.001$) were characteristic for cows from group B, that is, individuals with suspected inflammatory processes in the mammary gland, however, SCC in group B was below 200 thousands per milliliter. This may be due to an increase in the number of PMN and leukocytes (78.3%, $p \leq 0.001$) at an early stage of bacterial infection and the subsequent immune response to phagocytosis. It is expected that after the absorption of the pathogen, a gradual increase in the activity of macrophages will occur followed by normalization of the udder function.

2. Parameters of milk productivity and composition of Holsteinized Black-and-White cows as influenced by the udder condition (*M*±SEM, PZ Ladoga — a branch of the Ernst FRC VIZH, Krasnodar Territory, 2020-2021)

Parameter	Group			
	A (n = 96)	B (n = 40)	C (n = 115)	D (n = 62)
Number of observations, %	29.5	6.4	50.7	13.4
Daily milk yield, kg	25.7±0.3***	27.7±0.6$^{**/***}$	25.5±0.3***	23.3±0.5
MFF, %	3.39±0.04	3.32±0.08	3.57±0.04***	3.69±0.06***
MFP, %	3.10±0.02	3.05±0.04	3.22±0.02***	3.34±0.03***
MFC, %	2.62±0.02	2.58±0.03	2.70±0.01***	2.80±0.02***
MFL, %	4.93±0.01***	4.92±0.02***	4.78±0.01***	4.74±0.01
Dry matter, %	12.51±0.06	12.37±0.10	12.66±0.05$^{*/**}$	12.89±0.08$^{**/***}$
DSMR, %	9.09±0.02	9.01±0.04	9.06±0.02	9.16±0.03***
Acetone, mmol/l	0.063±0.004*	0.064±0.008	0.052±0.004	0.065±0.006
BHB, mmol/l	0.010±0.002	0.012±0.004	0.012±0.002	0.023±0.003$^{*/***}$
Urea, mg/100 ml	37.9±0.3	37.8±0.4	37.4±0.2	37.8±0.3
$C_{14:0}$, g/100 g	0.318±0.004	0.315±0.008	0.337±0.004***	0.343±0.006***
$C_{16:0}$, g/100 g	0.823±0.012	0.811±0.021	0.897±0.010***	0.907±0.016***
$C_{18:0}$, g/100 g	0.320±0.006	0.308±0.010	0.327±0.005	0.342±0.008**
$C_{18:1}$, g/100 g	1.108±0.015	1.078±0.027	1.131±0.013	1.186±0.020$^{*/**}$
LCFA, g/100 g	1.375±0.021	1.330±0.037	1.384±0.018	1.447±0.028*
MCFA, g/100 g	1.256±0.018	1.236±0.032	1.372±0.015***	1.401±0.024***
SCFA, g/100 g	0.444±0.007	0.438±0.012	0.471±0.006**	0.492±0.009***
MUFAs, g/100 g	1.035±0.015	1.009±0.026	1.056±0.012	1.104±0.019$^{*/**}$
PUFAs, g/100 g	0.125±0.001	0.122±0.002	0.127±0.001	0.131±0.002**
SFAs, g/100 g	2.203±0.031	2.160±0.056	2.361±0.027$^{**/***}$	2.444±0.041***
SCC, thousand cells per ml	23.7±67.6	184.8±120.4	1315.9±57.5***	467.8±89.5***
DSCC, %	33.7±0.8	78.3±1.4***	80.2±0.7***	59.4±1.0***
EMV, rub. · day^{-1} · head^{-1}	714±10***	744±19***	516±9	585±14***

N o t e. DSCC/SCC — proportion of lymphocytes + polymorphonuclear neutrophils (DSCC) in total somatic cell counts (SCC), MFF — mass fraction of fat, MFP — mass fraction of protein, MFC — mass fraction of casein, MFL — mass fraction of lactose, DSMR — dry skimmed milk residue, BHB — beta-hydroxybutyrate, fatty acids $C_{14:0}$ — myristic, $C_{16:0}$ — palmitic, $C_{18:0}$ — stearic, $C_{18:1}$ — oleic, LCFA — long-chain fatty acids, MCFA — medium-chain fatty acids, SCFA — short-chain fatty acids, MUFAs — monounsaturated FAs, PUFAs — polyunsaturated FAs, SFAs — saturated FAs, EMV — estimated milk value. For a description of the groups, see the Materials and methods section. Sequential pairwise comparison is performed.
* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Cows of group B were inferior to the rest in percentage of milk fat, protein, fatty acids, BHB ($p \leq 0.001$), except for lactose. However, in terms of EMV, these animals outperformed others by 30-228 rub. Cows of group A (healthy individuals) showed the best scores for SCC and DSCC, 23.7 thousand cells per ml and 33.7%,

respectively, with a higher daily milk yield (25.7 kg) compared to groups C and D. For the main milk components, these cows were inferior to those with high SCC value, except for MDL ($p \leq 0.001$) and traces of acetone ($p \leq 0.05$); the EMV values were close to group B, 714 rub. $\cdot$ day^{-1} $\cdot$ head^{-1}.

The cows of groups C (with subclinical or clinical active mastitis) and D (with chronic mastitis) exceeded other groups in the milk components, while have a relatively lower daily milk production. In animals from group C, MFF was 0.18 and 0.25% higher ($p \leq 0.001$) compared to groups A and B, in cows of group D 0.30 and 0.37% higher. MFP increased by 0.12 and 0.17%, respectively, and by 0.24 and 0.29%, respectively. The fatty acid content and casein also changed.

It should be noted that in cows of group D, the amount of milk ketone bodies increased, while for BHB, there was a highly significant difference, +0.011 (A/D, $p \leq 0.001$) and +0.013 mmol/l (B/D, $p \leq 0.05$), which is consistent with the results for the Holstein cattle population in Canada [24].

Despite higher MFF and MFP parameters, the economic efficiency of groups C and D was only 516 and 585 rub. $\cdot$ day^{-1} $\cdot$ head^{-1}. We believe that in our case, increased fat and protein secretion could be due to the active phase of the inflammation at the beginning (after calving and before the peak of daily milk yield) and the end of lactation (from day 210 after calving to the dry period), when there was a physiological maximum production of milk components. Schwarz et al. [25-27] noted similar patterns for populations of Holstein, Simmental and Brown Swiss breeds in Austria, China, Estonia, Germany and Spain.

We compared these parameters with respect to DSCC/SCC gradation for eight subgroups for a more detailed characterization of the changes in the daily productivity and milk composition (Table 3). It was found that the average daily milk yield of animals with SCC $\leq$ 200 thousand cells per/ml was 1.8-5.8 kg higher ($p \leq 0.001$) for DSCC $\leq$ 70% and 0.9-3.0 kg higher ($p \leq 0.001$) for DSCC > 70% compared to subgroups with other SCC scores. For the main milk component parameterss, e.g., MFF, MFP, and MFC, there was a significant superiority of cows' subgroups with CSC > 200 thousand cells per ml.

We concluded that an increased number of somatic cells in milk (in particular, > 1 million cells) changes the proportion of some FAs, regardless of the DSCC threshold. So, when comparing the contrasting subgroups with SCC $\leq$ 200 and $\geq$ 1001 thousand cells per ml, the greatest differences were found in the total fats, from +1.1 to +1.4 p.p. for SFAs, from +0.4 to +1.2 p.p. for palmitic FA, and from +1.0 to +1.4 p.p. for MCFAs. There also was a decrease in stearic and oleic fatty acids (by 0.6 and 1.0 p.p.), long-chain fatty acids (by 1.7-2.5 p.p.), monounsaturated fatty acids (0 .9-1.2 p.p.), and polyunsaturated fatty acids (by 0.2 p.p.). An increase in the fraction of saturated fatty acids with a simultaneous decrease in MUFA and PUFA leads to a decrease in the quality of processed products, since fatty acids with many double carbon bonds are recognized as the most useful for humans. Significantly higher values of EMV were in animals with SCC $\leq$ 200 thousand cells per ml (by 423-444 rubles compared to the cows with SCC $\leq$ 1001 thousand cells per ml).

Figure 2 shows the daily productivity vs. the normalized SCC estimates. In daily milk yield, cows with a SCC score of 1 point were statistically significantly ($p \leq 0.1$-0.001) superior by 1.5-6.0 kg to cows with 4 points and higher. The distribution of LS-estimates for the fat, protein and lactose fractions was similar to that for the DSCC/SCC control groups, that is, with an increase in the score, the MFF and MFP increased from 3.34 to 4.24% and from 3.02 to 3.57%. The dynamics of the MFL had an inverse pattern and averaged –0.062 p.p. for each point of the SCC score. In our opinion, this may be due to the more active use of milk sugar by the bacterial microbiota of the mammary gland.

3. Milk productivity and milk composition of Holsteinized Black-and-White cows depending on the SCC score gradations and DSCC values based on the LS-estimates and (*M*±SEM, PZ Ladoga — a branch of the Ernst FRC VIZH, Krasnodar Territory, 2020-2021)

Parameter	SCC, thousand cells per ml							
	DSCC ≤ 70 % (*n* = 829)				DSCC > 70 % (*n* = 1102)			
	≤ 200	201-500	501-1000	≥ 1001	≤ 200	201-500	501-1000	≥ 1001
Number of observations, %	29,5	7,8	4,3	1,4	6,4	15,3	14,5	20,9
Daily milk yield, kg	25,7±0,3***	23,9±0,6***	22,9±0,7*	19,9±1,2	27,7±0,6***	26,8±0,4***	25,2±0,4	24,7±0,4
MFF, %	3,39±0,04	3,71±0,07***	3,65±0,09*	3,95±0,16***	3,31±0,08	3,41±0,05	3,53±0,06*	3,72±0,05**/***
MFP, %	3,10±0,02	3,33±0,03***	3,37±0,04***	3,41±0,07***	3,05±0,04	3,13±0,02	3,24±0,03***	3,29±0,02***
MFC, %	2,62±0,02	2,80±0,03***	2,81±0,04***	2,85±0,06***	2,58±0,03	2,64±0,02	2,73±0,02***	2,74±0,02***
MFL, %	4,93±0,01***	4,80±0,02***	4,67±0,02***	4,55±0,04	4,93±0,02**/***	4,87±0,01***	4,81±0,01***	4,67±0,01
Dry matter, %	12,51±0,06	12,95±0,09***	12,80±0,12*	13,04±0,21**	12,36±0,10	12,50±0,07	12,68±0,07**	12,77±0,06***
DSMR, %	9,09±0,02	9,21±0,04***	9,11±0,05	9,05±0,09	9,01±0,04	9,05±0,03	9,11±0,03*	9,03±0,03
Acetone, mmol/l	0,063±0,004	0,056±0,007	0,071±0,009	0,097±0,016*/**	0,064±0,008*	0,049±0,005	0,046±0,006	0,060±0,005*
BHB, mmol/l	0,011±0,002	0,015±0,003	0,025±0,004**	0,068±0,008***	0,012±0,004	0,008±0,003	0,006±0,003	0,021±0,002***
Urea, mg/100 ml	37,9±0,3	37,8±0,4	37,4±0,5	38,5±0,9	37,9±0,4	38,0±0,3**	37,4±0,3	37,0±0,3
$C_{14:0}$, g/100 g	0,318±0,004	0,347±0,007***	0,331±0,009	0,366±0,016**	0,314±0,008	0,327±0,005	0,338±0,005**	0,344±0,005**
$C_{16:0}$, g/100 g	0,827±0,012	0,910±0,019***	0,897±0,025**	0,987±0,042***	0,808±0,021	0,844±0,014	0,879±0,015**	0,954±0,013***
$C_{18:0}$, g/100 g	0,320±0,006	0,345±0,009*	0,341±0,012	0,354±0,021	0,307±0,010	0,311±0,007	0,317±0,007	0,348±0,006**/***
$C_{18:1}$, g/100 g	1,108±0,015	1,184±0,025**	1,184±0,032*	1,263±0,055***	1,076±0,027	1,085±0,019	1,116±0,019	1,178±0,017**/***
LCFA, g/100 g	1,376±0,021	1,455±0,034*	1,429±0,044	1,519±0,075	1,327±0,037	1,336±0,025	1,368±0,026	1,434±0,023**
MCFA, g/100 g	1,256±0,018	1,404±0,029***	1,384±0,037**	1,516±0,063***	1,232±0,031	1,300±0,022	1,354±0,022**	1,441±0,020**/***
SCFA, g/100 g	0,444±0,007	0,498±0,011***	0,477±0,015*	0,532±0,025***	0,437±0,012	0,458±0,008	0,467±0,009*	0,483±0,008*/**
MUFAs, g/100 g	1,035±0,014	1,102±0,024**	1,102±0,031*	1,171±0,052**	1,006±0,026	1,009±0,018	1,042±0,018	1,102±0,016**/***
PUFAs, g/100 g	0,125±0,001	0,133±0,002**	0,127±0,003	0,138±0,005*	0,122±0,002	0,124±0,002	0,127±0,002	0,130±0,002**
SFAs, g/100 g	2,204±0,031	2,453±0,051***	2,409±0,066**	2,646±0,111***	2,154±0,055	2,249±0,038	2,329±0,039**	2,472±0,035**/***
SCC, thousand cells per ml	28±53	281±86*	586±112*/***	2039±189***	119±94	312±64	661±66***	2622±59***
DSCC, %	33,7±0,8	59,2±1,3***	60,4±1,7***	59,3±2,8***	78,1±1,4	78,2±1,0	79,7±1,0	82,3±0,9*/**
EMV, rub. · day^{-1} · $head^{-1}$	713±8***	636±14***	551±18***	269±30	753±15***	671±10***	593±11***	330±9

N o t e. DSCC/SCC — proportion of lymphocytes + polymorphonuclear neutrophils (DSCC) in total somatic cell counts (SCC), MFF — mass fraction of fat, MFP — mass fraction of protein, MFC — mass fraction of casein, MFL — mass fraction of lactose, DSMR — dry skimmed milk residue, BHB — beta-hydroxybutyrate, fatty acids $C_{14:0}$ — myristic, $C_{16:0}$ — palmitic, $C_{18:0}$ — stearic, $C_{18:1}$ — oleic, LCFA — long-chain fatty acids, MCFA — medium-chain fatty acids, SCFA — short-chain fatty acids, MUFAs — monounsaturated FAs, PUFAs — polyunsaturated FAs, SFAs — saturated FAs, EMV — estimated milk value. For a description of the groups, see the Materials and methods section. Sequential pairwise comparison is performed.
* $p \le 0.05$, * $p \le 0.01$, *** $p \le 0.001$.

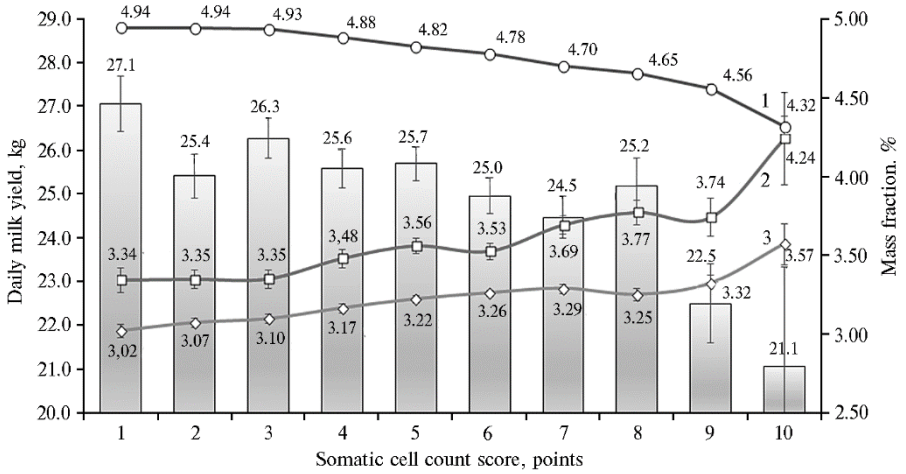


Fig. 2. Distribution of the least squares method-based LS-estimates for parameters of milk productivity and milk composition in Holsteinized Black-and-White cows depending on the somatic cell counts (SCC): diagram shows daily milk yield, 1 — mass fraction of lactose (MFL), 2 — mass fraction of fat (MFF), 3 — mass fraction of protein (MFP) (PZ Ladoga — a branch of the Ernst FRC VIZH, Krasnodar Territory, 2020-2021).

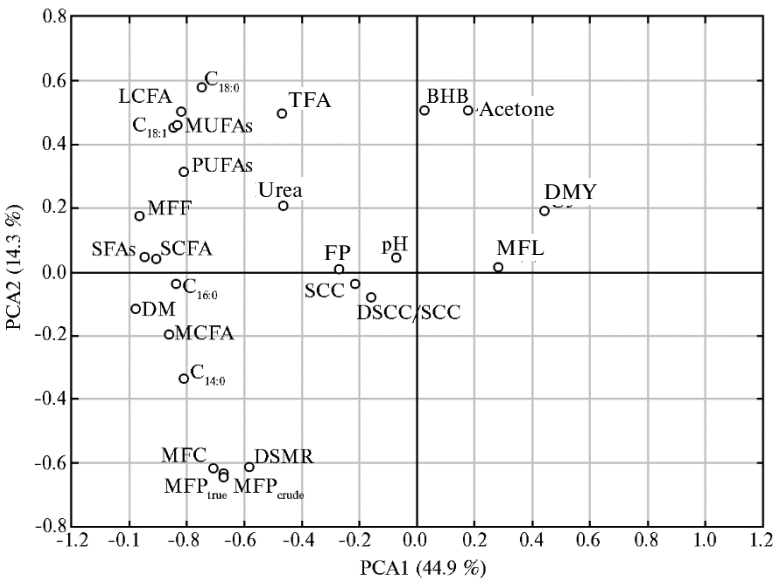


Fig. 3. Principal component analysis (PCA) **of milk parameter distribution in Holsteinized Black-and-White cows depending on variability along two factor axes:** DM — dry matter, DMY — daily milk yield, MFF — mass fraction of fat, MFP_{true} — mass fraction of true protein, MFP_{crude} — mass fraction of crude protein MFC — mass fraction of casein, MFL — mass fraction of lactose, DSMR — dry skimmed milk residue, BHB — beta-hydroxybutyrate, Acetone, Urea, fatty acids $C_{14:0}$ — myristic, $C_{16:0}$ — palmitic, $C_{18:0}$ — stearic, $C_{18:1}$ — oleic, LCFA — long-chain fatty acids, MCFA — medium-chain fatty acids, SCFA — short-chain fatty acids, MUFAs — monounsaturated FAs, PUFAs — polyunsaturated FAs, SFAs — saturated FAs, TFA — trans fatty acid isomers, sSCC — total somatic cell counts, FP — freezing point, DSCC — lymphocytes + polymorphonuclear neutrophils in SCC (PZ Ladoga — a branch of the Ernst FRC VIZH, Krasnodar Territory, 2020-2021).

We used principal component analysis (PCA) method to reveal co-variation of parameters characterizing milk composition and mechanisms underlying its formation (Fig. 3). The most significant variables turned out to be MFF, SFA and dry matter ($P \geq 0.99$), that is, their scatter of variables was minimized or evenly ordered, and the variance was maximized with respect to component 2. Using visualization based on two components of variability, it was shown that the protein

and fat fractions of milk formed their own clusters, while for a number of fatty acids, there was structural differentiation by the groups of medium- and long-chain fatty acids, mono- and polyunsaturated fatty acids. Urea as one of the factors of variability was closer to fatty acids. For biomarkers of metabolism (acetone and BHB), technological qualities (TQ and pH) and somatic cells (SCC, DSCC), their clusters were designated, not associated with other indicators of milk composition. The first component singled out the daily milk yield and the mass fraction of lactose (together with ketone bodies) into a separate group, thus denoting the independent (and at the same time complex) nature of the variability of these parameters.

The approach in which somatic cells are differentiated according to their types is of interest for accurate diagnosis of mammary gland physiology disorders, product quality control, and optimization of economic costs in the herd. Our findings have shown that the gradation of animals by groups based on DSCC and SCC makes it possible to distinguish individuals according to the likelihood of a subclinical form of mastitis. The obtained distribution of the milk productivity trait values and economic efficiency of cows indicated a significant influence of the studied factors. Schwarz et al. [26] reported similar data. On a sample of animals of different breeds from the dairy cattle populations of Austria, China, Estonia, Germany, and Spain, similar patterns were found in the composition of cow milk (percentage of fat, protein, lactose, traces of urea) and EMV. Previous studies [9, 25] have shown a close relationship between DSCC and SCC (both in combination and separately) with the presence of an infectious form of mastitis. With an increase in DCSC, the sensitivity of this biomarker in predicting the manifestation of infection increased.

It is also promising to study the structure of milk microbiota depending on milk composition, the number of somatic cells and their types. In Russia, an analysis of the association of *Staphylococcus aureus* isolates from milk with the manifestation of mastitis in cows has already been carried out [28]. We believe that the control over the change in the composition of cow's milk in the herd can be carried out using a complex of biomarkers, e.g., the amount of lactose, traces of acetone and BHB, which is currently used routinely in the farms of the Moscow region to predict the occurrence of mastitis and ketosis [22]. Principal component analysis has shown its effectiveness for determining the boundaries of interdependent variability of the component composition of milk in order to identify signs with independent variability.

Thus, the somatic cell counts in milk and the SCC differentiation by species can serve as additional criteria for predicting and monitoring the spread of mastitis. Our findings confirm the possibility of individual assessment of the mammary gland state based on the proportion of lymphocytes and polymorphonuclear neutrophils (differentiated somati cell count, DSCC) in the total amount of somatic cells in milk (SCC). Animals assigned to healthy individuals and individuals with suspected mastitis (predisposed to the onset of infection) had the desired indicators of milk production and economic efficiency. Animals with high SCC values, as well as with chronic mastitis were the most prone to metabolic disorders and ketosis, regardless of the SCC value. An increase in the percentage of fat in the milk of animals having SCC $\geq$ 1 million cells per ml led to a change in the ratio of fatty acids with an increase in the amount of saturated, medium chain and palmitic FAs. The normalized SCC estimates (SCCE) more clearly show the observed patterns in the change of cows' milk productivity parameters. An increase in SCCE by one point (limits from 1 to 10) led to a drop in daily milk yield by 0.6 kg of milk, in lactose by 0.062 p.p. and to an increase in the fat and protein levels by 0.090 and 0.055 p.p, respectively. Principal component analysis revealed structural clustering of milk composition parameter for fat and protein fractions, traces of metabolites (acetone, beta-

hydroxybutyrate), and somatic cell counts. Further study of the relationship between the synthesis of milk components in the mammary gland and the animal physiological status will clarify breeding parameters and genetic background of productivity traits. The development of methods for express diagnostics of animal health based on an expanded analysis of the milk component composition is one of the priorities for practical application of our research in the future.

REFERENCES

1. Hanuš O., Samková E., Křížová L., Hasoňová L., Kala R. Role of fatty acids in milk fat and the influence of selected factors on their variability — a review. *Molecules*, 2018, 23(7): 1636 (doi: 10.3390/molecules23071636).
2. Zaalberg R.M., Shetty N., Janss L., Buitenhuis A.J. Genetic analysis of Fourier transform infrared milk spectra in Danish Holstein and Danish Jersey. *Journal of Dairy Science*, 2019, 102(1): 503-510 (doi: 10.3168/jds.2018-14464).
3. Carvalho-Sombra T.C.F., Fernandes D.D., Bezerra B.M.O., Nunes-Pinheiro D.C.S. Systemic inflammatory biomarkers and somatic cell count in dairy cows with subclinical mastitis. *Veterinary and Animal Science*, 2021, 11: 100165 (doi: 10.1016/j.vas.2021.100165).
4. Pillai S.R., Kunze E., Sordillo L.M., Jayarao B.M. Application of differential inflammatory cell count as a tool to monitor udder health. *Journal of Dairy Science*, 2001, 84(6): 1413-1420 (doi: 10.3168/jds.S0022-0302(01)70173-7).
5. Rivas A.L., Quimby F.W., Blue J., Coksaygan O. Longitudinal evaluation of bovine mammary gland health status by somatic cell counting, flow cytometry, and cytology. *Journal of Veterinary Diagnostic Investigation,* 2001, 13(5): 399-407 (doi: 10.1177/104063870101300506).
6. Pilla R., Malvisi M., Snel G., Schwarz D., König S., Czerny C.-P., Piccinini R. Differential cell count as an alternative method to diagnose dairy cow mastitis. *Journal of Dairy Science*, 2013, 96(3): 1653-1660 (doi: 10.3168/jds.2012-6298).
7. Sordillo L.M., Shafer-Weaver K., DeRosa D. Immunobiology of the mammary gland. *Journal of Dairy Science*, 1997, 80(8): 1851-1865 (doi: 10.3168/jds.S0022-0302(97)76121-6).
8. Oviedo-Boyso J., Valdez-Alarcón J.J., Cajero-Juárez M., Ochoa-Zarzosa A., López-Meza J.E., Bravo-Patiño A., Baizabal-Aguirre V.M. Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *Journal of Infection*, 2007, 54(4): 399-409 (doi: 10.1016/j.jinf.2006.06.010).
9. Halasa T., Kirkeby C. Differential somatic cell count: value for udder health management. *Frontiers in Veterinary Science*, 2020, 7: 609055 (doi: 10.3389/fvets.2020.609055).
10. Lee C., Wooding F., Kemp P. Identification, properties, and differential counts of cell populations using electron microscopy of dry cows secretions, colostrum and milk from normal cows. *Journal of Dairy Research*, 1980, 47(1): 39-50 (doi: 10.1017/S0022029900020860).
11. Schwarz D., Diesterbeck U.S., König S., Brügemann K., Schlez K., Zschöck M., Wolter W. Czerny C.P. Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. *Journal of Dairy Science*, 2011, 94(10): 5033-5044 (doi: 10.3168/jds.2011-4348).
12. Schwarz D., Diesterbeck U.S., König S., Brügemann K., Schlez K., Zschöck M., Wolter W., Czerny C.-P. Microscopic differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. *Journal of Dairy Research*, 2011, 78(4): 448-455 (doi: 10.1017/S0022029911000574).
13. Pilla R., Schwarz D., König S., Piccinini R. Microscopic differential cell counting to identify inflammatory reactions in dairy cow quarter milk samples. *Journal of Dairy Science*, 2012, 95(8): 4410-4420 (doi: 10.3168/jds.2012-5331).
14. Paape M.J., Mehrzad J., Zhao X., Detilleux J., Burvenich C. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocyte. *Journal of Mammary Gland Biology and Neoplasia,* 2002, 7(2): 109-121 (doi: 10.1023/a:1020343717817).
15. Schwarz D. Differential somatic cell count — a new biomarker for mastitis screening. *Proc. of the 40th ICAR Biennial Session held in Puerto Varas, Chile, 24-28 October 2016.* ICAR, Rome, Italy, 2017: 105-113.
16. Damm M., Holm C., Blaabjerg M., Novak Bro M., Schwarz D. Differential somatic cell count—a novel method for routine mastitis screening in the frame of dairy herd improvement testing programs. *Journal of Dairy Science*, 2017, 100(6): 4926-4940 (doi: 10.3168/jds.2016-12409).
17. Sermyagin A.A., Gladyr E.A., Kharzhau A.A., Plemyashov K.V., Tyurenkova E.N., Reyer H., Wimmers K., Brem G., Zinovieva N.A. 167 Genetic and genomic estimation for somatic cell score in relation with milk production traits of Russian Holstein dairy cattle. *Journal of Dairy Science*, 2017, 95(4): 82-83 (doi: 10.2527/asasann.2017.167).
18. Naryshkina E.N., Sermyagin A.A., Vinogradova I.V., Khripyakova E.N. V sbornike: *Puti*

prodleniya produktivnoi zhizni molochnykh korov na osnove optimizatsii razvedeniya, tekhnologii soderzhaniya i kormleniya zhivotnykh. Materialy mezhdunarodnoi nauchno-prakticheskoi konferentsii [In: Ways to prolong the productive life of dairy cows by the optimization of breeding, keeping and feeding technologies. Materials of the international scientific and practical conference]. Dubrovitsy, 2015: 69-73 (in Russ.).
19. Wiggans G.R., Shook G.E.A Lactation measure of somatic cell count. *Journal of Dairy Science*, 1987, 70(12): 2666-2672 (doi: 10.3168/jds.S0022-0302(87)80337-5).
20. StatSoft, Inc. STATISTICA (data analysis software system), version 12. (2014). www.statsoft.com.
21. Chasovshchikova M.A., Gubanov M.V. *Vestnik KrasGAU*, 2021, 9(174): 132-137 (doi: 10.36718/1819-4036-2021-9-132-137) (in Russ.).
22. Sermyagin A.A., Belous A.A., Kornelaeva M.V., Filipchenko A.A., Kisel' E.E., Bukarov N.G., Ermilov A.N., YAnchukov I.N., Zinov'eva N.A. V knige: *Plemennaya rabota v zhivotnovodstve Moskovskoi oblasti i g. Moskvy (2017 g.)* [In: Pedigree work in animal husbandry in the Moscow region and Moscow (2017)]. Moscow, 2018: 11-22 (in Russ.).
23. Lashneva I.A., Sermyagin A.A. *Dostizheniya nauki i tekhniki APK*, 2020, 34(3): 46-50 (doi: 10.24411/0235-2451-2020-10309) (in Russ.).
24. Santschi D.E., Lacroix R., Durocher J., Duplessis M., Moore R.K., Lefebvre D.M. Prevalence of elevated milk b-hydroxybutyrate concentrations in Holstein cows measured by Fourier-transform infrared analysis in dairy herd improvement milk samples and association with milk yield and components. *Journal of Dairy Science,* 2016, 99(11): 9263-9270 (doi: 10.3168/jds.2016-11128).
25. Schwarz D., Lipkens Z., Piepers S., De Vliegher S. Investigation of differential somatic cell count as a potential new supplementary indicator to somatic cell count for identification of intramammary infection in dairy cows at the end of the lactation period. *Preventive Veterinary Medicine*, 2019, 172: 104803 (doi: 10.1016/j.prevetmed.2019.104803).
26. Schwarz D., Kleinhans S., Reimann G., Stückler P., Reith F., Ilves K., Pedastsaar K., Yan L., Zhang Z., Valdivieso M., Barreal M.L., Fouz R. Investigation of dairy cow performance in different udder health groups defined based on a combination of somatic cell count and differential somatic cell count. *Preventive Veterinary Medicine*, 2020, 183: 105123 (doi: 10.1016/j.prevetmed.2020.105123).
27. Schwarz D., Santschi D.E., Durocher J., Lefebvre D.M. Evaluation of the new differential somatic cell count parameter as a rapid and inexpensive supplementary tool for udder health management through regular milk recording. *Preventive Veterinary Medicine*, 2020, 181: 105079 (doi: 10.1016/j.prevetmed.2020.105079).
28. Fursova K., Sorokin A., Sokolov S., Dzhelyadin T., Shulcheva I., Shchannikova M., Nikanova D., Artem'eva O., Zinovieva N., Brovko F. Virulence Factors and Phylogeny of *Staphylococcus aureus* associated with bovine mastitis in Russia based on genome sequences. *Frontiers in Veterinary Science*, 2020, 7: 135 (doi: 10.3389/fvets.2020.00135).

AGRICULTURAL BIOLOGY,
2021, V. 56, Iss. 6, pp. 1199-1208
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

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

UDC 636.12:614.9

doi: 10.15389/agrobiology.2021.6.1199eng
doi: 10.15389/agrobiology.2021.6.1199rus

BIOLUMINESCENT SPORT HORSE SALIVA TEST: PROSPECTS FOR USE

L.Yu. SHTUMPF[1], O.V. KOLESNIK[1, 4], L.V. STEPANOVA[1] ✉, O.A. KOLENCHUKOVA[1, 2], A.S. FEDOTOVA[3], A.V. KOLOMEYTSEV[3], A.V. MAKAROV[1], V.A. KRATASYUK[1, 4]

[1]*Siberian Federal University*, 79, pr. Svobodnii, Krasnoyarsk, 660041 Russia, e-mail liubovs23@mail.ru, olga.kolesnik.krsk@gmail.com, slyudmila@mail.ru (✉ corresponding author), kalina-chyikova@mail.ru, andmak83@yandex.ru, valkrat@mail.ru;
[2]*Research Institute of Medical Problems of the North — Subdivision of Federal Research Center Krasnoyarsk Scientific Center SB RAS,* 3G, ul. Partizana Zheleznyaka, Krasnoyarsk, 660022 Russia, e-mail kalina-chyikova@mail.ru;
[3]*Krasnoyarsk State Agrarian University,* 90, pr. Mira, Krasnoyarsk, 660049 Russia, e-mail krasfas@mail.ru, avk1978@list.ru;
[4]*Institute of Biophysics SB RAS — Subdivision of Federal Research Center Krasnoyarsk Scientific Center SB RAS,* 50/50, Akademgorodok, Krasnoyarsk, 660036 Russia
ORCID:
Shtumpf L.Yu. orcid.org/0000-0001-6055-963X
Kolesnik O.V. orcid.org/0000-0002-4637-875X
Stepanova L.V. orcid.org/0000-0001-5503-4898
Kolenchukova O.A. orcid.org/0000-0001-9552-447X
Fedotova A.S. orcid.org/0000-0003-1630-2444
Kolomeytsev A.V. orcid.org/0000-0002-2203-3415
Makarov A.V. orcid.org/0000-0002-2593-207X
Kratasyuk V.A. orcid.org/0000-0001-6764-5231

The authors declare no conflict of interests
Acknowledgements:
Funded by the Russian Foundation for Basic Research and the Krasnoyarsk Regional Science Foundation, project No. 19-416-240001
Received May 5, 2021

Abstract

Assessment of the physiological state of horses in training is a relevant problem of sport horse breeding worldwide. Existing clinical methods do not provide reliable parameters of the functional state of animals at rest and in physical activity. The reported standards of the physiological state of horses and veterinary guidelines for clinical diagnosis and equestrian sports are contradictory. Therefore, objective tests are necessary to assess the body's response to physical activity. We propose simple, fast, non-invasive method based on an inhibitory effect of saliva on the enzyme activity of luminous bacteria as a screening testing. Changes in the luminescence of the bioluminescent enzymatic system under influence of small amounts of saliva can reveal changes in the body of sport horses as a response to the maximum permissible loads. This study proves for the first time that the bioluminescent enzyme test can track changes in the body condition of sport horses during training. The method uses the integral bioluminescent indicator which depends on the biochemical composition of saliva. The aim of this study is to substantiate suitability of the bioluminescent-based method for testing the functional state of sports horses in training. Trakenen sport horses (*Eguus caballus*) for dressage (n = 12) kept under standard conditions in the Training Center of Horse Breeding Complex (Krasnoyarsk State Agrarian University) were subjected to low, medium and high intensity training before the competition (February-June 2019-2020). Saliva and blood were sampled before (in the morning) and after training. The respiratory rate (RR) and heart rate (HR) were measured. The electrocardiography (ECG) was carried out according to a common method, including assessmsnt of the heart rhythm parameters and the ventricular systolic functional parameters. Hematological test were performed, and blood concentrations of protein and glucose were measured. The saliva was tested by colorimetric, chemi-, and bioluminescent methods. As the intensity of physical activity increased, there was an increase in heart rate, respiration rate, atrial excitation and a decrease in the time of cardiac diastole while hematological and biochemical blood parameters varied within normal limits. The effect of saliva on the intensity of bioluminescence depended on the physical activity. The residual luminescence signal decreased under low and medium intensity training and increased under high intensity training. During low intensity training, a high percentage of luminescence inhibition correlated with an increase in the total blood protein concentration (r = 0.6, p = 0.05) and a decrease in the blood glucose content (r = –0.7, p = 0.05) and the number of erythrocytes (r = –0.6, p = 0.05). Under moderate physical activity, an increase in bioluminescent fluorescence correlated with an increase in RR (r = 0.5, p = 0.1) and in the QRS interval (r = 0.8, p = 0.05). Under high intensity training, a low percentage of luminescence inhibition correlated with the lactate concentration in saliva (r = –0.58, p = 0.1), a reduction in

catalase activity in saliva (r = –0.7, p = 0.05), and a higher amplitude of the P wave on the electrocardiogram (r = 0.8, p = 0.05). Therefore, the bioluminescent analysis of saliva using a coupled enzyme system, NADH:FMN-oxidoreductase and bacterial luciferase can detect the effect of stressful physical activity during horse training of various intensity. The inhibition of bioluminescence can be an indicator of a horse performance in training. The test can be also applicable in sport horse breeding to prevent overtraining.

Keywords: sport horses, saliva, lactate, catalase, NADH:FMN-oxidoreductase, luciferase, bacterial bioluminescence, functional status, hematological parameters, blood biochemistry

Modern domestic horse breeding is a fairly stable, well-structured agricultural sector that can confidently compete both in the global market for horse resources and among livestock industries within the country [1-3]. In the modern world ranking of sports horses, the pets of Russian studs occupy a fairly high position. Russian horses have repeatedly been champions, winners of international equestrian competitions in the USA, UAE [4, 5]. Around the world, the number of horses is rapidly increasing and interest in national breeds is growing [6-8].

Physiological and clinical methods for assessing the functional state of sports horses are objective, but lengthy and difficult to interpret [9-11]. Physiological studies in general do not give a general picture of the condition of the horse [12, 13]. The information presented in the literature on the standards of the physiological state of horses and in veterinary guidelines for clinical diagnostics and equestrian sport is very contradictory. For example, there are significant discrepancies in the values of the main physiological and clinical indicators (temperature, heart rate, number of respiratory movements) and there are no standards for assessing the state of a sports horse both during rest and after muscle work of varying tension [14, 15].

Today, the basic principles of sport horse breeding have undergone a significant transformation based on the principles of Welfare Quality®, according to which the well-being of the horse becomes an object of paramount importance and should never be subordinated to competitive or commercial interests [3, 16]. In this regard, it is of interest to develop non-invasive methods for assessing the functional state of the body [17, 18]. The use of saliva as a material for research removes restrictions on the frequency and availability of measurements during the training or competitive process and allows you to create a convenient tool for the daily work of a rider, trainer, veterinarian. It is also possible to individually control the assessment of the body's response to physical activity and adjust the training process to the response of a sports horse in real time [19, 20].

As a screening test for the saliva of a sports horse, we propose to use a bioluminescent method using a bacterial enzyme system [21, 22], which has proven effective in testing the condition of the human body [23, 24]. A change in the luminescence of a bioluminescent test system when exposed to a small amount of saliva can indicate deviations in the body of sports horses that occur in response to maximum permissible loads, and allow the rider to restructure the training process. An important characteristic of bioluminescent testing of horse saliva is non-invasiveness, which allows painless and quick testing during training.

In the present work, it is shown for the first time that changes in the state of the body of a sports horse can be controlled by an integral bioluminescent indicator, which depends on the biochemical composition of saliva.

The purpose of the work is to evaluate the possibility of using the bioluminescent method for testing the functional state of sports horses in training.

Materials and methods. The study was performed on a group of sports horses (*Eguus caballus*) of the Trakehner breed (n = 12) with a specialization in dressage. The horses were kept under standard conditions of the horse breeding training and sports complex of the Krasnoyarsk State Agrarian University. Testing

of each horse, collecting saliva and blood was carried out before and after training at low, medium and high intensity during the preparation for the competition (February-June 2019-2020).

Low-intensity physical activity included horse training for 1 hour, medium-intensity exercise for 1.5 hours, and high-intensity exercise for 2 hours on a lunge or under saddle. The training program consisted of the following stages: on a free rein, in collection at a trot with the inclusion of lateral elements, shortening and spreading of the gaits and transitions from one gait to another, a hitch at a trot on a long rein, a step.

Saliva samples (1.0-1.5 ml) were collected in disposable sterile plastic tubes. Sampling before exercise was carried out in the morning (before feeding). After physical exercise, saliva, which was formed in sufficient quantities, was taken immediately after the completion of training. Basically, this procedure did not affect the emotional state of the horses.

The functional state of the animal was assessed by respiratory rate (RR), heart rate (HR), and electrocardiogram (ECG). Respiratory rate was determined visually, heart rate was determined by ECG on an EK3T-01-R-D electrocardiograph (Monitor, Russia). ECG was recorded in three standard and three enhanced limb leads. ECG analysis was carried out according to the generally accepted method, including the determination of the nature of the heart rhythm: ventricular systolic index (AVS), the height and width of the teeth, and the duration of the intervals (25).

Hematological studies were performed according to the generally accepted method with the counting of erythrocytes and leukocytes in the Goryaev chamber, the hemoglobin content was determined by the method of Sahli [25]. Biochemical analysis of blood serum was carried out according to the generally accepted method, the content of protein and glucose was measured [25].

Before studying saliva, the samples were centrifuged for 15 min at 5000 rpm (Eppendorf Centrifuge 5810 r, Eppendorf, Germany).

The concentration of lactate (lactic acid) in saliva samples was measured by the photometric method (colorimetry) (a UV-1800 spectrophotometer, Shimadzu, Japan) in accordance with the description [26, 27].

Chemiluminescent and bioluminescent testing of saliva was performed on a TriStar LB 941 plate luminometer (Berthold Technologies, Germany).

Catalase activity in saliva was determined by the H_2O_2-luminol-dependent chemiluminescent method using 25 rl of luminol (AppliChem, Germany) and 25 μl of 3% hydrogen peroxide (H_2O_2) (MCD Chemical, Russia) [28]. The dynamics of luminescence was recorded in the presence of saliva for 5 min. When analyzing the activity of the enzyme, the time of onset of the chemiluminescent reaction (t_0), the maximum intensity of the chemiluminescent reaction (I_{max}), and the maximum area of the chemiluminescent curve (S_{max}) were accounted.

For bioluminescent testing of saliva, a bienzymatic system NADH:FMN-oxidoreductase + luciferase was used, which is included in the kit of analytical bioluminescence reagents (CRAB) (Institute of Biophysics, Siberian Branch, Russian Academy of Sciences, Krasnoyarsk). The kit contains lyophilized preparations of highly purified luciferase enzymes EC 1.14.14.3 (0.4 mg/ml) from a recombinant strain of Escherichia coli and NADH:FMN-oxidoreductase EC 1.5.1.29 (*Photobacterium leiognathi*) (0.18 units of activity). The composition of the reaction mixture for the bioluminescent reaction was as follows: 80 μl of 0.05 M potassium phosphate buffer (pH 6.8-7.0), 5 μl of CRAB solution, 10 μl of 0.0025% tetradecanal solution (Merck, Germany), 50 μl of 0.4 mM NADH solution (Sigma, USA), 10 μl of 0.5 mM FMN solution (Serva, Germany). The reaction mixture was added to the cell of the tablet, and the value of the maximum luminescence

intensity was recorded (control measurement). In the experimental measurement, 40 μl of buffer was replaced by 40 μl of saliva diluted 60 times in buffer. The luminescence intensity was measured in duplicate. The ratio of the average maximum bioluminescence intensities of the experimental measurement (I) to the control (I0) was used to calculate the value of the residual luminescence (T, %).

Statistical data processing was performed using the Statistica 10 program (StatSoft, Inc., USA) with the calculation of the median (*Me*) and interquartile ranges (C_{25}-C_{75} percentiles). Differences between the indicators of dependent samples were assessed using the nonparametric Mann-Whitney U test, and correlation was assessed using Spearman's rank correlation coefficient; significance level of $p \leq 0.1$.

Results. Testing the functional parameters of the body of sports horses in training revealed trends to a significant increase in NPV ($p = 0.1$) and heart rate ($p = 0.1$) with an increase in physical activity from low to high relative to the values obtained before training (Fig. 1).

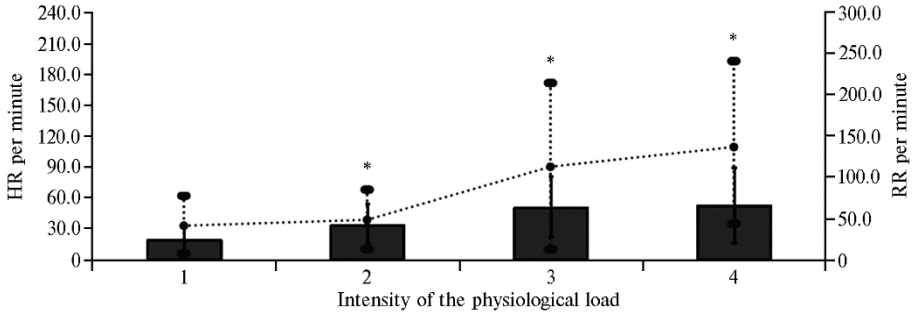


Fig. 1. Heart rate (HR, graph) **and respiratory rate** (RR, diagram) **in Trakehner sports horses (*Eguus caballus*) depending on the intensity of physical activity:** 1 — before training, 2 — low, 3 — medium, 4 — high intensity. The median (*Me*) and percentiles (C_{25}-C_{75}) are given ($n = 12$).
* Differences with pre-training value ($p = 0.1$).

The heart rate of the studied horses remained sinus regular. Prior to training, electrocardiological parameters corresponded to the average standard.

Low-intensity exercise increased P wave amplitudes to 4.0 mm (3.0-4.0 mm) and R wave amplitudes to 13.0 mm (12.0-14.0 mm) relative to pre-training values. There was also a shortening of the P-Q intervals up to 0.07 s (0.06-0.1 s), QRS up to 0.06 s (0.06-0.08 s), QPST up to 0.5 s (0.5-0.5 s), TP up to 0.6 s (0.5-0.8 s), RR up to 1.3 s (1.0-1.5 s), life expectancy up to 36.1% (31.1-46.2%). Physical activity of moderate intensity did not change the P wave amplitude, which was 2.0 mm (2.0-2.0 mm), and increased the R wave amplitude up to 11.0 mm (7.0-12.0 mm) compared to the values up to workouts. Shortening of QPST intervals to 0.4 s (0.4-0.5 s), TP to 0.4 s (0.01-0.7 s), RR to 1.2 s (1.1-1.5 s) were recorded. Life expectancy increased to 35.9% (33.3-37.0%). High-intensity physical activity increased P wave amplitudes up to 4.0 mm (4.0-4.0 mm) and R wave amplitudes up to 24.0 mm (23.0-26.0 mm) compared to pre-training values. The intervals TP were also shortened to 0.2 s (0.2-0.2 s), RR to 0.9 s (0.8-0.9 s). Life expectancy increased to 49.3% (31.0-46.9%).

With low-intensity exercise, the systolic index (P wave) in horses increased ($p = 0.004$) relative to that with moderate-intensity exercise. Physical activity of great intensity affected systolic and diastolic parameters. There was a statistically significant increase in the amplitude of the P ($p = 0.0043$) and R ($p = 0.0043$) waves and a decrease in the intervals P-Q ($p = 0.0043$), TP ($p = 0.017$) and RR ($p = 0.017$) relative to the corresponding values at loading of average intensity.

Hematological and biochemical parameters were in the range of the

physiological norm both before training and after exercise. The amount of hemoglobin increased to 14.0 g% (11.8-15.6 g%) at low intensity exercise and up to 13.1 g% (11.8-13.8 g%) at high intensity. The content of erythrocytes decreased with increasing load up to 10.0 million/μl (8.6-11.8 million/μl) at its low intensity and up to 7.5 million/μl (7.2-9.0 million/μl) at medium and high intensity. The number of leukocytes, on the contrary, increased to 5.7 thousand/μl (4.1-5.8 thousand/μl) with a low load, up to 6.8 thousand/μl (5.9-8.1 thousand/μl) with an average and up to 8.3 thousand/μl (7.9-9.8 thousand/μl) with high intensity. There was an increase in the concentration of total protein and a decrease in glucose with increasing physical activity. The index of total protein after exercise of low, medium and high intensity was 64.4 g/l (61.8-65.1 g/l), 62.5 g/l (60.9-63.6 g/l), and 66.9 g/l (63.9-66.9 g/l), respectively, For glucose, it was 4.9 mmol/l (4.8-5.1 mmol/l) for low and 4.4 mmol/l (4.1-4.8 mmol/l) for medium and high intensity. We did not find statistically significant differences in changes in the quantitative composition of cells, concentrations of total protein and glucose, depending on the intensity of the loads.

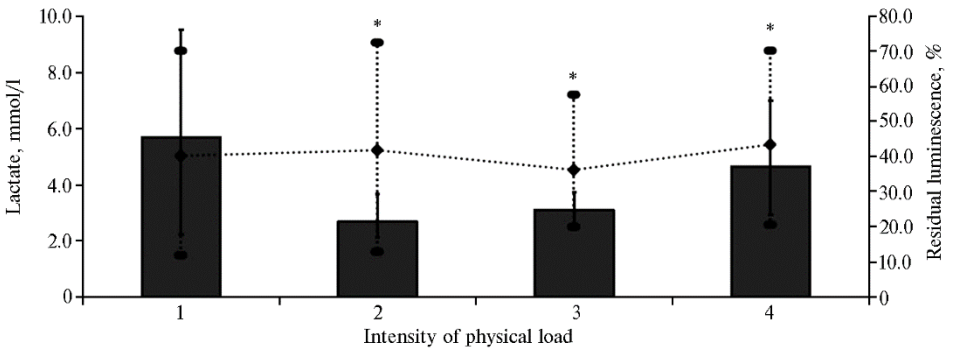


Fig. 2. Residual luminescence (diagram) and lactate concentration (graph) **in saliva of Trakehner sports horses (*Eguus caballus*) depending on the intensity of physical activity:** 1 — before training, 2 — low, 3 — medium, 4 — high intensity. The median (*Me*) and percentiles (C_{25}-C_{75}) are given (n = 12).
* Differences with pre-training value (p = 0.1).

The concentration of lactate in saliva tended to increase during physical activity of low and high intensity and amounted to 5.2 mmol/l (4.7-5.9 mmol/l) and 5.4 mmol/l (3.8-7.2 mmol/l), and at medium load it corresponded to the indicators obtained before training, 4.5 mmol/l (3.8-5.0 mmol/l) and 5.0 mmol/l (4.1-5.4 mmol/l) (Fig. 2).

The results of saliva testing also showed the dependence of the bioluminescent glow on physical activity. There was a tendency to its decrease relative to the indicators before training with low-intensity physical activity, followed by an increase with increasing load (see Fig. 2). We believe that the quenching of the bioluminescent glow during physical activity of different intensities is due to a change in the metabolic composition of saliva, which is caused by the functional state of the horse's body during exercise.

At low, medium and high physical activity, catalase production was activated relative to pre-workout levels. The highest intensity of the luminol-dependent chemiluminescent luminescence was observed at a low load, while the minimum intensity was observed at a high load. A statistically significant increase in the intensity of the luminol-dependent chemiluminescent luminescence of saliva indicated an increase in free radical oxidation processes (Fig. 3).

Therefore, in horses with an increase in physical activity, the functional indicators of the body increased, which indicated the activation of the respiratory, cardiovascular and enzymatic systems during training. Excitation of the atria and a

reduction in the time of cardiac diastole during physical exertion indicated a rapid blood filling of the ventricular volumes of the heart, which was explained by the intensive work of the skeletal muscles. A high physical load, in contrast to a low one, increased the rate of propagation of excitation through the muscles of the right and left ventricles, which characterized the intensive work of a healthy horse's heart in a state of active training [29].

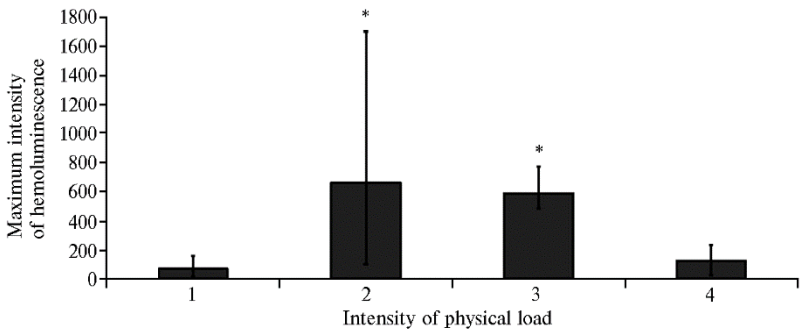


Fig. 3. Intensity of luminol-dependent hemoluminescence in the presence of H_2O_2) in saliva of Trakehner sports horses (*Eguus caballus*) depending on the intensity of physical activity: 1 — before training, 2 — low, 3 — medium, 4 — high intensity. The median (*Me*) and percentiles (C_{25}-C_{75}) are given (n = 12). * Differences with pre-training value (p = 0.1).

As reported, in endurance sport horses, haematological parameters and salivary chemistry are associated with heart rate [30, 31]. Our results of the clinical analysis of the blood of Trakehner sports horses confirmed the change in hematological parameters in connection with the heart rate or NPV during exercise. However, the identified changes remained within the normal range, which indicated a good functional state of the horses [30] or, possibly, a high degree of their preparedness. The latter was not noted in other studies, since they considered the effect of physical activity of the same intensity.

Studies of racehorses with low performance in Italy and Ukraine showed that increased exercise intensity affected the change in cardiomyocyte permeability and the release of enzymes into the blood [32, 33]. In our experiments, physical activity of different intensity caused a change in the concentration of glucose and total protein in the blood serum, that is, they influenced the dynamics of carbohydrate metabolism [30].

In similar studies of horses of various sports specializations, an increase in the concentrations of lactate and pyruvate in the blood was noted at different periods of the training cycle [11, 12, 32]. Our data show that lactate concentration in horse saliva remained stable at low and high levels of exercise. An increase in the maximum concentration of lactate with increasing load may be due to insufficient oxygen saturation of the body, which indicates a poor performance of the horse [8, 11, 32]. Therefore, the stable increase in lactate concentration observed by us during low and high intensity training compared to the pre-training index indicated the absence of hypoxia and indicated a high fitness of the horses.

The ability of horses to overcome high physical loads is confirmed by changes in catalase activity, which characterizes the state of oxidative systems and aerobic oxidation processes [14]. On horses of different breeds, it was found that the current physical load could be assessed by the reduced, increased or multidirectional dynamics of the catalase content [17-19]. We found an increase in the production of salivary catalase in horses with an increase in the intensity of physical activity, which can be explained by the activation of free radical processes due to oxidative stress.

According to the presented data, horse saliva is as informative for the analysis of the functional state of the body as blood. Saliva, as a dynamic biological fluid, rapidly changes its composition depending on the increase in physical activity [14, 31]. The bioluminescent index collectively takes into account such changes. It is of importance to identify the factors that determine the intensity of the bioluminescence of saliva in physical exertion and act as the reasons for its change. For these data, we analyzed the correlation between the magnitude of the residual luminescence and the functional parameters, cardiac hemodynamics, blood biochemical parameters, the content of lactate and catalase in saliva.

With low-intensity exercise, a high percentage of luminescence inhibition correlated with an increase in total protein concentration ($r = 0.6$, $p = 0.05$), a decrease in glucose content ($r = -0.7$, $p = 0.05$), and blood erythrocyte counts ($r = -0.6$, $p = 0.05$). During moderate exercise, the increase in bioluminescence correlated with an increase in RR ($r = 0.5$, $p = 0.1$) and lengthening of the QRS interval ($r = 0.8$, $p = 0.05$). During high-intensity exercise, a low percentage of luminescence inhibition correlated with salivary lactate ($r = -0.58$, $p = 0.1$), decreased salivary catalase ($r = -0.7$, $p = 0.05$), and increase in P wave amplitude ($r = 0.8$, $p = 0.05$).

So, bioluminescent testing of saliva of horse in training using the NADH:FMN-oxidoreductase + luciferase bienzyme system showed that the observed luminescence intensity is related to changes in the concentration of metabolites in saliva and depends on the physical load experienced by the animal. The value of the residual glow decreases with low physical activity and increases with high physical activity. It was found that the intensity of the luminescence correlates with the concentration of catalase and lactate in saliva. Thus, our finding have proved potential use of non-invasive saliva testing to assess the impact of physical activity on sports horses in training. The obtained preliminary results make it possible to assess the physiological state of horses under physical loads of various volume and intensity.

REFERENCES

1. Korobko A.V., Rachikova O.V. *Aktual'nye problemy intensivnogo razvitiya zhivotnovodstva*, 2013, 16(2): 3-10 (in Russ.).
2. Lutsenko M.V., Petrushko N.P. *Aktual'nye problemy intensivnogo razvitiya zhivotnovodstva*, 2016, 19(1): 281-289 (in Russ.).
3. German Yu.I., Gorbukov M.A., Rudak A.N., Sadykov E.V. *Konevodstvo i konnyi sport*, 2019, 5: 37-40 (in Russ.).
4. Zarubezhnye gastroli rossiiskikh skakunov. *Zolotoi mustang*, 2000, 6. Available: http://www.gold-mustang.ru/magazine/ippodrom/234.html. Accessed: 15.06.2020 (in Russ.).
5. de Mare L., Boshuizen B., Plancke L., de Meeus C., de Bruijn M., Delesalle C. Standardized exercise tests in horses: current situation and future perspectives. *Vlaams Diergeneeskundig Tijdschrift*, 2017, 86(2): 63-72 (doi: 10.21825/vdt.v86i2.16290).
6. Allen K.J., van Erck-Westergren E., Franklin S.H. Exercise testing in the equine athlete. *Equine Veterinary Education,* 2016, 28(2): 89-98 (doi: 10.1111/eve.12410).
7. Munsters C.C.B.M., van den Broek J., Welling E., van Weeren R., Sloet van Oldruitenborgh-Oosterbaan M.M. Prospective study on a cohort of horses and ponies selected for participation in the European Eventing Championship: reasons for withdrawal and predictive value of fitness tests. *BMC Veterinary Research*, 2013, 9(1): 182 (doi: 10.1186/1746-6148-9-182).
8. Kabasova I.A., Petrushko N.P. *Aktual'nye problemy intensivnogo razvitiya zhivotnovodstva*, 2018, 21(2): 306-312 (in Russ.).
9. Hartmann E., Søndergaard E., Keeling L.J. Keeping horses in groups: a review. *Applied Animal Behaviour Science*, 2012, 136(2-4): 77-87 (doi: 10.1016/j.applanim.2011.10.004).
10. Stefánsdóttir G.J., Ragnarsson S., Gunnarsson V., Jansson A. Physiological response to a breed evaluation field test in Icelandic horses. *Animal*, 2014, 8(3): 431-439 (doi: 10.1017/S1751731113002309).

11. Stefánsdóttir G.J., Ragnarsson S., Gunnarsson V., Roepstorff L., Jansson A. A comparison of the physiological response to tölt and trot in the Icelandic horse. *Journal of Animal Science*, 2015, 93(8): 3862-3870 (doi: 10.2527/jas.2015-9141).
12. Larsson J., Pilborg P.H., Johansen M., Christophersen M.T., Holte A., Roepstorff L., Olsen L.H., Harrison A.P. Physiological parameters of endurance horses precompared to post-race, correlated with performance: a two race study from Scandinavia. *International Scholarly Research Notices. Veterinary Science*, 2013, 2013: 684353 (doi: 10.1155/2013/684353).
13. Janczarek I., Wilk I., Zalewska E., Bocian K. Correlations between the behavior of recreational horses, the physiological parameters and summer atmospheric conditions. *Animal Science Journal*, 2015, 86(7): 721-728 (doi: 10.1111/asj.12343).
14. Strzelec K., Kankofer M., Pietrzak S. Cortisol concentration in the saliva of horses subjected to different kinds of exercise. *Acta Veterinaria Brno*, 2011, 80(1): 101-105 (doi: 10.2754/avb201180010101).
15. Costa E.D., Dai F., Lebelt D., Scholz P., Barbieri S., Canali E., Zanella A.J., Minero M. Welfare assessment of horses: the AWIN approach. *Animal Welfare*, 2016, 25(4): 481-488 (doi: 10.7120/09627286.25.4.481).
16. Denoix J.-M. *Biomechanics and physical training of the horse*. Manson Publishing Ltd, London, United Kingdom, 2014.
17. Munk R., Jensen R.B., Palme R., Munksgaard L., Christensen J.W. An exploratory study of competition scores and salivary cortisol concentrations in Warmblood horses. *Domestic Animal Endocrinology*, 2017, 61: 108-116 (doi: 10.1016/j.domaniend.2017.06.007).
18. Janczarek I., Bereznowski A., Srtzelec K. The influence of selected factors and sport results of endurance horses on their saliva cortisol concentration. *Polish Journal of Veterinary Sciences*, 2013, 16(3): 533-541 (doi: 10.2478/pjvs-2013-0074).
19. Kędzierski W., Cywińska A., Strzelec K., Kowalik S. Changes in salivary and plasma cortisol levels in Purebred Arabian horses during race training session. *Animal Science Journal*, 2014, 85(3): 313-317 (doi: 10.1111/asj.12146).
20. Palm A.E., Watte O., Lundström T., Wattrang E. Secretory immunoglobulin A and immunoglobulin G in horse saliva. *Veterinary Immunology and Immunopathology*, 2016, 180: 59-65 (doi: 10.1016/j.vetimm.2016.09.001).
21. Sorokina E.V., Zarubina A.P. *Uspekhi sovremennoi biologii*, 2017, 137(6): 613-620 (doi: 10.7868/s0042132417060084) (in Russ.).
22. Esimbekova E.N., Torgashina I.G., Kalyabina V.P., Kratasyuk V.A. Enzymatic biotesting: scientific basis and application. *Contemporary Problems of Ecology*, 2021, 14(3): 290-304 (doi: 10.1134/S1995425521030069).
23. Kratasyuk V., Esimbekova E. Applications of luminous bacteria enzymes in toxicology. *Combinatorial Chemistry & High Throughput Screening*, 2015, 18(10): 952-959 (doi: 10.2174/1386207318666150917100257).
24. Kratasyuk V.A, Stepanova L.V., Ranjan R., Sutormin O.S., Pande S., Zhukova G.V., Miller O.M., Maznyak N.V., Kolenchukova O.A. A noninvasive and qualitative bioluminescent assay for express diagnostics of athletes' responses to physical exertion. *Luminescence*, 2020, 36(2): 384-390 (doi: 10.1002/bio.3954).
25. Kovalev S.P., Kurdeko A.P., Bratushkina E.L., Volkov A.A. *Klinicheskaya diagnostika vnutrennikh boleznei zhivotnykh* [Clinical diagnostics of internal diseases of animals]. St. Petersburg, 2016 (in Russ.).
26. Marchik L.A., Martynenko O.S. Effectiveness of different variants of getting sportsmen's skin extract for identifying the concentration of lactate. *Austrian Journal of Technical and Natural Sciences*, 2015, 3-4: 7-9.
27. Borshchevskaya L.N., Gordeeva T.L., Kalinina A.N., Sineokii S.P. Spectrophotometric determination of lactic acid. *Journal of Analytical Chemistry*, 2016, 71(8): 755-758 (doi: 10.1134/S1061934816080037).
28. Vinnik Yu.S., Savchenko A.A., Per'yanova O.V., Teplyakova O.V., Yakimov S.V., Teplyakov E.Yu., Meshkova O.S. *Sibirskoe meditsinskoe obozrenie*, 2006, 3(40): 3-6 (in Russ.).
29. Chernenok V.V., Simonova L.N., Simonov Yu.I. *Vestnik FGOU VPO Bryanskaya GSKHA*, 2017, 3(61): 41-44 (in Russ.).
30. Andriichuk A.V., Tkachenko G.M., Tkachova I.V. *Izvestiya KGTU*, 2016, 43: 145-153 (in Russ.).
31. Contreras-Aguilar M.D., Cerón J.J., Muñoz A, Ayala I. Changes in saliva biomarkers during a standardized increasing intensity field exercise test in endurance horses. *Animal*, 2021, 15(6): 100236 (doi: 10.1016/j.animal.2021.100236).
32. Maksymovych I., Slivinska L. Metabolic responses in endurance horses at exhausted syndrome. *Ukrainian Journal of Veterinary and Agricultural Sciences*, 2018, 1(1): 17-22 (doi: 10.32718/ujvas1-1.03).
33. Alberti E., Stucchi L., Lo Feudo C.M., Stancari G., Conturba B., Ferrucci F., Zucca E. Evaluation of cardiac arrhythmias before, during, and after treadmill exercise testing in poorly performing standardbred racehorses. *Animals*, 2021, 11(8): 2413 (doi: 10.3390/ani11082413).