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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)

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Abstract

Diagnosis and prevention of the retroviral infection spread among farm animals still remain poorly developed primarily due to the fact that a hierarchic cascade of the events which underlie the retrovirus—host interactions involves molecular, intracellular levels, including cell organelles, and extracellular levels associated with the function of cellular immune networks. This paper presents an overview of own and literature data on the interaction of retroviral pathogen on the example of bovine leukemia virus (BLV) with intracellular structures of target cells. Here we consider four stages of the cascade of the events promoting pathogen, including i) introduction into the cell cytoplasm, ii) the synthesis of DNA copies of the viral genome RNA, iii) their transport into the cell nucleus, and iv) provirus DNA introduction into the host genome. The host genes interacting with viral structures are revealed at each stage. Two key processes contribute to genetic variability of retrovirus genome during infectious cycle: two viral RNAs dimerization needed for reverse transcription increases the frequency of recombination between RNA chains (N. Dubois et al., 2018), and provirus cDNA integration into the host genome can lead to activation of mutational and epigenetic events in both the pathogen genome and the host genome (A. Melamed et al., 2018). BLV pathogenesis is divided into two steps, the infectious cycle of mass infection of host target cells and sequential selection of individual infected cell clones. The peculiarity of the integration sites of the host genome is an increased frequency of mobile genetic elements originally closely related to exogenous retroviral infections (N.A. Gillet et al., 2013; T. Miyasaka et al., 2015). The high density of mobile genetic elements is characteristic of the host genomic DNA fragments flanked by inverted repeats of microsatellite AGC and identification sequence of the DNA transposon Helitron. The multiplicity of intracellular targets, whose polymorphism may be the basis of resistance to retroviral infections, allowed us to assume for the first time that the universal critical factor of the infectious process is the integration of proviral DNA into the host genome. It is suggested that the increased sensitivity of cells to productive BLV infection is due to a decrease in the activity of mechanisms involved in the genome protection from transposition activity. In the next communication, we will discuss the relationship between BLV-infected cells and host immune cell networks, which can also have a determining effect on the development of retroviral-induced infection.

Keywords: retrovirus, bovine leukemia virus, infectious cycle, B lymphocytes, bovine leukemia virus receptor, reverse transcriptase, integrase, mobile genetic elements

Retroviruses (*Retroviridae*) are widely spread mammal pathogens regularly causing multi-million damages to the agriculture. A problem in prevention of the infection incidences is low vaccination effectiveness which is poorly researched. Vaccination principle presupposes that in the processes involved in pathogen—host organism interaction there is a point blocking of which by activa-

tion of relevant host's antibody response may interrupt the infection. However, as indicated by a large number of experimental data, retroviral infection is a cascade of events where retrovirus-host interaction occurs at different levels, from intracellular to supracellular. Accordingly, retrovirus resistance may be due to host's intracellular signal, transport, and enzymatic proteins, as well as to characteristic features of host's leukocyte populations determining adaptive and innate immunity.

Present review is aimed to identify key BLV-host interaction stages. In particular, in the first communication we consider events ensuring BLV entering into the target cells and integration of proviral DNA in host genome, and the second communication will scope effects of BLV-proviral DNA integration toward certain components of the host innate and adaptive immunity.

Structure and functioning of Bovine leukemia virus (BLV) genome, stage of induced BLV pathogenesis, and effect on expression of numerous host genes have been described in sufficient detail. However, retrovirus-host interaction mechanisms are complicated and still require further understanding. For instance, from 7 to 22 % cattle have antibodies to BLV, despite the absence of B-cell clones bearing proviral DNA [1, 2]. Up to date, milestone metabolic pathways resulting in lymphatic leukemia under BLV infection, their diversity, and the reasons leading to ineffective immunity against BLV upon vaccination remain unclear. The ideas about various mechanisms determining resistance to BLV are not yet developed. This adversely interfere preventive measures. Besides, lack of such information challenges and impairs the accuracy of diagnostics and indication, as well as prognosis of infected animal danger to others and individual peculiarities of pathogenesis.

Present communication fills up this gap in part. Analysis of the sources of potential resistance during BLV infection at cellular level which we have performed denotes variety of retrovirus-target cell interactions and multiplicity of cellular mechanisms of retroviral infection resistance. Herewith, it appears that events associated with proviral DNA-host genome integration have crucial significance. We believe that reduction of intracellular control over transposition of mobile genetic elements closely related to retroviral infections by their origin play the main role.

Bovine leukemia virus entry into a target cells. BLV (*Deltaretrovirus* genus, *Orthoretrovirinae* subfamily, *Retroviridae* family) is phylogenetically closely related to Human T-lymphotropic virus type 1 (HTLV-1) [3]. HTLV-1 infects human CD4+ T-cells, while BLV infects bovine B-cells; both viruses integrate DNA copies of their genomic RNA into the host genome as a proviral DNA [4, 5]. In BLV infected animals, the pathology is initially symptomless, then an increased proliferation of B-cells in some animals occures and, finally, nearly 5 % animals form B-cell lymphomas. BLV causes significant economic damage to dairy and beef cattle farming [6]. Development of effective immunization methods is so far unsuccessful [7]. In addition to the most common 8 serotypes of BLV derived from mutations in gene *g51*, encoding the surface glucoprotein [8], variants of nucleotide substitutions not related to these mutations have been described. According to this, 28 fully sequenced BLV genomes were divided into A, B and C groups with high, average, and low pathogenicity, respectively, as assessed by in vitro virus replication and by in vivo virus load in cows [9].

Proviral DNA integration results in significant changes in both virus and host cell genomes and may be considered as an example of insertional mutagenesis [10-12]. There are two main steps of BLV-induced infection process: massive integration of proviral DNA into B-lymphocyte genone and prolifration of several infected B-clones [13]. Peripheral blood cells with proviral DNA which are detected in cattle infected with BLV are less than 1 % [3]. Following the first infection

steges, proviral DNA massively integrates into actively transcribed regions of the host genome, but, in furtherance, the integrated copies survive only in single cells and only few of them result in tumorogenesis [13]. Infection process is accompanied by massive death of cells with integrated proviral genome [14]. This is due both to a response of the host's immune system to virus antigens and to proviral DNA integration into actively transcribed host DNA regions, which may decrease cell viability, especially when proviral DNA is incorporated into the regulatory or coding sequences of vital structural genes [13].

Generally speaking, natural selection acts against cell populations with actively expressed proviral DNA. Besides, it was found that BLV infected B-cells are divided into two subpopulations, in one of which IgM and BLV RNA are actively expressed, whereas the other one lacks both IgM and viral RNA expressions. It is assumed that it is the second cell population which becomes predecessor of lymphoma, since proviral DNA (plus-strand) is not expressed in tumor cells [15]. The accumulating data show the expression of micro-RNA of BLV proviral DNA, transcription of which occurs in the B-lymphocytes not expressing full-size BLV genome (positive strand), to be the key regulatory factor promoting initiation of B-cell neoplastic transformation [16-18].

Mechanisms to ensure BLV penetration into target cells. BLV is spreading together with liquids containing cell component (blood, milk) and mainly infects CD5+ IgM+ B-lymphocytes [19]. The accumulated experimental data shows that BLV mainly enter target cells indirectly (i.e. not by direct penetration of free viral particles), but through integration of virus-infected and not infected cells [20]. Intercellular transfer of infection is ensured by close interaction of two glycoproteins bound to lipid layer of virion envelope, the surface protein (SU) and transmembrane protein (TM). Both glycoproteins are products of post-translational breakdown of the predecessor encoded by *eny* gene, which have a unique disulphide bond between the cysteic motifs of SU (CXXC) and TM (CX_6CC). Analysis of retrovirus spread events give a scheme of the fusion induced by the interaction between SU receptor-binding domain (RBD) and receptor in the target cells (21, 22) (Fig. 1).

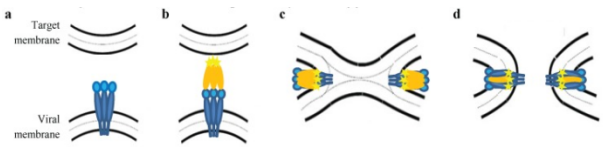

Fig. 1. Bovine leukemia virus (BLV) coat protein membrane fusion (according to the model for murine leukemia virus) [22]. Fusion incompentent state of the envelope complex formed by the receptor-binding (surface protein (SU), gp51 in light blue) and the fusion (transmembrane protein (TM), gp30 in dark blue) subunits (a). After receptor binding, a conformational change exposes the fusion peptide (yellow star) to the target cell membrane (b). Insertion of the fusion peptide into the lipid bilayer mediates formation of a hemifusion diaphragm and blending of viral and cellular lipids (gray dots) (c). Fusion structure after refolding. In this state the fusion peptide and the TM are anchored into the same membrane in an anti-parallel conformation (d) [22].

SU receptor-binding domain (RBD) interacts with specific receptor(s) in target cells. This interaction induces conformational changes initiating TM-directed fusion process. Breaking of disulfide bridge between SU and TM after binding to the receptors provides TM conformation required for membrane fusion. The fusion peptide located at the NH2 end of the TM destabilizes the cell membrane, which allows the virus nucleocapsid to enter the cytoplasm of the host cell [22]. It is assumed (and there is a number of experimental proof) that adaptor-related protein complex-3 (AP-3) participating in protein transport is the main RBD BLV cell receptor [23-25]. This complex participates in formation of transport vesicles to unite the Golgi apparatus and lysosomes and is highly con-

servative among mammal species (88 % identity of amino acid sequences in humans and cattle, and 99 % identity in sheep and goats). There are two main domains in human AP3D1, the adaptine and BLV receptor (Fig. 2). Comparison of amino acid sequences of these domains revealed 15 differences in the second domain (virus receptor) with full absence thereof in the adaptine domain [25].

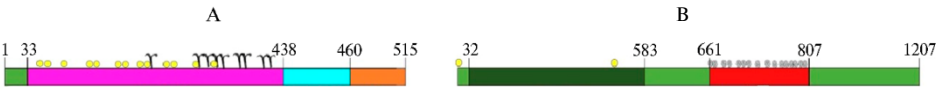

Fig. 2. Bovine leukemia virus coat protein (BLV, *env* gene) (A) **and human adaptive protein complex AP3D1** (B) **[25].** Positions 1-33 — signal peptide (SP), 34-438 — surface protein (SU, gp51), 439-460 — transmembrane protein (TM, gp30), 461-515 —cytoplasmatic region of protein molecule (CR). Zn^{2+} ion binding sites containing cysteine and histidine amino acid residues are shown by yellow circles, N-glycosilation sites (ϒ) are marked (A). Protein AP3D1 (1207 amino acids) contains two domains, the adaptine (32-583) and BLV receptor (661-807). AP3D1 bears two sites of binding Zn^{2+} ions (yellow circles) and 16 conservative sites in BLV receptor domain (grey circles) (B) [25].

Previously we have shown that expression of BLV receptor AP3D1 gene in BLV-infected cows is higher, but does not correlate with an increase in the number of lymphocytes [2]. The research data evidence on expression of this gene in junior B-lymphocytes which reduces with aging [24]. Regardless of the lack of statistically significant correlations between the number of lymphocytes and *ap3d1* gene expression, we have found statistically significant ($p < 0.05$) increase of *ap3d1* expression in animals with BLV proviral DNA as compared to the infection-free individuals. These suggest a relatively increased proportion of junior B-lymphocytes in cell population of BLV-infected animals.

Given wide occurrence of AP3D1 in organs and tissues, its conservatism in the considered mammal types, and pleiotropic mutation effects in gene encoding this central sub-unit of AP-3 complex ensuring transport of membrane proteins to lysosomes [26], one can hardly conceive mechanisms of AP-3 participation in BLV selective penetration exactly into B-lymphocytes. Nevertheless, a number of experimental papers points out to availability of such mechanisms [25]. Complexity of dynamic protein exchange processes between endosomes, plasmatic membrane, and lysosomes allows us to suggests that various ligands, cell elements of cytoskeleton, and other factors which are difficult to control in direct experiments may ensure specific interaction between

Thus, BLV gets into cells of various tissues in different mammalians but can produce full virus progeny only in several of them and only in B-lymphocytes. Accordingly, it is possible to block BLV reproduction at next steps of infection (reversal transcription, integration nto host gene, multiplication of infected cells) [27] that, nevertheless, does not excludes cell resistance to BLV penetration into target cells.

Formation of proviral DNA. Once BLV gets into cell cytoplasm, the viral RNA genome acts as a matrix to synthesize DNA copies (cDNA). Effectiveness of cDNA synthesis significantly depends on successful dimerization of two molecules of virus RNA [28]. At this stage, recombination frequency between two strands of virus RNA is high, and may cause wide spectrum of aberrant proviral genomes.

Interaction between the host's tRNA and long terminal repeats (LTR) of virus genome plays a significant role in initiation of retrotranscription. 5′- and 3′-LTRs are complexes of regulatory motifs required for production of DNA copy of virus RNA and further transcription of proviral DNA. Mutations in LTR, as well as changes in their methylation pattern, may significantly affect the BLV-induced infection (Fig. 3) [29]. 5′- and 3′-LTR regions of virus genome

are initially identical, and differences in their nucleotide sequences (e.g. nucleotide replacements and indels) appear after integration into the host genome. The sequence of LTR retrovirus includes three main functional units, i.e. TG...CA box with TG at 5′-end of 5′-LTR and CA at 3′-end of 3′-LTR; TSR (target site repeat) region of ~ 4-6 bps (short direct repeat flanking 5′- and 3′-ends; this is a "signature" of sites involved in insertion of proviral DNA into the host genome); PBS (primer binding site), a 18 bps sequence complementary to 3′-end of some tRNA located near 3′-end of 5′-LTR (this site is very important since revers transcription starts from binding with tRNA).

Transport of proviral DNA into nucleus of the host cell. Many researches had experimentally shown that replication, formation of pre-integration complex, and further interaction with proteins of nuclear pores during proviral cDNA transfer through the nuclear membrane is due to activity of microtubules of cytoskeleton and microtubule-organizing centre (Fig. 4) [30]. Involvement of cytoskeleton and many host proteins in transport of pre-integration complex to nucleus is characteristic of all retroviruses. Genome of members of *Retroviridae* family including two subfamilies, *Spumaretrovirinae* (*Spumavirus* genus) and *Orthoretrovinae* (*Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus,* and *Lentivirus* genus), consists of two copies of single-stranded RNA. As it was already noted, virion upon entry into a cell is bound by specific receptor surface cell protein, and virion and host membranes are fused (the event occurs either on cell surface or after internalization in endosomes). As a result, virus capside bearing viral genome and reverse transcriptase gets into cytoplasm. In the cytoplasm, reverse transcriptase, after activation by interaction with nucleoside triphosphates, retrotranscribes two-stranded DNA from RNA genome. Resultant cDNA is transported to cell nucleus as part of pre-integration complex (PIC). PIC composition varies in different retroviruses and different cell types. Although, PIC components are poorly studied, it is known that PIC contains proviral DNA, integrase, and capside proteins.

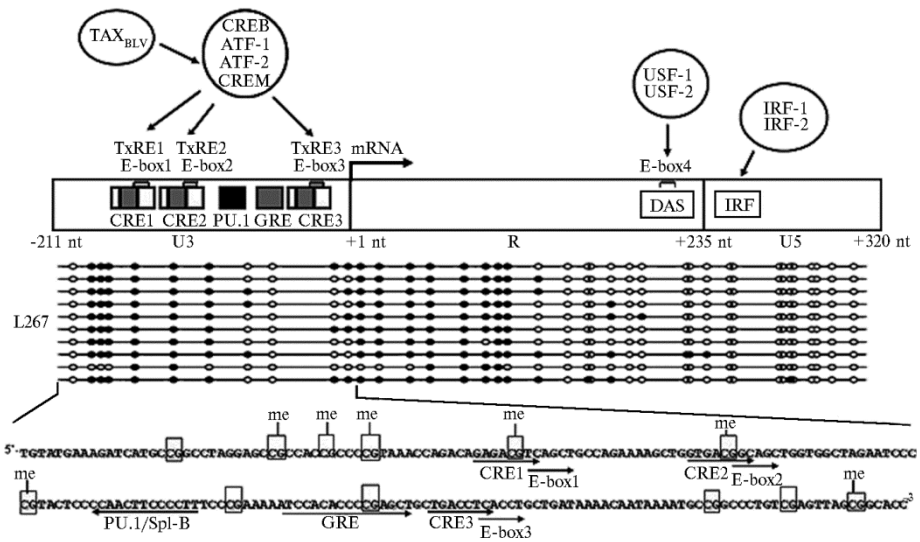

Fig. 3. 5′-LTR of bovine leukemia (BLV) provirus with methylation in CpG (cytosine-phosphate-guanine) motif in sheep cell line L267 at latent BLV infection [29]. mRNA transcription initiation site in region U3—R in 5′-LTR (+1 nucleotide, nt) is marked by arrow. TxRE1, TxRE2, and TxRE3 — three main transcription enhancer sequences of 21 bps each, interacting with host transcription factors CREB, CREM, ATF-1, and ATF-2, which is required for activation of BLV transcription by virus transactivator Tax$_{BLV}$. Each enhancer contains sequences which are homologous to E-box sequence consensus (E-box1, E-box2 and E-box3) and overlap CRE (CRE1, CRE2, and CRE3 elements regulated by cyclic AMP, i.e. cyclic-AMP responsive element, CRE). U3 region

includes glycocorticoidal regulation element (GRE) (binding of hormone-receptor complex) and site PU.1/Spi-B. USF-1/USF-2 binding site (E-box 4) and interferon-regulating factor binding site (IRF-1/IRF-2) are located in R section of U5 region. Full nucleotide sequence of U3 region with transcription factor binding sites (marked by arrows) and CpG dinucleotides (methylation sites me are highlighted by rectangular) is provided [29].

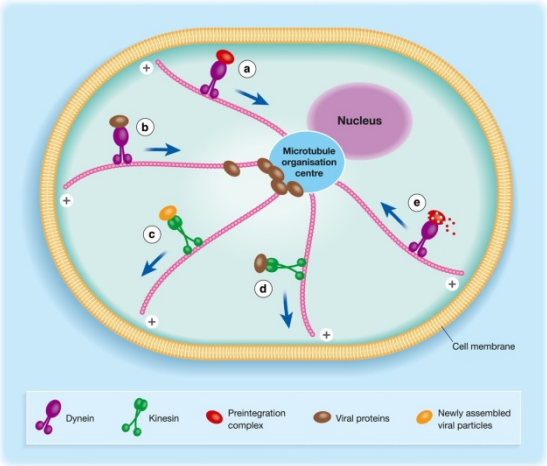

Fig. 4. Motor proteins of microtubules involved in replication of retroviruses [30]. Preintegration retrovirus complexes use dynein for transport along microtubules (a). Newly synthesized retrovirus proteins (Gag and Env) with dynein move to perinuclear zone for virion formation (b). Formed virus particles are further moved by kinesin to cell membrane to exit the cell (c). Viruses assembling at plasma membrane use kinesin for proteins and genome RNA transport (d). Dynein activity is also required to remove virus envelope (E). MOC stands for microtubule-organizing centre. Positive end of microtubules is marked (+), arrows show direction of macromolecule movement through microtubules [30].

Integrated proviral DNA is transcribed by the host's RNA-polymerase II, and virus mRNA is delivered to cytoplasm. Afterwards, they are translated into virus proteins Gag, Pol, and Env (and into auxiliary proteins, if virus genome possesses their genetic determinants), which are later moved to plasma membrane by vesicules, cytoskeleton or otherwise. Following the assembly, immature virus particles bud off the cell membrane. Virion maturing is initiated by viral protease which mediates cleavage of Gag and Gag-Pol proteins. In each described step of this strictly regulated process many host proteins are involved, including the cell cytoskeleton elements. Mutation of genes encoding such proteins may significantly influence the proviral DNA infection success.

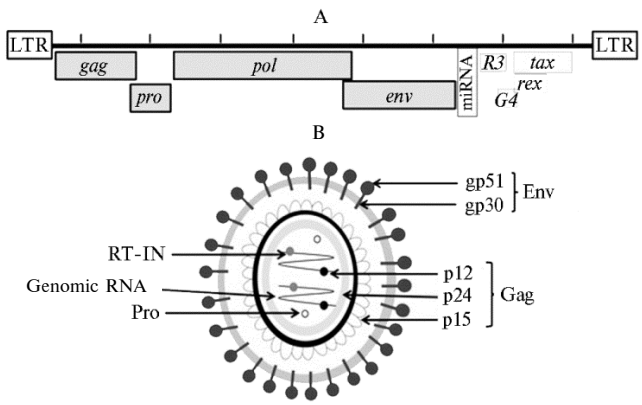

Fig. 5. Genome (A) **and virion structure** (B) **of bovine leukemia virus (BLV) [31].** Structural genes and enzyme genes *env*, *gag*, *pro* and *pol*; regulatory genes *tax* and *rex*; auxiliary genes *R3* and *G4*; microRNA (miRNA) (A). Structural proteins and enzymes: extracellular and transmembrane glycoproteins gp51 and gp30 (Env), Gag proteins — p12 (nucleocapside), p24 (capside) and p15 (matrix), reverse transcripase and integrase (RT-IN) encoded by *pol* gene, protease (Pro) (B).

Structure of BLV genome has been described in detail quite long time ago (Fig. 5) [31]. BLV genome consists of 8714 nucleotides, includes main genes encoding structural proteins and enzymes (mainly, *gag*, *pro*, *pol,* and *env*), and pX region flanked by two identical LTRs. Gene *gag* is translated to the predecessor protein Pr45 which is processed into three mature proteins, i.e. matrix protein P15, which binds virus genome RNA and interacts with lipid bilayer of virus membrane, capside protein P24 (the blood level of antibodies to this protein is high in BLV-infected animals) and nucleocapside protein P12 which is necessary to pack genome RNA (see Fig. 5). Gene *env* encodes mature extracellular pro-

tein gp51 and transmembrane protein gp30. Region pX located between *env* and 3′-LTR encodes regulatory Tax and Rex proteins, as well as auxiliary R3 and G4 proteins (see Fig. 5). Regulatory proteins are involved in control of transcription and export of virus RNA to cytoplasm. In particular, G4 significantly increases virus production [32]. Region encoding five is located between *env* and pX. These microRNAs are transcribed by RNA-polymerase III. Rex and Gag proteins participate in transport of proviral cDNA into cell nucleus. Note, Gag is also bound with centrosome proteins of spindle apparatus, which may lead to multicentric mitosis [33, 34].

Many host proteins, which are partially studied for HIV-1 retrovirus, participate in virion uncoating, transport and cDNA delivery into cell nucleus (Fig. 6) [35].

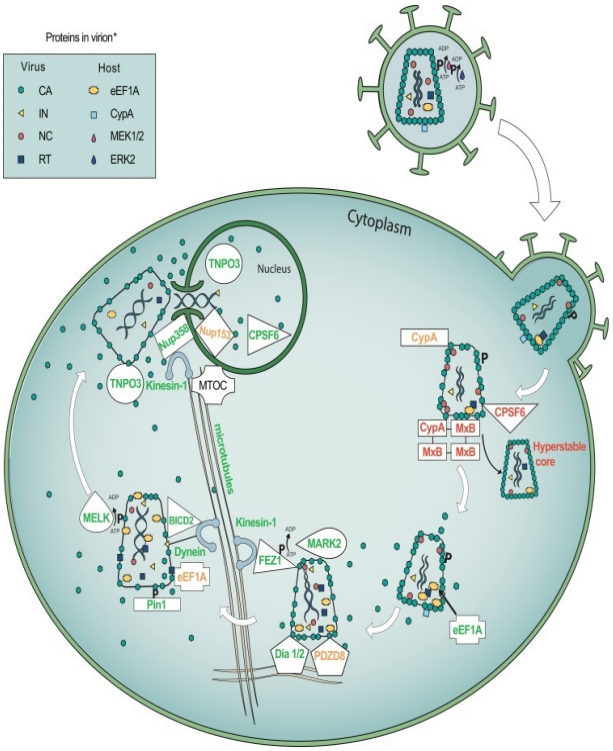

Fig. 6. Factors of host cells involved in uncoating of HIV-1 protein envelope [35]. CA — capside; CPSF6 — specific cutting and polyadenylation factor 6; CypA — cyclophyline A; BICD2 — bicaudal D2; Dia1/2 — formin 1 and formin 2, bound to diaphane; eEF1A — eucariotic elongation factor 1A; ERK2 — kinase 2 regulated by extracellular signals; FEZ1 — fasciculation and elongation factor zeta 1; IN — integrase; KIF5B — kinesin 1 (heavy strand); NC — nucleocapside; NPC — nuclear pore complex; Nup — nucleoporine; MARK2 — kinase 2, regulates affinity of microtubules; MEK1/2 — mitogen-activating protein 1 and protein 2; MELK — maternal embryonic leucine binding kinase; MTOC — microtubule-organizing centres; MxB — Myxovirus B resistance protein; PDZD8 — PDZ domain containing protein 8 involved in interaction between the endoplasmic reticulum and mitochondria; PIC — preintegration complex; RT — reverse transcriptase; RTC — reverse transcription complex; TNPO3 — transportin 3.

Factors of host cell regulate the infection activity of HIV-1. HIV-1 virion contains host proteins CypA, ERK2, and eEF1A regulating fusion with host cell membrane and virion exit to its cytoplasm. PDZD8 and CypA bind CA to stabilize virion, which is required for development of infection; however the infection process is blocked if CypA is bound with MxB forming the complex in which MxB oligomers cause hyperstabilization of virion. Cellular eEF1A interacts with virus RT and activates it. Dia1 and Dia2 bind CA—NC complexes which facilitates their removal (possibly due to local stabilization of microtubules). Dynein interacts with virion through the adapter protein BICD2 or by direct interaction with IN; kinesin 1 interacts with virion through adapter FEZ1 (phosphorylated MARK2). This is important for transfer of replication complexes through cytoplasm to MTOC at core periphery. Pin1 binds CA. Phosphorylated MEK1/2, activated by ERK2 at virion maturing, facilitates its uncoating. MELK also phosphorylates CA, which accelerates this process. Virus RT binds eEF1A for stabilization of RTC. Nuclear pore proteins Nup358 and Nup153 promote importation of PIC, Nup358 moves to cytoplasm with the use of KIF5B, and Nup153 prolongs virion-PIC association in the nucleus. TNPO3 retains CPSF6 in the nucleus to prevent CPSF6-dependent virion hyperstabilization; TNPO3 also facilitates separation of CA from replication complexes in the nucleus. Proteins colored in green ensure optimal virion uncoating kinetics, proteins colored in orange delay uncoating, proteins colored in red cause virion hyperstabilization that suppresses infection. The figure shows only virus proteins and host proteins essential for uncoating and transport of virion [35].

The next crucial step of infection process relates to interaction of pre-integration complex with chromatine. In murine leukemia virus (MOVE) retrotransposon, this interaction is mediated by the product of GAG protein cutting by p12 protein [36]. It is assumed that interaction of virus integrase with cellular serine-treonine phosphatase 2A (PP2A) makes certain contribution to integration of proviral DNA into the host genome [37].

BLV proviral DNA is preferably inserted into the host genome regions rich in CpG (which is typical for promoter regions and protein encoding genes) close to tRNA genes and pseudogenes. tRNA genes (unlike genes encoding proteins and transcribed by RNA polymerase II to mRNA) are constitutively transcribed by RNA polymerase III (Pol III). tRNA pseudogenes lose their ability to produce functional tRNA, but their transcription is still associated with Pol III activity. No predominant integration of BLV proviral DNA in regions with dispersed repeats LINE BovB, SINE BOV-A2, SINE ART2A, and LTR ERV was found [13]. Preferred retrovirus integration occurs into host genome regions with high frequency of palindrome structures [38, 39)], in sites of transcription initiation [41] or in regions having other structural and functional peculiarities, in particular, propensity for interaction with virus integrase [42-44]. Similarity of BLV and HTLV-1 retroviruses is approved by comparison of the parameters of integration sites; for instance, both proviruses are located in actively transcribed regions close to tRNA genes and pseudogenes [13]. According to another research paper based on analysis of 264 sites of BLV integration into bovine genomes, it was concluded that more often such sites are rich in AT-nucleotides and long interspersed nuclear elements (LINE) [45]. Previously, we have shown in own research that density of mobile genetic elements and their recombination products increases in BLV-infected individuals in sequences flanked by AGC microsatellite inverted repeat, as well as in the Helitron transposon identification sequence [46, 47].

Own experimental data allows us to assume that general decrease in activity of protective mechanisms preventing transposition of mobile genetic elements is a factor of propensity for integration of proviral DNA into nuclear genome. It should be noted that preferred location of BLV proviral DNA in the regions of tDNA genes and pseudogenes supports our assumption since multiplication and spreading of pseudogenes is due to transposition of mobile genetic elements, and tRNA pseudogenes are predecessors of a large group of non-autonomous mobile genetic elements of SINE (Short Interspersed Nuclear Element). It is notable that length of found fragments of genome DNA flanked by the inverted repeats of AGC microsatellites, as well as the identification Helitron sequence, varies from 300 to 1500 bps in size and is predisposed to formation of short loops due to flange complementarity.

Transcription of proviral DNA. Transcription of proviral DNA involves i) many factors of cell origins controlled by genes located near the integration region of proviral DNA which interacts with host cell chromatine during integration, as well as ii) regulatory factors encoded by retrovirus genome. Among them, Tax is a key factor [48]. U3 region in 5′-LTR (see Fig. 3) contains canonic promoter of CAT-box (CCAACT in coordinates –97 to –92) and TATA-boxes (GATAAAT between –44 and –38 positions). TxRE is an anchoring motifs containing the elements, functioning of which is regulated by cyclic AMP (cyclic-AMP responsive element, CRE). 21-nucleotide motif also serves as a target to bind Tax protein, a key proviral DNA transcription activator enhancing association between CREB and DNA. In fact, internal CRE-similar motifs (GACGTCA, TGACG, TGAC, and TCA) are close to consensus TGACGTCA. E-box motif (5′-CACGTG-3′) located in front of the site of transcription initiation connects basic transcription factors USF1 and USF2. Many genes of such factors are lo-

cated in motif R. Interferon-regulating factor (IRF-1 and IRF-2) binding sites stimulating the basal expressions without Tax are located in U5.

At post-transcription level, BLV expression is regulated by virus protein Rex which interacts with RNA sequence in 3′-LTR located between AATAAA signal and polyadenilation site. This site with increased frequency of a stable hairpin is bound with two signals of transcription termination. Export of viral transcripts from the nucleus to cytoplasm requires Rex association.

As it was already noted, tumor cells lack Pol II mediated transcription of proviral DNA, while Pol III mediated transcription of DNA regions encoding five BLV microRNAs occurs. It was shown that microRNAs are involved in regulation of many key cellular processes in normal state and during various diseases, including oncopathologies [49]. Transcription of microRNAs in BLV-infected tumor cells causes changes in expression of a set of genes related to signal functions, immune system, and oncogenesis [16, 17, 50]. It was also found that second strand of BLV proviral DNA is also actively transcribed in the infected B-lymphocytes and tumor cells, provided that microRNA participates in destruction of antisense transcript [51]. It is assumed that the balance between transcription of positive and negative strands of proviral DNA in BLV and HTLV-1 defines latent or reactivated viral forms [52].

Therefore, the available data provide the evidence of diverse interactions between the BLV retrovirus genome and host cells. Herewith, key events occur when virion invades a cell and interacts with transport host systems which provide the exchanges between plasma cell membrane and intracellular organelles. The next step is reverse transcription of virus RNA, resulting in cDNA which are further delivered into the nucleus to multiply integrate into the host genome as proviral cDNA (infection cycle). The proviral cDNA is intracellularly transcribed and reproduced, and, finally, predominant replication of several infected cell clones occurs. Given such diversity, infection process may be stopped at any of these steps due to polymorphism of genes (either in pathogen, or in host) which products are involved in a cascade of such events. Infection itself increases frequency of mutagenic events (both during reverse transcription of two strands of virus RNA, and during proviral DNA integration). As a result, nearly 1 % of the infected target cells initiate actively proliferating clones. Since detection of antibodies induced by viral envelope proteins disallows reliable identification of the infected individuals and highly efficient vaccination, it is apparent that events related to integration of proviral DNA with host gene are very important for the infection cycle. The available experimental data allows us to presuppose that key factor at this step is a decrease in intracellular control of transposition of the host's mobile genetic elements closely related, by their origin, to retrovirus. Such assumption is supported by data on i) preferred insertion of BLV proviral DNA into regions with high density of tRNA pseudogenes, LINE, and on ii) higher frequency of mobile genetic elements in genome regions flanked by the inverted repeats of several microsatellites and identification Helitron motifs in BLV-infected cows as compared to infection-free animals. In the next communication we will focus on interactions of BLV-infected cell and cellular networks of the host's immune system which may have controlling influence on proceeding of the retrovirus-induced infection.

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GENETIC MARKERS OF MEAT PRODUCTIVITY OF SHEEP (*Ovis aries* L.). I. MYOSTATIN, CALPAIN, CALPASTATIN

(review)

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Abstract

The study of genetic and biochemical bases of phenotypic polymorphism that determine meat productivity of agricultural animals is relevant for animal breeding. Breeders of USA, Europe and Australia use genes associated with quantitative and qualitative traits of meat cattle, such as *CAPN* and *CAST* (calpactin and calpain cascade), *MSTN* (myostatin), *GDF5* (growth differentiating factor), *TG5* (thyreoglobulin), *LEP* (leptin), *FABP4* (protein binding fatty acids) in selection programs (A.V. Eenennaam, 2006; Y.F. Liu et al., 2010; U. Singha et al., 2014; A.Ciepłoch et al., 2017). The main trend in the development of sheep breeding in recent decades throughout the world is a steady growth in mutton production, which determines an increase in the proportion of specialized meat breeds and increasingly growing requirements to parameters of meat productivity of meat sheep and meat wool sheep (A.M. Holmanov et al., 2015; M.I. Selionova, 2015). In this regard, search for candidate genes associated with these parameters is given more atttantion (D.W. Pethick et al., 2014). The presented review summarizes data on several factors which affect meat productivity in shhep. First, myostatin biological activity, gene structure and effect on the indices of sheep meat productivity are under consideration. Myostatin gene located on chromosome 2 and includes three exons and two introns is highly polymorphic (J.G. Hickford et al., 2010; M.R. Ansary, 2011; H. Han et al., 2013). Its mutations g+6723G>A and g+2449G>C have positive effects on the development of muscles and lead to a significant increase in meat with a decrease in fat content in the carcass (A. Clop et al., 2006; P.L. Johnson et al., 2009; I.A. Boman et al., 2010; A.Y. Masri et al., 2011; M. Hope et al., 2013; J. Wang et al., 2016). Another factor determining meat productivity in sheep is a proteolytic calpain-calpastatin system (CCS) (D.E. Goll et al., 2003; H.Y Chung, 2003). Calpastatin gene is located on chromosome 5 and includes 4 exons and 3 introns (B.R. Palmer, 1998). Calpain and calpastatin genes are presented by a variety of alleles, which differ in the frequency in different breeds (F.E. Shahroudi et al., 2006; S.O. Byun et al., 2009; M.A. Azari et al., 2012; G. Shahabodin et al., 2012; R.R. Arora et al., 2014; N. Shahram et al., 2014; N.S. Kumar et al., 2015). There is a relationship between point mutations in *CAPN* gene and fatty hips, kidneys, heart and a significant association of these mutations with lower fat deposition in the carcass. Intensity of growth rate in sheep young is primarily due to a greater increase in muscular weight which also correlates with *CAST* gene (M.R. Nassiry et al., 2006; A. Mahdavi Mamaghani et al., 2008; M. Tahmoorespur et al., 2012; Q. Fang et al., 2013). These results testify to expedience for myostatin, calpain and calpastatin genes typing in breeding genotypes with higher meat productivity.

Keywords: *Ovis aries* L., sheep, meat productivity, myostatin, *MSTN*, calpain, *CAPN*, calpastatin, *CAST*, genetic polymorphism, SNP, genome editing

Studies of the genetic and biochemical basis of phenotypic polymorphism characteristics of meat productivity have already been conducted for many decades. It is known that most productivity indicators are under the joint control of a significant number of genes. Polymorphism of candidate genes involved in the

formation of certain indicators of productivity and desirable genotypes is detected using the standard methods of molecular genetic analysis (AFLP — amplified fragment length polymorphism, ISSR — inter-simple sequence repeats) [1]. The greatest success has been achieved in dairy cattle breeding. The main genes determining the quantitative and qualitative indicators of milk productivity are identified. Marker-assisted selection (MAS) in many countries with developed dairy farming has become an integral part of national breeding programs [2].

The identification of genes associated with the meat productivity of animals was somewhat slower, however, also successful. For example, in the countries of America, Europe and Australia, the test for genes of calpactin and calpain cascade (*CAPN1*, *CAST*), myostatin (*MSTN*), growth differentiation factor (*GDF5*), thyroglobulin (*TG5*), leptin (*LEP*), a protein binding fatty acids (*FABP4*) is applied [3-6].

A constant increase in the production of mutton, which determines the increase in the share of specialized meat breeds and the increasing requirements for meat productivity for sheep of meat-wool and wool breeds, has become the main trend in the development of sheep breeding in recent decades worldwide [7, 8]. However, the increase in sheep meat productivity on the basis of MAS should be recognized as the least developed topic so far. By now, the use of molecular genetic tests in breeding programs has not led to a noticeable improvement in the economically significant indicators in the production of lamb. At the same time, the identification of and direct selection for the corresponding genes can be promising, since the indicators of meat productivity are characterized by low heritability. For example, for merino breeds and their hybrids with meat breeds, the coefficient of heritability of carcass mass, its yield, and the content of meat in it is 0.20-0.40, for specialized meat breeds — 0.38-0.54 [9-11].

The work objective of this review was to analyze the current state of research on the identification of genes that control the meat productivity of sheep and consider the main directions of the application of molecular genetic tests in sheep breeding to increase the qualitative meat production.

To understand the mechanism of genetic variability that affects the development of skeletal muscles of sheep, it is important to consider its functioning and features of normal development. It is known that muscle tissue is one of the main components of the body of superior vertebrates (it accounts for up to 40 % of the weight), 25 % of protein metabolism reactions flow in it [12]. The muscle tissue is dominated by the so-called 'slow' muscle fibers (type I). They are characterized by the fact that they slowly get tired, contain a large number of mitochondria and myoglobin, which gives the tissue a reddish color and determines the oxidative type of metabolism. On the contrary, 'fast' fibers (types IIb and IIx) have a glycolytic anaerobic type of metabolism, contain less myoglobin and mitochondria that causes the lighter color of these fibers [13]. The type and number of muscle fibers are laid in the early and middle stages of fetal development. In the late stages of embryogenesis and the first period of postnatal development, hypertrophy of all muscle fibers occurs due to the fusion of multinuclear myofibrils with mononuclear satellite cells [13]. These structural transformations determine the maximum similarity of the skeletal muscles of newborns and adults, including the type of muscle fibers [14]. In this case, mutations that affect the development of muscle tissue, as a rule, change the amount, composition of muscle fibers, as well as the degree of their hypertrophy.

The myostatin gene (*MSTN*) is one of the best-known candidate genes for meat productivity in sheep [15], goats [16], and cattle [17, 18]. Interest in *MSTN* appeared in the study of its mechanism of action in mammals and fish [19] and participation in ensuring the racing efficiency of dogs [20]. Myostatin is also

known as growth and differentiation factor 8 (GDF8) [21, 22]. A.C. McPherron et al. [23], studying the mouse genome and coding proteins belonging to one of the most important families of growth factors (transforming growth factor β-family, *TGF-β*), conducted one of the first detailed studies of the myostatin gene and mechanisms that ensure its biological activity. Later, the structure of the myostatin gene was determined for other species of farm animals [24, 25].

In the study of the *MSTN* gene structure in sheep, Y.F. Gong et al. [26] found that it is located on the 2nd chromosome and consists of three exons and two introns. In exons 1, 2 and 3, 508 nucleotides (including 373 coding), 374 and 1893 nucleotides (381 coding) are located respectively, in introns 1 and 2 — 1833 and 2030 nucleotides, respectively. The coding regions of the gene are highly conserved. In the sheep breeds of New Zealand Romney, Texel, Corriedale, Dorper, Perendale, Suffolk, Poll Dorset, Dorset Down, Merino, 28 nucleotide substitutions (data from NCBI GenBank, access DQ530260), including a well-described mutation c.*1232G<A (MSTN g+6227G<A), are revealed. Among these 28 substitutions, only one is in exon 1 (C.101G<A) and potentially leads to the replacement of glutamic acid (Glu) with glycine (Gly) in the codon 34. Three SNPs are located in the promoter area, three in the 5′-UTR area, 11 in intron 1, five in intron 2, and five in the 3′-UTR [27]. In Soviet Merino sheep, 28 SNPs are also described, two of which were discovered for the first time: c.940G>T in exon 3 and c.*16C>A in the 3-flanking region of the gene. Mutation c.940G>T converts the 314th codon of glutamic acid into a stop codon (GAA>TAA), which leads to the shortening of the protein product by 62 amino acids [28]. It has been reported about additional but not yet described mutations in the promoter region, introns 1 and 2 and the 3′-UTR region [29-31]. The obtained data show that the genetic variability of myostatin may be greater and a more detailed study of the impact of identified SNPs on the development of skeletal muscle of sheep is necessary.

In sheep of different breeds and animals of other species, the myostatin gene is highly homologous. The areas with affinity to the genes of the *MEF2* (myocyte enhancer factor-2, a key promyogenic transcription factor) and *AR* (androgen) were found in the promoter of *MSTN* in sheep, which indicates the possibility of participation of the protein products of these genes in the regulation of myostatin expression [32]. The regulation of transcription of the *MSTN* gene also involves the MyoD, Myf5, Mrf4, and P21, Smad factors [33-35]. Blocking the pathway from the myostatin gene to its product and further to the target muscle cells having an appropriate transmembrane receptor is accompanied by the expressed positive effect on the metabolism of skeletal muscle cells [36-38]. During embryonic myogenesis, myoblast progenitors are activated by the MyoD factors. After the interaction with the myostatin, the factor 21 (p21) starts; it inhibits the activity of proteins of the Cdk2 cyclin and the Rb retinoblastoma, which suppresses the proliferation of myoblasts and satellite cells in the G_1 phase. Reduction of myoblast proliferation in vitro under the influence of different myostatin concentrations was proved. In the absence of myostatin, the Rb protein is retained in the hyperphosphorylated form, which leads to an increase in the degree of myoblast proliferation [39]. Confirmation of the described mechanism was obtained by C. Liu et al. [40]. In their work, a vector with a built-in short RNA region (short-hairpin, shRNA) was used to block the myostatin expression in the cell culture of sheep primary myoblasts. It allowed reducing the activity of endogenous MSTN by 73.3 %, increase the proliferation of primary myoblasts by 28.3 % and significantly reduce the expression of MyoD proteins (by 37.6 %, $p = 0.025$), myogenin (by 33.1 %, $p = 0.049$), p21 (by 49.3 %, $p = 0.046$) and Smad3 (by 50.0 %, $p = 0.007$).

The negative regulatory role of myostatin in the development of skeletal muscles was demonstrated in the experiment to produce sheep with the expressed phenotypic effect of 'double muscles'. The knock-out method (KO) by means of microinjection of the genetic vector shRNA and the somatic cell nuclear transfer technology (SCNT) was used to block the expression of the *MSTN* gene. Five live lambs were received from 429 KO-embryos; three of them have reached the age of productive use. In transgenic animals with the myostatin gene knockout, the diameter of myofibrils (muscle fibers) and live weight were significantly higher at the age of 6 months [41].

The high efficiency of simultaneous use of nuclease acting as a transcription activator (transcription activator-like effector nucleases, TALENs) and single-stranded oligonucleotide DNA sequence (single-stranded DNA oligonucleotides, ssODN) for genome editing in sheep was revealed [42]. Testing on the cell line of primary fibroblasts of sheep NEK 293T showed that the joint transfection of TALENs and ssODN induced precise editing of the myostatin gene. *MSTN*-modified cells were successfully used as donor nuclei for embryo cloning [42]. With the help of TALENs and ssODN, cattle and sheep with KO-myostatin (knock-out MSTN) were also derived in the UK and the USA. The authors used the btGDF83.1L + 83.1 NR [43] construction and the RCIscript-GoldyTALEN transcription vector (Addgene ID 38142, Addgene, USA). Twelve lambs were derived from 26 edited blastocysts, 9 of which proved to be viable and demonstrated significantly higher growth energy due to greater growth of myofibrils [44]. M. Crispo et al. [45] demonstrated the high efficiency of the CRISPR/Cas9 system for editing *MSTN* and producing knockout sheep with increased body weight and expressed skeletal muscle development. A total of 53 MSTN-KO blastocysts were transplanted to 29 recipients, 22 viable lambs were received from them, 10 of which confirmed the genetic mutation of myostatin. In lambs with myostatin expression disabled, the average daily muscle mass growth was significantly higher [45].

Genetic manipulations that inactivate MSTN in transgenic mice and fish cause the same effect: in knockout individuals, body weight and its growth were significantly higher than in the control [23, 46]. Thus, by microinjection of the vector with antisense RNA of the myostatin gene, transgenic fish with the phenotype of 'double musculature' was obtained [47]. In KO-homozygous transgenic individuals, the number of myostatin and protein mRNAs was 33 % and 26 % respectively of their content in non-transgenic individuals, while the number of mRNAs myogenic regulatory factors, the MyoD, myogenin (MyoG), Mrf4, and Myf5, was significantly higher. Blocking the expression of myostatin in knockouts caused an increase in the area of muscle fibers by 2 times and body weight by 45% compared to non-transgenic individuals [47]. In transgenic mice, the *MSTN* knockout resulted in an almost 4-fold increase in skeletal muscle mass, and excessive expression of follistatin activated activin receptors regulating the synthesis of the llb type of fibers and led to myoblast hypertrophy with a significant increase in skeletal muscle plasticity [48-51].

The revealed biological role of myostatin and demonstration of genomic editing prospects for obtaining animals with high meat productivity without embedding recombinant DNA in the genome determined the interest in this protein and its gene as a potential genetic marker. The first studies of the effect of the myostatin gene on meat productivity were performed on sheep of the Texel breed with more developed muscles than in other breeds. The aim of the experiment was to determine a genetic mechanism controlling this distinctive phenotypic feature of the breed [15]. In the 3′-region mRNA of the myostatin gene, SNP.*1232G>A was revealed (previously referred to as g+6723G>A),

which is significant for the formation of muscle fibers. It was found that it or a mutation leading to the replacement of nucleotide G to A (allele *A*) creates the tenor of the recognition for the three miRNAs (miR-1, miR-206, and miR-122), thereby blocking the point of translation initiation of mRNA and the myostatin gene is inhibited. It leads to muscle hypertrophy and an increase in the amount of flesh in the carcasses. Similar results were obtained on the hybrids of Texel with the breeds of Poll Dorset and Welsh Mountain [52-54]. This effect of the mutation is confirmed on other breeds of sheep. Thus, at genotyping of 338 Charolais lambs by two SNPs in *GDF8* (g+2449G>C and g+6723G>a), comparison of SNP-genotypes, productivity indicators, and phenotypic traits in 56,500 lambs revealed a significant relationship of SNPs with muscle depth ($p < 0.001$). The muscle depth in animals with g.+6723AA compared to having the genotypes g+6723GG and g+6723AG was more with high confidence ($p < 0.002$). The additive and dominant effects of the allele g+6723A were 1.20±0.30 mm and –0.73±0.36 mm [55], respectively.

The positive effect of mutation g+6723G> of the A myostatin gene in a homozygous state is reported [56]. The authors revealed a significant advantage of homozygotes *AA* in carcass and flesh yield in the lumbar region and hind limbs. No differences in physical and chemical parameters of muscles musculus longissimus lumborum (LL) and musculus semimembranosus (SM) in sheep of different genotypes were found. At the same time, after cooking, steaks from SM from animals with the *AA* genotype received a much higher tasting score. The connection of mononucleotide substitutions revealed in the amplicon 304 bps from the promoter region of sheep *MSTN* with the signs of meat productivity in New Zealand Romney sheep was found [57]. General linear models of the mixed effect showed that the individuals with the genotype c.-2449GC had higher flesh yield ($p = 0.032$) and the bone-flesh ratio ($p = 0.028$) than those with the genotype c.-2449GG. The genotype c.-2379CC was associated with an increase in birth weight ($p = 0.003$) and weaning weight ($p = 0.028$), whereas the genotype c.-2379TC is not associated with growth rate. The haplotype H3 was associated with a decrease in birth weight ($p = 0.002$) and at weaning ($p = 0.011$), and the haplotype H2 was associated with increased flesh yield in the carcass ($p = 0.012$). The authors highlight the prospects for the selection of carriers of c.-2449GC for increasing the meat productivity of sheep [57].

In studies on Norwegian White Sheep breed, a new mutation (c.960delG) in the coding region of the myostatin gene was described. It was found that it shifts the reading frame at position 320 and leads to the premature stop codon at position 359, which causes greater muscle development and lower fat content. The detected mutation had a greater phenotypic effect than c.*1232G>a (g+6723G>A) [58]. In Makuei sheep, bred in Iran, in intron 1 of the myostatin gene, new mononucleotide polymorphisms are described and registered in the NCBI GenBank under the number KJ526625, which are in the positions 224 bps, 226 bps, and 242 bps and lead, respectively, to replacements of c.224C>T, c.226A>G, and c.242g>T. Genetic and statistical analysis showed that the replacement of c.226A>G is related to the heart size and the girth of the metacarpus, while this and other described mutations are not associated with the height at the withers and sacrum and with the body length [59]. In the Baluchi breed, the mutation in intron 1 of *MSTN* significantly influenced the live mass of animals and the flesh yield in the carcass [60].

Discussing the prospects of breeding on certain alleles and genotypes on *MSTN* to increase the meat productivity of sheep, some negative aspects should be noted. Getting animals with the effect of ‘double muscles’ was accompanied by a decrease in the size of vital organs (heart, lungs, kidneys) and the fragility

of the bones of the hind limbs. The animals were more susceptible to respiratory diseases, urolithiasis, alveolar hypoxia, hypoxemia and dystocia at the same time. In addition, rapid muscle growth had a negative impact on reproduction, increased embryonic mortality, abortion, and stillbirth rates [61]. The authors highlight that strict genetic control in the selection of parent pairs is necessary to avoid undesirable effects [6, 62].

The proteolytic calpain-calpastatin system (CCS) is involved in the realization of signs of meat productivity of sheep. It is represented by a family of Ca^{2+}-dependent neutral proteases, which are present in most animal tissues and involved in the regulation of cellular processes, including signaling and synthesis of cytoskeletal proteins, and muscle tissue homeostasis. The increase in the rate of skeletal muscle growth is regulated by a decrease in the rate of muscle protein degradation by reducing the activity of the calpain locus (calpain, *CAPN*) and simultaneously increasing the activity of calpastatin cascade proteins (calpastatin, *CAST*). In addition, the calpain-calpastatin complex regulates proteolytic and cytolytic reactions after slaughter, determining the rate of destruction of Z-discs of skeletal muscles and weakening of the bonds between muscle fibers, which plays a key role in the decay of tubulin and titin during maturation and the formation of the so-called tenderness of meat [63, 64]. The biological role of the calpain-calpastatin system in protein autolysis makes the calpain and calpastatin genes important candidates in the development of genetic approaches to the production of tender texture meat [65, 66].

The first protein of the calpain family was studied in 1976. It was found that calpain is represented by two heterodimeric types, each of which contains similar and very different subunits, the K30 and K80, respectively. Calpain A, or μ-calpain, is maximally activated in vitro at a concentration of Ca^{2+} 50-100 μm, calpain B, or m-calpain, at a concentration of 1-2 μm. However, at the optimal concentration of Ca^{2+}, the activity of calpain B is higher than that of calpain A [65, 67]. Calpain activity and Ca^{2+} concentration vary depending on the temperature and storage time of the muscles after slaughter. After 120 h at 30 °C, the content of free calcium in the longest back muscle was 40% higher than at 2 °C. The activity of m-calpain decreased more slowly than that of μ-calpain [68]. Calpastatin, the endogenous calpain inhibitor, binds in the presence of Ca^{2+} only with heterodimeric calpain molecules. Single-chain calpains are not inactivated by calpastatin due to the lack of a second binding site. Amino acid sequences of μ-and m-calpain in vertebrates are highly conservative (mammalian calpain homology is more than 90 %), and the set of substrates they split is similar and sometimes identical [68]. Genome-wide sequencing of the calpain gene and the study of regulatory and conservative subunits of this protein allowed determining their high similarity in many animal species. Thus, the identity of the nucleotide sequence in sheep of 192 bps in the calpain gene (exons 5 and 6 with intermediate intron) with a similar sequence in the goat, cattle, bison, and pig was 99, 97, 97, and 89 %, respectively [69]. For amplification of a portion of the cattle calpain 3 gene, primers designed to detect the genome of the sheep were used, and comparable results were obtained [70].

On different breeds of sheep, it was shown that the calpain gene is polymorphic, and the frequency of genotypes varies. In the Iranian breed Bandur, three genotypes — *AA*, *AB*, and *BB* with a frequency of 0.672; 0.295 and 0.033, respectively, were revealed [68]. In the Dalagh breed, the distribution of these genotypes, designated as G1, G2, and G3, was 0.082; 0.891 and 0.027 [71]. Similar results were obtained for fat-tailed and Karakul sheep [72, 73]. In the study of the genetic structure of 11 phenotypically dissimilar breeds of sheep in India with different geographical distribution for a number of candidate genes of

meat productivity (*CAPN4*, *CAST*, *FABP3*, and *DGAT1*), the average heterozygosity for them and their haplotypes was determined, 0.328 and 0.545, respectively [74].

The study of the influence of the allelic state in the calpain gene on the parameters of meat productivity of sheep allowed finding significant differences in the fat content in carcasses obtained from animals of different genotypes. Thus, the association of point mutations in exon 10 *CAPN3* with the fat layer around the thighs, kidneys, and heart in animals with different SNP was found [75]. In experiments carried out on lambs of the Romney breed in New Zealand, a reliable association of one of the genotypes with a lower fat content (in the absence of connection with the parameters of carcasses by weight and measurements) was revealed in exon 10 *CAPN3*, which determined a higher grade and price. The data obtained allowed the authors to conclude on the prospects of using the *CAPN3* gene in the breeding program of this breed to improve the consumer qualities of lamb [76]. At the same time, Y. Muto et al. [77], assessing the biochemical value of polymorphism in the *CAPN3* gene in sheep, did not found a significant effect of mutations on the studied signs. None of the four identified mononucleotide substitutions, including exon 10, did cause changes in the amino acid sequence of the protein *CAPN3*. No significant differences in the degree of mRNA expression and the amount of the *CAPN3* protein in animals of different genotypes were found [77].

As already noted, calpastatin is a specific inhibitor of m- and μ-calpains in mammalian tissues, causing the tenderness of meat when ripe after slaughter. The structure of the sheep calpastatin gene was first studied in 1998 [78]. It is localized on the 5th chromosome, includes 4 exons, and its size is about 100 thousand bps. Sequencing of consensus sequences of the calpastatin gene of sheep showed high similarity with those of goats, cattle, and pigs (94-98, 92-93 and 82-83 %, respectively) [79]. In sheep of the Dorset Horn breed, two alleles (*M* and *N*) of the calpastatin gene, which occur at a frequency of 0.77 and 0.23, respectively, were revealed [78]. In populations of sheep from Iran and Pakistan, two alleles were also detected, the frequency of genotypes *MM*, *MN*, *NN* in the breeds of Dalagh was 0.082, 0.891, 0.027; Lori — 0.320, 0.630, 0.050; Zel — 0.620, 0.260, 0.120; Lohi — 0.77, 0.20, 0.03; Kajli — 0.68, 0.26, 0.06, and Thalli — 0.80 (*MM*) and 0.20 (*MN*) [71, 80-82]. In subsequent studies, it was found that the calpastatin gene in sheep is genetically more diverse and represented by many other alleles. A study of the nucleotide sequence of exon 6 by PCR-ISSR analysis and subsequent DNA sequencing allowed determining five new alleles, one of which was a missense mutation and led to the replacement of glycine by leucine (Gln/Leu), which could potentially affect the function of the protein [83]. In the New Zealand Romney breed, in the study of exon 6 and introns 5 and 12 of the calpastatin gene, in addition to those described earlier, four new alleles in exon 12 were found. Generalization of the results obtained by other researchers and their own data allowed the authors to distinguish nine different haplotypes for the calpastatin gene of sheep and make an assumption about their greater informative value in comparison with single nucleotide substitutions (SNPs) for the analysis of the relationship between genes and such a polygenic factor as meat tenderness [84].

Analysis of the gene sequence of calpastatin in the ancient Iranian breeds, differing in the number and form of fat deposits along the caudal vertebrae (thin-tailed sheep Zel; fat-tailed breeds Lori-Bakhtiari; Chall; hybrids with an average severity of tail Zel-Atabay), revealed four SNP in intron 5 (C24T, G62A, G65T, and T69–) and three in exon 6 (c.197A>T, c.282 G>T, and c.296

C>G) [78]. All three polymorphisms in exon 6 were missense mutations that resulted in the replacement of glycine with leucine (Gln/Leu at position 66), glutamine with asparagine (Glu/Asp at position 94) and proline with arginine (Pro/Arg at position 99). The authors suggested that amino acid substitutions may affect the physical and chemical properties of the CAST protein, including hydrophobicity, amphiphilicity, total charge and activity of Ca^{2+}-channels. In total, eight haplotypes (*CAST-1*, *CAST-2*, *CAST-3*, *CAST-4*, *CAST-6*, *CAST-8*, *CAST-10*, *CAST-11*) were identified, with *CAST-1* and *CAST-2* occurring at a frequency of 0.365 and 0.295, previously not described haplotype *CAST-8* – with a frequency of 0.129. The highest heterozygosity (0.802) in the studied haplotypes was found in the Lori-Bakhtiari breed. Differences in the frequency of *CAST-10* and *CAST-8* between the fat-tailed breed of Lori-Bakhtiari and thin-tailed Zel were highly significant ($p < 0.001$), which indicates the possibility of using these haplotypes as genetic markers in the study of the specificity of breeds [79]. The study of the polymorphism of exon 1 of the calpastatin gene of sheep of the fat-tailed Kurdi breed using PCR-ISSR allows revealing the genotypes *aa*, *ab*, and *ac*, occurring at a frequency of 0.55; 0.32 and 0.13 respectively. The average daily weight gain in lambs with the genotype *ab* was 215.2 g, which was significantly higher than for the genotypes *aa* (204.9 g, $p < 0.05$), and *ac* (172.6 g, $p < 0.01$) [85]. The connection of *CAST* genotypes with the growth rate of young sheep is reported, primarily due to a greater increase in muscle mass [86-88].

Thus, the volume of conducting research indicates an increased interest in the search for candidate genes that mark meat productivity in sheep, and the use of genomic editing methods to obtain animals with the expressed effect of increasing muscle mass. In this regard, the genes of myostatin, calpain, and calpastatin are promising for further study in different breeds, the development of reliable DNA tests and their use for genotyping animals as a mandatory reception in breeding programs.

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MICROBIOLOGICAL RISKS RELATED TO THE INDUSTRIAL POULTRY AND ANIMAL PRODUCTION

(review)

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Abstract

The intensive poultry and animal farming generate substantial amounts of bioaerosols, dust, and harmful gases entering the environment daily, which pollutes the adjacent area with the radius ca. 3 km (M.V. Vlasov et al., 2010). Average dust load in the air of a poultry house can reach 10 mg/m^3 (M. Saleh et al., 2014), with median concentration of endotoxins reaching 257.6 ng/m^3 (K. Radon et al., 2002). Microbial load in the deposited dust can reach 3.2×10^9 CFU/m^3, fungal load is 1.2×10^6 CFU/m^3 (J. Skora et al., 2016). Concentrations of mesophilic bacteria in the air can reach 8.8×10^4 CFU/m^3 in an animal house (E. Karwowska, 2005) and 1.89×10^8 CFU/m^3 in a poultry house (K. Roque et al., 2016). Microbial population in the air within the premises for cattle growing can include pathogenic strains *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Candida* spp., *Aspergillus* spp. (V.Yu. Morozov et al., 2016). A bioaerosol in a poultry house can contain different species from genera *Pseudomonas*, *Bacillus*, *Corynebacterium*, *Pasteurella*, *Vibrio*, *Enterobacter*, *Salmonella*, *Brucella*, *Leptospira*, *Haemophilus*, *Mycoplasma*, *Yersinia*, *Staphyloccocus*, *Streptococcus*, *Micrococcus*, *Pantoea*, *Sarcina* (E. Lonc et al., 2010). In the air of poultry houses over 30 microbial species were identified including 13 fungal species dominated by *Aspergillus* and *Penicillium* species (A. Lugauskas et al., 2004). Concentrations of aerobic fungi were found to vary from 4.4×10^3 to 6.2×10^5 CFU/m^3 (V. Agranovski et al., 2007). Inhalation of large amounts of this bioaerosol by farm personnel can promote respiratory inflammations, asthma, various allergic responces (B. Bakutis et al., 2004; D. Pomorska et al., 2009; L.A. Melnikova et al., 2009). Dust mites are among the most active allergen in poultry production (E. Lonc et al., 2010). Over 20 % of farm personnel were reported to complain about the work-related symptoms of respiratory diseases (J. Hartung et al., 2007). The acute enteric infections (colibacteriosis, salmonellosis) are another important problem for the poultry production. High flock densities in the intensive production systems and especially different technological disruptions can lead to the emergence and transmission of diseases affecting livability and productivity of animals (O.R. Ilyasov et al., 2017). In these conditions a constant risk of the outbreaks of infectious diseases which can affect the whole flock is inevitable; the risk of the transmission of zoonotic diseases to humans is therefore high (A.G. Vozmilov et al., 2013). Occasionally the outbreaks of the diseases hazardous for human occur: bovine tuberculosis, rabies, leptospirosis, brucellosis, anthrax. Therefore, modern methods of poultry and animal production are of potential risk for the health status in animals and poultry and in the farm personnel while contamination of the adjacent air space can compromise the welfare of the habitants. Disinfection and decontamination methods, such as aerosol and UV disinfection, filtration of supply and exhaust air should be used to minimize these risks.

Keywords: animal production, poultry production, bioaerosol, pathogenic microflora, microorganisms, fungi, viruses, dust load, infectious diseases, air pollution, air medium

Modern technologies used in animal and poultry farming are economi-

cally efficient and facilitate supplying the locals with products as soon as possible. In most cases, it is achieved due to high density of animals and poultry in restricted areas. As a consequence, atmospheric air is intensively polluted at production territories and well beyond. Since negative changes in quality of aerial environment may have negative impact on public health, air protection is of primary concern [1-4].

The problem of potential risks for public health and for livestock health in commercial animal farming and poultry is relevant in all countries with developed technologies and large-scale food production. Experimental data on organic, biological, chemical, and other pollutions is constantly replenished and systemized. Present review considers comparative research outcomes on quantitative and specific structure of pathogenic microorganisms, viruses, as well as harmful substances in the aerial environment of animal and poultry farming complexes of Russia and other countries.

Aerial bacteria may be a part of dripping (liquid) or dust (solid) aerosols [5]. Mainly, these bacteria are soil-derived saprophytes getting into the air from the soil. Naturally, nearly 40 thousand species of fern, moss, and mushroom seeds, and nearly 1200 species of bacteria and actinomycetes are found in the air. Air flows may widely dessiminate such microorganisms and seeds [6].

Main pollutant of the air basin at the territory of animal and poultry farms is manure (litter). Enormous amount of untreated manure (litter) is accumulated failing special storage areas, whereby it is contaminated by pathogens in the disadvantaged by chronic infections farms. In dry weather and in high wind the infected particles get into the air in form of dust [7]. All of this challenges epizootic and epidemic situation and creates prerequisites for pollution of environment by biological waste [8].

Air containing microorganisms, organic substances, and dust is daily expelled from the animal and poultry farming areas through ventilation system. Transfer from one production facility to the other threatens to give rise to diseases caused by microorganism association [9, 10]. It was shown that form 4.6 to 83.4 billion microorganisms and from 0.2 and 6.1 kg dust get into the air per hour through the exhaust ventilation system of pig-breeding complexes with population of 10-40 thousand animals, with up to 174.8 billion and 41.4 kg, accordingly, at poultry farm with 720 thousand birds in the flock [11]. Substances getting into the air from the animal breeding areas may be felt in windless weather at 1-1.5 km distance from such areas, and by wind direction at distance of 2-3 km and more [12]. Even those pathogens that survive in the air within a few minutes are spreading during this time at great distances, e.g. up to 500 m for *Staphylococcus* [13].

Main microorganism source in the air of animal breeding areas is livestock. It was proven that 2 million and sometimes more microbial cells (including pathogenic) are present in 1 m^3 of such air [6]. Significant increase of the bacterial load in the air, equipment surfaces, feed, and water occurs when keeping and feeding technologies are violated [14].

The highest dustiness of the air is noted in the industrial poultry farming: pen-type, downy, and epithelial dust is formed in breeding young and adult birds, especially during molting. Besides, microorganisms discharged from the upper respiratory airways in poultry get into the air together with intestinal waste after drying. Grained feed becomes the main source of herbal dust [9, 15]. Feed dust often contains antibacterial substances and antibiotics, including those of broad spectrum. Their constant presence in the air may result in antibiotic-resistant strains of microorganisms [16, 17]. Dust at poultry farms contains 3-6 % of fiber, up to 70 % of raw protein, and 7-10 % of substances extracted by ether,

including particles of feather, down hair, litter, fungus, and microbes [18]. Such dust serves both the carrier and growth medium for the microbes. Dust is referred to as solid particles in the air of up to 100 μm. Particles with diameter over 100 μm settle on the surface rapidly, whereas particles with smaller diameter do this very slow. Speed of their transfer fully depends on wind force. Dust with particles of over 10 μm is present in the air in form of suspended matter [6].

High concentration of inorganic and organic substances, biologically sources components, as well as pathogenic microorganisms in the dust bears potential hazard for workers of poultry and animal breeding complexes and causes respiratory diseases, including complicated diseases [5, 16, 19, 20]. Up to 20 % of farmers and agricultural workers complain of symptoms of respiratory diseases related to their professional activity. Spread of obstructive pulmonary disorders is increased with increased duration of the impact of polluted air [13, 21].

At 13 poultry farms with population of 8000 and 42000 birds, total concentration of dust in the air averaged 1.44 mg/m^3 (with high percentage of particles of less than 10 μm in diameter). Bacteria and fungi in the deposited dust amount 3.2×10^9 CFU/m^3 and 1.2×10^6 CFU/m^3, respectively [22]. According to K. Radon et al. [2], dust concentration at poultry farms is 7.01 mg/m^3. M. Saleh et al. [23] note that the highest concentration of inhalable dust (up to 10 mg/m^3) was in broiler chicken breeding by the end of week 4 of fattening.

Dust in animal and poultry farming areas contains significant number of bacterial endotoxins which are released to the air upon lysis of bacterial cell [24]. According to communication of K. Roque et al. [25], the highest content of endotoxins was found in dust of poultry farms (588.8 ± 138.1 EU/m^3), while the lowest indicator was in cattle keeping areas (57.0 ± 32.1 EU/m^3). According to studies of R. Schierl et al. [26], endotoxin concentration in the aerial and deposited dust varied and comprised 16.9 EU/m^3 for dairy livestock, 557.9 EU/m^3 for beef cattle, 668.7 EU/m^3 for pigs, 463.2 EU/m^3 for egg-laying hens, and 1902 EU/m^3 for turkey. Frequent inhalation of endotoxins results in acute inflammations in human respiratory tract, obstructive lung diseases, and asthma widely spread among workers of poultry farming industry [25, 26]. Having got inside the bird's body, endotoxins weaken the immune system decreasing productive performance of poultry; significant strengthening of immune response may cause septic shock [27].

Mechanic mixing of air upon creation of equal temperature conditions over poultry farming area, as well as active movements of animals and poultry promotes increased concentration of suspended dust containing microorganisms [28-30]. According to Russian approved recommendations of technological design of poultry farming units, concentration of dust in the air of poultry farm shall not exceed 5 mg/m^3 for adult bird, 1 mg/m^3 for 1-4-week old young birds, 2 mg/m^3 for birds aged 5 to 9 weeks, 3 mg/m^3 for birds aged 10 to 14 weeks, and 4 mg/m^3 for aged 15 to 22 weeks. Increase of dust concentration by 2 mg/m^3 is acceptable at collection of eggs and poultry feeding [31]. When dustiness exceeds 5 mg/m^3, microorganisms getting into the bird's respiratory tract causes inflammation thereof [19]. Maximum permissible concentration (MPC) of microorganisms in 1 m^3 of air is 250 th. CFU for adult bird, 30 th. for young birds aged from 1 to 4 weeks, 50 th. for bids aged from 5 to 9 weeks, 100 th. for 10-14-week old poultry, and 150 th. CFU for 15-22-week old birds [31]. Aerial bacteria concentration in animal farming areas must be less than 500-1000 CFU/m^3 [6].

According to E. Karwowska [32], number of microorganisms in animal farming areas varies from 1.7×10^3 to 8.8×10^4 CFU/m^3 for mesophilic bacteria, from 3.5×10^1 to 8.3×10^2 — for hemolytic bacteria, from 1.5×10^3 to 4.6×10^4 —

for staphylococcus, from 5.0×10^0 to 2.0×10^2 — for coliforms, and from 1.7×10^2 to 2.4×10^4 — for fungi *Aspergillus* (*A. niger*, *A. nidulans*, *A. ochraceus*), *Penicillium notatum*, *Penicillium* sp., *Cladosporium* sp., and *Alternaria* sp. genus. K. Roque et al. [25] have identified in the air of animal and poultry spaces six genus of gram-negative bacteria, 31 genus of gram-positive, and 11 fungi genus, with predominance of gram-positive *Staphylococcus lentus*, *S. chromogenes*, *Bacillus cereus*, *B. licheniformis*, and *E. faecalis*, fungi *Candida albicans* and gram-negative bacteria *Sphingomonas paucimobilis*. All these organisms are dangerous pathogens, especially for animals and humans with weak immunity. Upon studying microbial concentration in the air and microflora species composition in calf herd, the identified pathogenic strains were *Staphylococcus aureus* (46.1 %), *Streptococcus faecalis* (23.1 %), *Escherichia coli* (15.3 %) and *Candida* spp. (15.3 %), and in block of young animal raising there were *Escherichia coli* (29.4 %), *Streptococcus faecalis* (23.5 %), *Candida* spp. (17.6 %), *Staphylococcus aureus* (17.6 %) and *Aspergillus* spp. (11.8 %) [33].

Comparison of the microbial patterns depending on the season and distance from the farm had shown the least number of bacteria of *Enterobacteriaceae* family in the air inside and outside of poultry farm during winter and autumn (averaged nearly 5.0×10^0 CFU/m^3), provided that during spring the number of such bacteria was maximum (5.2×10^3 CFU/m^3). Staphylococcus was the most widespread microorganism during the entire year (nearly 81 %). Heterotrophic bacteria and fungi comprised 12 and 6 %, accordingly. Concentration of bacteria in the air was determined at distance of 10, 50 and 100 meters from poultry farms. At distance of over 10 m from the farm, number of bacteria has decreased in several times as compared to the values at poultry farms and was minimal at 100 m distance [4].

Studies of the changes in air pollution in farms of broiler chicks, accounting for their age and productiveness, had shown [34-36] that concentration of aerobes increases with bird ageing. The highest concentrations were in air of areas where 5-week old chicks were kept (6.4×10^6 CFU/m^3). According to K. Bródka et al. [37], total concentrations of aerobic mesophilic bacteria varied inside poultry farms from 4.74×10^4 to 1.89×10^8 CFU/m^3. Gram-negative bacteria ranged from 4.33×10^2 to 4.29×10^6 CFU/m^3, *Enterococcus* genus ranged within 1.53×10^4-1.09×10^7 CFU/m^3, and gram-positive bacteria ranged from 3.78×10^4 to 6.65×10^7 CFU/m^3. Due to the fact that mechanical ventilation has been decreasing microbial concentration (by over 2 times), the lowest values for each of the studied microorganism groups were fixed at intensification of the air exchange at poultry farms. Concentration of aerial microorganisms is an important indicator of epizootic state of poultry farm since spread of pathogenic microflora by air is one of the most rapid ways to emergence of massive diseases in poultry. Bioaerosol in air of poultry farms may contain members of *Pseudomonas*, *Pasteurella*, *Streptococcus*, *Salmonella*, *Bacillus*, *Enterobacter*, *Corynebacterium*, *Haemophilus*, *Vibrio*, *Yersinia*, *Brucella*, *Leptospira*, *Mycoplasma*, *Staphyloccocus*, *Sarcina, Micrococcus*, *Pantoea* genus, etc. [39]. If MPC of microorganism in the air is exceeded, average daily body weight gain and survivability in chicks is reduced with valid decrease in humoral immunity [33, 39, 40].

Upon assessment of the impact of microbial aerosols on the immune system of meat-type ducks under controlled ventilation G. Yu et al. [41] had established strong correlation between the concentration of aerobes, gram-negative bacteria, fungi, endotoxins in the air, and titers of antibodies to avian influenza virus serotype H5 (H5 AIV), concentration of immunoglobulin G, interleukin 2, transformation of T-lymphocytes, lysozyme concentration and indices of thymus, spleen, and bursa (calculated as percentage ratio of organ weight

to live weight of an individual). It means that high concentration of microbial aerosol negatively impacted the immune status of ducks. V. Agranovski et al. [42] had also assessed air quality at poultry farms. According to their studies, concentration of bacteria was 1.12×10^5-6.38×10^6 CFU/m^3. Approximately 85 % of bacteria were gram-positive. Number of aerobic fungi varied from 4.4×10^3 to 6.2×10^5 CFU/m^3. They had found members of *Cladosporium*, *Aspergillus*, *Penicillium*, *Scopulariopsis*, *Fusarium*, *Epicoccum*, *Mucor*, *Trichophyton*, *Alternaria*, *Ulocladium*, *Basidiospores*, *Acremonium*, *Aureobasidium*, *Drechslera*, *Pithomyces*, *Crysosporium*, *Geomyces*, and *Rhizomucor* genus.

Fungal spores have micrometer sizes and are also classified as bioaerosol. They are always present in the atmospheric air, where its concentration varies depending on environmental conditions. Just like bacteria, *Stachybotrys chartarum*, *Alternaria alternate*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Fusarium* sp., *Penicillium* sp., *Rhizopus* sp., *Mucor* sp., *Trichoderma* sp. and *Trichothecium* sp. are found in soil, dust, feed, and ground litter, but at least in birds or animals [43]. Conditions of poultry and animal farming areas are favorable for fungal reproduction [34]. Here, their maximum counts were noted during autumn, where 88 % of specific composition was accounted for mold fungi [44].

Viable forms of fungi, as well as their metabolites (micotoxins) in birds, animals, and human beings may cause a number of pathologies, mainly in respiratory organs (irritation of mucous membrane, invasive lung mycosis, allergic rhinitis, allergic lung alveolitis, asthma) and skin (dermatomycosis, onychomycosis) [45]. A. Lugauskas et al. [46] reporte about 31 fungal species of 13 genus found in the air of poultry farms. They isolated and identified 6 species of *Aspergillus*, among which *A. oryzae* and *A. nidulans* (accordingly 15.1 and 9.7 %) prevailed. *Penicillium expansum*, *P. olivinoviride*, *P. claviforme* and *P. viridicatum* predominate among 12 species of *Penicillium* genus. As per K. Radon et al. [2], counts of fungi in poultry farms varied from 2.0×10^7 to 1.1×10^9 CFU/m^3, and bacteria were also abundant (from 4.7×10^9 to 4.2×10^{10} CFU/m^3). H. Shokri [45] had established that most spread fungal species in air of poultry farms are member of *Candida* genus (30.2 %) and *Aspergillus* genus (26.9 %) genus, outside poultry farms the prevailing species were *Alternaria* (37.6 %) and *Candida* (19.3 %) representatives.

Cell immunity participates in protection of human respiratory airways from microorganisms [13, 47]. *Bacillus*, *Aspergillus*, and *Penicillium* species cause nasal and bronchovascular inflammations because of increased migration of neutrophiles, macrophages, and lymphocytes. Gram-negative bacteria impact the metabolic activity of phagocytes, which results in decrease of the total amount of cell elements, mainly due to decrease in the number of neutrophiles and macrophages in lavage liquid of respiratory tract [48]. It was proven [3, 49-51] that inhalation of non-infectious microorganisms and their components may result in inflammation of respiratory tract, and antigens and allergens, having activating the immune system, may cause allergic reactions.

Modern intensified technologies of poultry and animals breeding presuppose high stocking density with maximum use of space to increase production yields per 1 m^2 and decrease energy costs, thus decreasing the cost of finished product [26]. However, this causes heavy pollution of inside air not only by dust, but also by organic compouns — ammonium, carbon dioxide, hydrogen sulphide, toxic products of putrescence and fermentation of organic substances [21, 52]. Increase of MAC of hazardous substances in the air renders negative effect on poultry. For instance, concentration of ammonium in the air exceeding 50 mg/m^3 causes reduced consumption of feed, conjunctivitis, and, consequently, moderating poultry growth, whilst increase of the concentration of carbon oxide up to 100 mg/m^3 is fraught with death [19].

Biological pollution of air is also due to presence of parasites. Most spread parasites in poultry farming are dust mites *Dermanyssus galline*, *Ornithonyssus sylvarium*, *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Knemidocoptes mutans* and *Acarus siro*, which often cause allergy and asthma [38].

Zoonotic diseases (Siberian plague, brucellosis, foot and mouth disease, tuberculosis, listeriosis, and tularemia) are transmitted not only from animal to animal, but also from animal to humans, and vice-versa [53]. Many diseases in humans and animals have common evolutionary origin. For instance, although 15 thousand years ago tuberculosis had invaded livestock, it, nevertheless, became one of the gravest human diseases with time [54]. According to Information and Analysis Center of the Administration of Veterinary Supervision of Federal Service for Veterinary and Phytosanitary Surveillance (Federal State Budgetary Institution All-Russian Research Institute for Animal Health), outbreaks that are dangerous for humans were continually registered in animals in Russia during the first six months of 2017: 4 new bovine tuberculosis foci, over 155 brucellosis foci, 404 unfortunate rubies cases, of which 439 animal deaths, 15 unfortunate cases of Stuttgart diseases. Eight foci of Siberian plague were identified in 2016 [55]. Pathogenic microflora excreted to the environment by sick animals and poultry is disseminated by aerial flows [56]. At insufficient and improper disinfection of animal farming area during prophylactic periods leads to accumulation of pathogenic microflora in the air, which, in its turn, results in contamination of newly located livestock [46, 57, 58]. High stock density causes high susceptibility to pathogens in animals and poultry, increasing probability of massive outbreak of infectious disease. Most dangerous infections are bovine plague, classic and African pig plague, Bluetongue virus, horse flu, Siberian plague, rabies, brucellosis, tuberculosis, bovine leukemia, Aujeszky's disease, leptospirosis, foot and mouth disease, bird flu, New Castle disease, and sheep and goat pox. Some infections are often registered in the Russian Federation. Three outbreaks of New Castle disease, 31 unfortunate bird flu cases, 188 African pig plague foci, and 43 cases of nodular dermatitis had occurred in 2017 [55].

Acute intestinal infections remain the pressing issue in poultry farming. They are characterized by polygenicity, variability of antigenous composition of agents, longstanding antigenic and toxic stimulation of the host's immunocompetent cells. Colibacillosis accounts for nearly 50-60 % of the total poultry loss [59-62]. Colibacillosis is rare as a standalone disease and more often occurs in combination with respiratory micoplasmosis, infectious bronchitis, pullorum disease (chicken fever), and infectious laryngotraheitis [63-65]. One more dangerous infectious disease is bird's salmonellosis. First of all, salmonellosis affects gastrointestinal tract, and, in subacute and chronic form, causes complications, such as pneumonia and arthritis. Salmonellosis in chickens usually comprises approximately 5 %, but this microorganism causes massive outbreaks of food intoxications in humans [66, 67].

Therefore, functioning of animal and poultry farming complexes is associated with threat for environment since hazardous gases, dust, and bioaerosols with high concentration of pathogenic bacteria, viruses, fungal spores, and endotoxins are daily emitted into the air. In most cases, these pollutants exceed the maximum allowable concentrations that can potentially harm the health of animals, poultry, farmers (working poultry farms), as well as people from neighboring regions. High density of animal stock creates conditions for outbreaks of massive infections, including zoonoses, with fast spread to the entire stock. Accordingly, aerosol and UV-disinfection, filtration of supplied and exhaust air are mandatory for animal and poultry farming.

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METABOLISM AND MECHANISMS OF CYTOTOXIC ACTION OF THE LEAD IN MAMMALS

(review)

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Abstract

The real ecological situation in the Russian Federation is characterized by environmental pollution with lead compounds (V.V. Snakin, 1998). The mode of action, intake, distribution in animal body and excretion of this toxic heavy metal are substantial to establish its permissible limits and biological effects. These data are constantly replenished and require updating to reflect changes in climatic and environmental conditions, anthropogenic impacts, and geographic differences. Absorption of lead in the gastrointestinal tract (GIT) of mammals depends on the permeability of the membrane of intestinal epithelial cells and is influenced by physicochemical properties of a compound (concentration, particle size, mineralogical composition, solubility in the liquid environment of GIT, ionic potential, atomic mass), physiological feaatures of an organism (metabolism, body weight, age, gender, pregnancy, lactation), the diet composition and levels of protein, cellulose, calcium, zinc, iron, manganese, and vitamin D (J.A. Jamieson et al., 2006, D.J. MacLachlan et al., 2016; O.A. Levander, 1979; C.J.C. Phillips et al., 2011). These factors characterize the parameters of uncertainty, which are partially excluded in determining the content of lead in the peripheral blood of mammals. In peripheral blood, lead is transported by red blood cells and accumulates mainly in the liver, kidneys and bones. In fact, the toxic effect of lead on mammals depends on its accumulation in organs and tissues. Lead is excreted from mammals with faeces and urine, as well as through wool, milk, sweat glands and fetus. The half-life of the metal from the soft tissues and peripheral blood is 24-40 days. The toxic effect of lead on the organs and tissues is due to a decrease in the cell number of (E.B. Mirzoev et al., 2015). Reducing of viable cell number to a certain critical level leads to functional violations and toxic effects. Activation of free radical lipid peroxidation (LPO) and violation of Ca^{2+} homeostasis are the main mechanisms of cytotoxic action of Pb^{2+} ions (G. Flora et al., 2012; A. Roy et al., 2016; E.A. Veal et al., 2007; A.W. Harman et al., 1995). Mechanisms of regulation of cellular metabolism include, on the one hand, changes in the intensity of the process of free radical LPO, and on the other hand, modifications of the lipid composition of membranes (E.B. Burlakova, 2007). Activation of free radical LPO by lead is due not only to the generation of reactive oxygen species, but also to a decrease in the activity of antioxidant enzymes, superoxide dismutase and catalase. Changes in the composition of biological membranes affect the activity of membrane-bound proteins, i.e. enzymes, channel-forming proteins, receptors, which affects Ca^{2+} homeostasis and cell functioning a whole (R. Jahn et al., 2003, A.H. Kahn-Kirby et al., 2004). Mitochondria which provide cells with energy play a role in the cytotoxic action of Pb^{2+} ions (M. Bragadin et al., 2007). The big data analysis on Pb pollution will determine the strategy for further study of lead action, as well as the methods to solve the problem.

Keywords: lead, cytotoxic effect, calcium, blood, organ, feed, absorption, lipid peroxidation

Technical progress is accompanied by the rise of man-made pollution by heavy metals, particularly lead and its compounds. Lead is a global pollutant and classic toxicant. Annually this element is released into the environment in Russia, 0.6-1.4 thousand tons with industrial waste, 0.05 thousand tons with waste water, and 4 thousand tons from motor transport [1]. In a number of regions, its con-

tent in the air, soil, and water exceeds the maximum acceptable concentrations that enhance the likelihood of getting the metal to animal and human body [2].

Studying of Pb effect is necessary for reasoning of the acceptance limits of its effect on mammals and for assessment of the biological effects underpinned by specific aspects of metabolism of this toxic heavy metal in body. Such data is constantly refilled and requires updating upon integration, accounting for changing of climate and ecologic conditions, man-made effects, and wider supervision geography.

Purpose of present review is to analyze data on patterns of Pb intake, distribution, and excretion, as well as on mechanisms of its cytotoxic action.

Lead mainly gets into the body of mammals with feed and water. Feed undergoes grinding and enzyme destruction in gastrointestinal tract, as a result of which metal is transformed into the state accessible for digestion. Lead absorption in gastrointestinal tract is controlled by nervous and endocrine systems and occurs by passive diffusion, active transport, pinocytosis and endocytosis. Absorption process depends on permeability of membrane of intestinal epithelial cells; its intensity is affected by physico-chemical properties of the lead compounds (size of particles, mineralogical composition, compound solubility in liquid medium of gastrointestinal tract) [3-5], and physiological properties (sex, age, weight, metabolism, pregnancy, and lactation) [6-8], as well as type of diet and content of calcium, lead, iron, manganese, and vitamin D [3, 9].

One of the main factors determining the lead resorption into the blood is ionic potential and atomic mass, increase of which causes decrease of the absorption percentage. For the lead such coefficient varies from 0.05 to 0.2 [10, 11]. Lead bioavailability is influenced by its chemical form: it is higher in lead acetate than in oxide and sulfide [12]. Having penetrated into the liquid medium of mammal's gastrointestinal tract, many toxic substances very soluble in potable water form insoluble hydroxides. At the same time, slightly soluble substances are very soluble in alkali medium of gastrointestinal tract and are absorbed into the blood stream through the intestinal epithelium tissue [13]. Experiments with rats had shown that increase of the dosage of lead acetate from 1 to 100 mg/kg results in decrease of its resorption in the gastrointestinal tract from 42 to 2 % [14]. Evidently, absorption intensity in mammal's gastrointestinal tract depends on quantity of supplied metal and has non-linear nature. Possibly, it is associated with the saturation process of active transport of Pb^{2+} ions in the intestinal epithelium tissue.

With aging, metal resorption in animal gastrointestinal tract is reduced due to sealing of the intestinal cell membranes and decrease of pore diameter in them. In young species such process is more active since growing body needs mineral substances and has increased permeability of cell wall membranes [11]. Upon injection of ^{203}Pb in stomach of rats aged 1, 3, 6, 16, and 54 weeks, specific activity of radionuclide in 3-7 days comprised accordingly 82-57 %; 2.3 %; 0.4-1.1 %; 0.6 %; 0.3-0.5 % of the total activity [15]. Possibly, found differences are due to permeability of the cell wall membranes and are associated with the state of regulatory and protective mechanisms of homeostasis (sympathoadrenal system, central and peripheral nervous system, metabolic detoxication systems) in animals of different age.

Metal absorption process in mammal's gastrointestinal tract depends of their body weight and intensity of metabolism. Heat loss in small animals grows faster due to increase of the body surface per weight unit. Thus, such value comprises 0.15 m^2/kg in rats and 0.072 m^2/kg in rabbits. Differences in metabolism activity are linked with differences in resorption of metals in gastrointestinal tract of males and females: absorption intensity is higher in male rats than in

female rats [16]. Absorption of lead compounds in pregnant and milking species is stronger [17, 18]. Possibly, the reason is in total activity of physiological processes since not only absorption of Pb^{2+} ions, but also Cu^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+}, Cd^{2+} ions is increased during such period [19]. Moreover, synthesis of metallothioneins (MT) participating in transport of elements from gastrointestinal tract to blood during pregnancy and lactation increases [20].

Diets with low and high content of protein increase lead absorption in gastrointestinal tract in rats as compared to its optimal content. Thus, lead accumulation in kidney, liver, and heart tissues in rats getting diet from 3 % of protein had accordingly increased by 52, 32, and 27 %. Similar situation was observed upon increase of the protein in diet up to 30 %: lead content in the studied organs exceeded the control samples by 36, 29, and 24 %. At the same time, when protein content in the diet (15 %) was optimal, accumulation of the lead in kidneys, liver, and heart had increased by 24, 14, and 13 %, respectively [21]. Increase of percentage of raw fiber in the livestock diet decreases metal content in milk [22]: evidently, lead in feed rich in fiber is weakly leached since great share of the metal transferred to gastrointestinal tract is not reabsorbed and is immediately released from the body.

Information about the effect of vitamin D on lead absorption in gastrointestinal tract is contradictory. It is found that upon increase of the physiological norm of vitamin D in feed (over 500 IU/kg of feed) content of the lead in blood and tissues of chicks decreased. Conversely, such indicator grows when content of vitamin D (up to 500 IU/kg of feed) is low [23]. At the same time, accumulation of the lead in chest muscles grows in broiler chicks with increase of the dosage of vitamin D in diet from 3000 to 5000 IU/kg [24]. Possibly, not only content of vitamin D, but also content of Ca^{2+} influence the lead absorption intensity from the feed, since resorption of Pb^{2+} in gastrointestinal tract is intensified by deficit of Ca^{2+} in the diet. High correlation between the content of Ca-binding protein (Ca-BP) in the intestinal mucosa and the lead intoxication was found. It is assumed that Ca-BP promotes not only absorption of Ca^{2+}, but also Pb^{2+} from the gastrointestinal tract [23]. It should be noted that Ca-BP is activated with participation of vitamin D. At excessive content in the diet, bivalent cations Ca^{2+}, Zn^{2+}, Fe^{2+} suppress Pb^{2+} absorption due to change of the ability of the later to attach to membrane. At the same time, deficit of Fe^{2+} causes intense absorption of Pb^{2+} in gastrointestinal tracts, which may be due to functioning of carrier proteins in charge for Fe^{2+} transport.

Generally speaking, 3-10 % of supplied metal is absorbed from the mammal's gastrointestinal tract (except the neonatal period) [25, 26]. Preliminary starvation increases lead absorption [27]. It was found that 3 % of metal injected in the stomach is absorbed in the small intestine after feeding, with 60 % on an empty stomach [28]. It was established, as demonstrated on the models of inverted pockets of intestinal sections in rats, that the highest quantity of the lead is absorbed in small intestines (702.6±4.16 μmol/g of wet weight), and slightly lower in duodenum and ileum (646.7±28.2 and 520.8±21.3 μmol/g of raw weight) [29]. Flowing off the small intestines, blood flows in the portal vein and afterwards in liver. Lead in mainly accumulated in liver cells (hepatocytes) in microsomal and mitochondrial fractions. It is established that single intraintestinal injection of the lead acetate (62.5 mg/kg) is characterized by metal accumulation in hepatocyte mitochondria in male rats on day 1. In furtherance, on day 5 and day 10, the amount of Pb^{2+} decreases with simultaneous increase in the lysosomal ultrastructures, especially lysosomes and residual bodies, which implies compensatory intensification of detoxication processes in cells [30]. Under the effect of intracellular enzymes, lead forms complex compounds

in hepatocytes with bile acids, with which lead is excreted to the small intestine. Part of metal is moved from the stomach with fecal masses, whereas the other part is reabsorbed (enterohepatic circulation process).

Total lead pool in the body may be divided into slowly and rapidly exchanging parts. Slowly exchanging part is located in bone tissue, whereas metal content increases during the entire life and comprises 80-90 %. Part of metal that is faster involved in metabolic processes is located in soft tissues, mainly in kidneys (8.29 %) and liver (2.20 %), as well as in the peripheral blood (1.00 %) [3, 25, 26]. It should be noted that erythrocytes in the peripheral blood contain 99 % of lead [31]. In cells, lead is mainly accumulated in cytoplasm, with δ-amino levuline acid dehydratase (ALAD) as the main binding protein (ALAD binds 35-84 % of the total quantity of the metal). Besides, there are two more proteins of 10 and 45 kDa [32]. Most part of the lead in blood is bound with albumin and γ-globulin. The balance forms a complex with low-molecule combinations containing sulfhydryl groups (MT, cysteine, transferrin). It should be noted that quantity of Pb^{2+} ions (i.e. unbound) in blood is insignificant [25, 26, 33].

During pregnancy, lead penetrates through placenta into foetus by simple diffusion. Concentration of the metal in the foetus blood comprises 85-90 % of its content of the mother's blood. By the end of antenatal period, lead is found in foetus organs and tissues with predominant localization in bones [25, 26, 34].

Therefore, toxic effect of the lead on mammals is determined by its accumulation in organs and tissues. Herewith, content of the lead in peripheral blood could be more reliable indicator allowing us to exclude in part the uncertainty of effect of physico-chemical properties of compound, physiological state of animal, and type of diet.

Lead is excreted with fecal masses and urine, as well as through hair, milk, sweat glands, and foetus. Main quantity of the metal entered to gastrointestinal tract is not retained in the body and is extracted with fecal masses. At average, 30 mkg is extracted through kidneys due to glomerular filtration, with tubular excretion at high lead concentrations. Semi-extraction of the metal from soft tissues and blood takes 24-40 days. Lead mobilizes from the depot for a number of reasons (lactation, calcium deficit), which increases its concentration in the blood and causes toxic effects [25, 26, 35].

Toxic effect of Pb on organs and tissues in mammals is characterized by decrease of the number of viable cells [36, 37]. Decrease of such number to the critical value results in destruction of physiological functions of the organ. Activation of free-radical lipid peroxidation (LPO) and Ca^{2+} homeostasis disorder are considered the main mechanisms of cytotoxic effect of Pb^{2+} ions [38-41].

Free-radical LPO occurs in all types of membranes and plays an important role in regulation of the normal cellular metabolism. Free radicals required for many biological processes are acting as regulatory molecules in biochemical reactions involved in signal transduction ways [42, 43]. In this system an important place is occupied by signal molecules of reactive oxygen species (ROS): superoxide anion-radical ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$), nitrogen oxide (NO^{-}) and hydrogen peroxide (H_2O_2). In particular, H_2O_2 generates hydroxyl radicals in the presence of Cu^{2+} and Fe^{2+} ions by Haber-Weiss (F. Haber, J. Weiss) or Fenton (H.J.H. Fenton) reactions. Such reasons proceed both in cytoplasm and also site-specifically [44].

Cell metabolism regulation mechanisms involve change of intensity of free-radical LPO and modification of membrane lipid content. Activation of LPO is characterized by accelerated extraction of easily oxidized lipids and enrichment of membranes by oxidation-resistant fractions [45]. Change in the

composition of biological membranes impacts the activity of membrane-binding proteins (enzymes, channel-forming proteins, receptors) that influences cell functioning in general [46, 47].

ROSs participate in a cascade of biochemical reactions which result in genome activation and adaptive synthesis of proteins ensuring compensatory metabolic changes [48]. Under the effect of lead, ROS is formed due to oxidation of ALAD, membrane lipids activation of nicotine amide-adenine dinucleotide phosphate oxidase NAD(P)H and inhibition of antioxidant protection enzymes. Lead is accumulated in erythrocytes in the peripheral blood and is bound with δ-ALAD, which has four lead-binding sites. Intracellular lead inhibits enzymes participating in haem synthesis, including δ-ALA-synthase, δ-ALAD and ferrochelatase [49]. Low concentration of metal (5-7 μg/dl) in the peripheral blood reduces activity of δ-ALAD, which results in increase of the concentration of δ-ALA in blood and urine [50, 51]. Oxidized δ-ALA generates ROS by reduction of ferrocytochrome activity and transfer of electron from oxyHb and metHb to other iron complexes [52, 53]. Increase of the lead concentration (8 μg/dl) in the peripheral blood of mammals is characterized by more intensive free-radical LPO and higher blood concentration of malondialdehyde (MDA) [54]. ROS generation occurs due to increase in activity of membrane-binding NAD(P)H-oxidase, which forms $O_2^{-\bullet}$ from the molecular oxygen [55]. Intensification of free-radical LPO affected by the lead is due not only to ROS generation, but also by reduction of activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). Their inhibition is associated with high lead affinity with thiol groups and its ability to replace essential elements in proteins, i.e. Cu^{2+}, Zn^{2+}, and Mg^{2+} ions in catalytic SOD centers and Fe^{2+} ions in CAT.

Ca^{2+} homeostasis in cells also refers to universal cytotoxicity mechanisms [56]. Concentration of ionized Ca^{2+} in cytoplasm is maintained at 10^{-7} mol/l by various mechanisms (Ca^{2+}- pump and Ca^{2+}-specific channels), which are localized both in cell membrane, and in membranes of intracellular organelles [57]. When activator interacts with a cell, concentration of ionized Ca^{2+} in cytoplasm is shortly increased by times that ensure formation of Ca^{2+}-calmodulin complex inducing relevant metabolic reactions. In particular, protein kinase C is activated and increases phosphorilation of cell proteins. Membrane proteins of Ca^{2+}-Na^{+}-exchange system and Ca^{2+}-Mg^{2+}-ATPase which perform transferring Ca^{2+} ions against the concentration gradient are activated simultaneously with formation of Ca^{2+}-calmodulin complex [58]. Cell membrane is a dynamically changing structure, and its damages may be induced by structural changes in a membrane as a whole or in the micro environment of Ca^{2+}-pump and Ca^{2+}-selective channels.

Incubation of human erythrocytes for 1 hour at 5 μmol/l lead in medium led to an increase in ionized Ca^{2+} and pro-coagulation cell activity [59]. Increase of the Ca^{2+} concentration was also observed in splenocytes of rats after 10 min incubation in the medium with 1 μmol/l lead [60]. Increase of intracellular concentration of Ca^{2+} is possibly due to, in particular, activity of membrane proteins (Ca^{2+}-Na^{+}-exchange system and Ca^{2+}-Mg^{2+}-ATPase). In fact, inhibition of activity of Na^{+}-K^{+}-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase was observed in liver and kidney cells in rats getting lead with potable water (750 mg/l) during 77 days. Herewith, concentration of this metal in the peripheral blood was 55.6±6.3 μg/dl [61]. Similar results were obtained with erythrocytes of rats which during 35 days drank 0.2 % lead salt solution instead of potable water, at that blood concentration of Pb^{2+} was 97.56±11.8 μg/dl [62]. Reduction of the protein activity was observed at intensification of free-radical LPO.

Pb^{2+} ions at $1\text{-}5\times10^{3}$ μmol/l block transport of Ca^{2+} ions in human erythrocytes by inhibition of activity of Ca^{2+}-Mg^{2+}-ATPase [63]. Similar results

were obtained at 0.1 to 100 μmol/l concentration of Pb^{2+} in the medium [64]. It is assumed that Pb^{2+} directly affects the sulfhydryl groups of ATPase if Pb^{2+} concentration is more than 1 μmol/l, and calmodulin (at less than 1 μmol/l). Pb^{2+} ions may impact transport of Ca^{2+} ions through plasmatic cell membranes, directly affecting the potential sensitive channels. Possibly, lead blocks regions of Ca^{2+} binding at external cell surface or disturbs Ca^{2+}- dependent dephosphorylation of channels [65]. Besides, Pb^{2+} ions render modifying effect on Ca^{2+}-dependent potassium channels. Pb^{2+} ions activate the channels if Pb^{2+} concentration is less than 10 μmol/l, and inhibit these channels if concentration is higher [66].

Assessment of protein kinase C activity at incubation of rat brain cells with Pb^{2+} ions had revealed its increase, provided lack of Ca^{2+} ions in the medium [67]. It was established that protein kinase C activation coefficient with Pb^{2+} is 4800 times lower than with Ca^{2+} (5.5×10^{-5} and 25 μmol/l), but maximum values of enzyme activity are registered with Ca^{2+} ions. This is due to the fact that protein kinase C has several Ca^{2+} ion binding sites, the first of which more effectively binds Pb^{2+} ions [68].

Pb^{2+} ions influence several cell functions of calmodulin, including activation of calmodulin-dependent phosphodiesterase, by inclusion in Ca^{2+}-binding regions. Affinity of Pb^{2+} ions to Ca^{2+}-binding calmodulin sites is comparable with such in Ca^{2+} ions [69], however demonstrates lower values [70].

Mitochondria supplying energy to cells play certain role in Pb^{2+} cytotoxicity. It should be noted that low concentration of ROS in cells is maintained due to oxidative phosphorylation in mitochondria. Toxic effect of Pb^{2+} ions on cells of renal tubule and epithelial cells is accompanied by changes in their form, structure, and size of mitochondria, which may be due to predominant metal accumulation in mitochondrial fraction [71, 72]. Besides, disturbance of transmembrane transport of ions causing changes of Ca^{2+} homeostasis was noted. Herewith, Pb^{2+} ions inhibit flow of Ca^{2+} ions in mitochondria at simultaneous stimulation of its release from organelles [73, 74]. Reduction of the membrane potential and swelling of mitochondria were noted under the effect of lead, resulting in opening of pores in the internal membrane [75]. It is assumed that Pb^{2+} ions are directly bound with Ca^{2+}-sites in mitochondria pores. It should be noted that opening of pores occurs due to increase of the concentration of $O_2^{-\bullet}$ or its products, whereas closing of pores is caused by reduction of its concentration. Longstanding opening of pores results in apoptosis and cell death [76, 77].

Thus, accumulated data shed the light on specific aspects of metabolism and mechanisms of cytotoxic effect of the lead on mammals and allows us to highlight a number of external and internal factors affecting such processes (physico-chemical properties of lead compounds, physiological features of organism, levels of protein, fiber, vitamin D, as well as micro and macro elements in the diet). The highest metal accumulation occurs in bones, kidneys, and liver, and in several cases the lead is mobilized from depot. Activation of free-radical lipid peroxidation and disturbance of Ca^{2+} homeostasis are main mechanisms of cytotoxic action of Pb^{2+} ions. Free-radical lipid peroxidation becomes more intensive under the effect of the lead due to generation of active oxygen species and reduction of superoxide dismutase and catalase activity. Biological membranes and mitochondria are involved in manifestation of Pb^{2+} cytotoxic effect . Strategy of control over this toxicant in the environment should be based on patterns of Pb entry, distribution, and excretion from mammal's body.

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CHOLESTEROL DEFICIENCY MUTATION HCD DOES NOT IMPACT MILK PRODUCTIVITY AND BLOOD LEVELS OF CHOLESTEROL AND TRIGLYCERIDES IN RUSSIAN HOLSTEIN BLACK AND WHITE CATTLE

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Abstract

The spread of lethal and semi-lethal mutations in cattle populations results in embryonic and postembryonic mortality of calves. The use of a limited number of sires creates the danger of wide spread of genetic abnormalities. Genetic markers identify carriers of a mutation in the absence of information about phenotypic manifestations of the disease. Cholesterol deficiency mutation (HCD, haplotype cholesterol deficiency), a recessive defect of Holstein cattle, is characterized by the death of calves in the first days or months of life. The extent of this genetic defect worldwide is currently very high, 6 to 17 %. In general, there is little information about the relationship of recessive mutations with dairy cattle productivity, and data on the effect of the HCD mutation, first described in 2015, on breeding traits are extremely limited. This paper is the first to report data on a genetic study of the APOB gene region on the BTA11 chromosome and milk production indices depending on the HCD status in a Russian dairy cow population. The obtained results indicate that in the studied population the HCD mutation does not reduce the pedigree value of animals in terms of milk production and milk quality (for fat and protein). The study was performed in a breeding farm of the Leningrad region in 2017. Random sample of Holstein black and white cattle include cows (n = 451) born in 2009-2015 and the calves (n = 7) with clinical signs of diarrhea and proven HCD carriers in pedigree (sires, sires of sires). Genotyping of animals was carried out by PCR using allele specific primers. The productivity of lactation 1 and 2 (milk, yield of milk fat and protein) was studied depending on the genotypes according to HCD. The ANOVA variance analysis and calculation of means were carried out with RStudio program on the basis of a single-effect model. Estimated breeding value of milk, fat and protein yields in kg was calculated using BLUP Animal Model. The concentration of triglycerides and cholesterol was determined with an automatic biochemical analyzer RX Daytona (Randox Laboratories, UK). According to the results of the study, 35 cows (7.76 %) of those tested are the HCD carriers. Among the calves, one calf was defined as a carrier and one heifer with homozygous HCD genotype for *APOB* gene had all symptoms of the disease. It is established that the HCD^+ cows are not inferior to their peers on milk productivity. The cows with the mutant allele of the *APOB* gene born in 2013 significantly exceeded healthy animals: during lactation 1 by 1219 kg ($p \le 0.01$) for milking, by 13.8 kg for milk fat yield, and by 19.9 kg for milk protein yield ($p \le 0.05$); during lactation 2 by 1392 kg ($p \le 0.001$) for milking, by 44 kg ($p \le 0.05$) for milk fat yield, and by 39.8 kg for milk protein yield ($p \le 0.01$). The average estimated breeding value (EBV) of HCD carriers is 6.8 % higher in milk yield, 8.1 % in fat and 4.8 % in protein compared to HCD^- animals. Monitoring of progeny of HCD carriers using Illumina Bovine IBDv3 (50k) did not reveal significant haploblocks in the *APOB* gene region, therefore, selection for increased milk productivity would not lead to a significant increase in the incidence of HCD carriers. Comparative analysis of biochemical indices in the first half

of the dry period did not reveal significant differences in the blood cholesterol (3.04±0.31 mmol/l and 3.33±0.12 mmol/l, respectively) and triglycerides (0.197±0.01 mmol/l, and 0.170±0.01 mmol/l) between groups of latent HCD carriers and cows free from this mutation. Our study has shown that the use of HCD carriers does not reduce productivity in the dairy herd. However, monitoring for this genetic defect is necessary, as incorrect selection of animals can lead to the birth of a sick and non-viable offspring, which in turn will cause economic losses in the farms

Keywords: cattle, genotyping, HCD, haplotype cholesterol deficiency, lethal recessive mutation, apolipoproteine B, gene *APOB*, milk yielding, triglycerides, cholesterol

Artificial insemination and use of limited number of sires creates a risk of lethal recessive mutations in cattle [1]. Not many abnormalities may be visually observed. DNA screening with the use of high density SNP chips identifies mutations without phenotypic disease manifestations [2]. Such method is used to determine fertility haplotypes found in cattle and becoming the mortality factor at different stages of animal growth. Screening of domestic Holstein and Black Pied Holstein cattle breeds had shown that frequency of known mutations CVM, BLAD, DUMPS, BY, HCD, HH1, HH3, HH4, and HH5 reaches 10 % in cows and 4 % in sires [3]. Monitoring of hazardous recessive mutations in the cattle herd is mandatory and allows for timely exclusion of mutation carriers from the breeding, significantly reducing the economic losses. Thus, inspection of Holstein sires and timely culling of BLAD and CVM carriers in the Leningrad region decreased the frequency of such mutations up to 1-2 % [4-6].

Haplotype cholesterol deficiency (HCD) is a new recessive genetic defect in Holstein cattle. Identification of such haplotype associated with loss of calves in early postnatal period due to occurrence of therapeutically incurable idiopathic diarrhea was reported by S. Kipp et al. in 2015 at Interbull conference in Orlando (USA). Homozygous animals have disturbance of lipid metabolism and hypocholesteremia. Low blood cholesterol level was detected in heterozygous calves lacking clinical signs. Search of genome associations by 44747SNP scanning (Illumina BovineSNP50 BeadChip, version 2; 54Kv2; llumina, Inc., USA) in the affected calves had resulted in identification of homozygous region of 1.01 Mb on BTA11 (positions from 77274120 bp to 78290130 bp), which indicated autosomal monogenic inheritance of such disorder by recessive or codominant type. Significant SNP is located in position 72248536 bp at distance of nearly 5 Mb from the defective haplotype [7]. Search for genome mutations by genome-wide association studies (GWAS) revealed 22 SNPs in total (from 64367438 to 83585365 bp), which had reached validity threshold near the defective haplotype. Genome studies based on 54K SNP Chip genotypes allowed identification of casual region on chromosome BTA11. Based on analysis of sick animal pedigrees, well-known Canadian Holstein sire Maughlin Storm was found to be the carrier of such disorder [8, 9]. Further studies had shown that this mutation results from an insertion of 1299 bp in exon 5 of *APOB* gene (apolipoprotein B) on BTA11 with a shift of the reading frame in codon region for amino acid residue 135 in APOB. This leads to 97 % truncation of the protein [10].

Other authors found an insertion of reduced endogenic retrovirus ERV2-1 in LTR (Long Terminal Repeats) on BTA11 in exon 5 of *APOB* gene, which resulted in stop-codon not far from the insertion. This preterm stop-codon in open *APOB* gene reading frame caused truncation of protein length by 140 amino acids. It was established that such preterm reduction results in inability to remove chylomicrons from the intestinal cells, which causes cholesterol malabsorption [11]. Apolipoprotein B (APOB) is necessary for synthesis of chylomicrons and lipoproteins of very low density in the intestines and liver. Apolipoproteins are protein components of lipoproteins, usually amphiphilic, which spe-

cifically bind lipids to form lipoprotein particle [12, 13].

High carriage percentage of HCD in Holstein herd was found in different countries: 5.07 % in China (sires, n =138) [14], 17.4 % in Germany (sires, n = 264) [10], 17 and 12 % in Canada for cows of 2012 and 2016 years of birth, respectively [15]. Based on pedigree analysis of 584 sires used for breeding in Russia, it was found that 10.3 % males (60 sires) were latent carriers of mutant allele of *APOB* gene [16]. Herewith, origin of sires varied (Canada, America, and Austria). Based on genotyping of 41 sires, whose fathers were HCD carriers, 17 animals (39 %) were latent carriers of the mutation. Timely screening of the populations for genetic defects in cattle and correct selection of animals could reduce economic losses. According to J.B. Cole et al. [17], economic losses from embryonic and post-embryonic deaths in USA due to lethal mutations comprised nearly 11 million US dollars annually.

In this paper, we had for the first time performed genetic assessment of the region of *APOB* gene and had compared diary output of cows in one of the Russian populations depending on the HCD status. Our findings show that HCD mutation does not reduce pedigree value of animals by milk yield and milk quality in terms of fat and protein.

Our purpose was to assess spread of HCD mutation in a population of Russian Black Pied Holstein cows as associated with milk yield and lipid metabolism indicators.

Techniques. Black Pied Holstein cows (*Bos taurus taurus*) of one of pedigree farming units in the Leningrad Region (2017, n = 451) were randomly sampled, animals were born in 2009-2015 years. Sampled calf (n = 7) had clinical diarrhea signs and HCD carriers in their pedigree (fathers, father's fathers).

DNA was extracted by phenol method [18] from blood taken from tail vein.

The primers used for PCR genotyping were 5′-GGTGACCATCCTCTCTCTGC-3′ (the universal forward primer), 5′-AGTGGAACCCAGCTCCATTA-3′ (the revers primer for amplification of 249 bp fragment) to identify wild allele, and 5′-CACCTTCCGCTATTCGAGAG-3′ (the forward primer for 436 bp fragment) to identify mutant allele of *APOB* gene (indel-polymorphism) [19]. PCR protocol was as follows: 1 min at 95 °C (initial denaturation); 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C (35 cycles); 10 min at 72 °C (amplifier Thermal Cycler T1000, Bio-Rad Laboratories, Inc., USA). Reaction mixture contained 67 mM Tris-HCl (pH 8.6), 1.5 mM $MgCl_2$, 16.6 mM NaOH, 0.125 mM each of dATP, dGTP, dCTP, and dTTP, 0.5 μm of each primer, 50-100 μg of matrix DNA and 2.5 U of Taq DNA polymesare (Sibenzyme LLC, Russia). Resulting DNA fragments were separated by horizontal electrophoresis at 10 V/cm in 1× TBE buffer with ethidium bromide (0.1 μg/ml) in 2.0 % agarose gel (Agarosa LE 2, Helicon, Russia). Amplicon sizes were determined with molecular weight marker ThermoScientific Gene Ruler Ultra Low Range DNA Ladder (Fermentas, Lithuania). A video system Gel Imager 2 (Helicon, Russia) was used for gel documentation and data processing.

Linkage disequilibrium (LD) was analyzed in a sample of daughters of HCD sires form Leningrad Region. Genotyping (BovineSNP50 BeadChip v. 3, Illumina, Inc., USA) was conducted as per the manufacturer's protocol. Genotype studies were limited by region of *APOB* gene on BTA11 chromosome, including 33 SNPs of nearly 2000 kbp in length. LD (R^2) was calculated with PLINK 1.9 software [20].

Cholesterol and triglyceride blood levels were determined in HCD^+ animals (n = 3) and in conventionally healthy animals (HCD^-, n = 14) of lactation 2 to lactation 4 in the first half of interlactation period. Blood for biochemical studies was collected from the tail vein with vacuum system Vacuette (Greiner

Bio-One, Austria) 2 hours after feed supply (from 10.00am to 11.00am). In 30-40 min afterwards, vials with blood were centrifuged for 20 min at 3000 rpm. Concentration of triglycerides and cholesterol was measured (a biochemical analyzer RX Daytona, Randox Laboratories, Great Britain) with the use of Cormay reagents (Poland).

Records of dairy yield in cows born in 2012-2014 years were taken from the pedigree cards (form 2MOL). Yield during lactation 1 and lactation 2, milk fat yield, and milk protein yield were accounted for.

Correlation between the genotypes of cows and analyzed traits was identified by statistical significance of differences between the mean values. Found value t_d was compared to *t*-Student table [21]. Dispersion analysis ANOVA and calculation of the means was conducted with RStudio software [22] based on the following model with one fixed effect: $y_{ij} = \mu + HCD_j + e_{ij}$, where y_{ij} is estimated breeding value (EBV) in cow *i* by production traits, μ is mean, HCD_j is fixed haplotype effect, e_{ij} is unknown residual. Data was statistically processed with Microsoft Excel and AtteStat (http://www.studmed.ru/programma-attestat-1205_1778bebd8f9.html) software. Mean (*M*) and standard error of mean (±SEM) are presented.

Estimated breeding value (EBV) for milk yield, fat and protein production was calculated by BLUP Animal Model based on phenotypic data of 2016 [23]. Haplotype effect was shown as 0 (HCD^-) and 1 (carrier HCD^+).

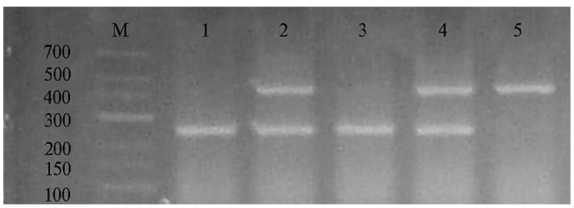

Fig. 1. Electrophoregram of PCR products for indel polymorphism in *APOB* gene in Black Pied Holstein cows: M — molecule weigh marker; 1, 3 — healthy animals (249 bp fragment), 2, 4 — heterozygous carriers (fragments of 249 bp and 436 bp), 5 — homozygous animal (436 bp fragment) (Leningrad Region, 2017).

Results. Figure 1 shows typical electrophoregram of PCR products for indel polymorphism in *APOB* gene.

Totally, we have tested 55.7 % of breeding herd suspected for mutant HCD allele of *APOB* gene. Based on study results, 35 cows (7.76 %) and one female calf were heterozygous carriers of HCD mutation.

Homozygous HCD genotype for *APOB* gene was found only in one heifer with sharp retardation in growth and development, debilitation, and incurable diarrhea (Fig. 2). The calf's weigh at birth was 39 kg, at 2-month age was 49 kg, and at 3-months age was 49 kg; average daily weight gain from the birth to 2 months of age was 166 g. Genotyping showed the mother cow to be HCD-positive, and the father also was the mutant allele carrier. As per parental pedigrees, Maughlin Storm 5457798 sire was among the mother's predecessors, whereas Breadale Goldwyn 10705608 sire was among the father's predecessors. These sires and their descendants are already used for many years in artificial insemination programs in Russia and are found to be latent HCD carriers.

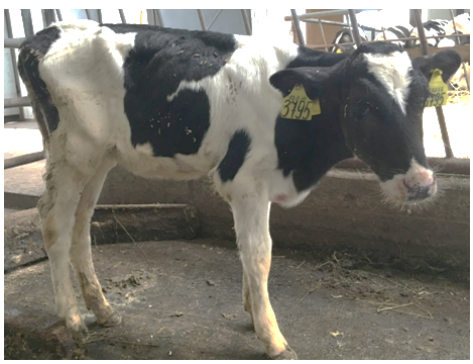

Fig. 2. Black Pied Holstein calf homozygous for mutant allele of *APOB* gene with typical clinical signs of cholesterol deficit syndrome (retardation in growth and development, debilitation, diarrhea) (Leningrad Region, 2017).

HCD^+ calves were as good as their peers and sometimes exceeded them by milk production values (Table 1). Thus, latent carriers of *APOB* gene mutant allele born in 2013 validly left be-

hind healthy animals. i.e. during the first lactation by 1219 kg milk yield ($p < 0.01$), by 13.8 kg milk fat yield, and by 19.9 kg milk protein yield ($p < 0.05$); during the second lactation by 1392 kg ($p < 0.001$), 44 kg ($p < 0.05$), and 39.8 kg ($p < 0.01$), respectively. No valid differences were found between the groups of calves born in 2012 and 2014.

1. Milk production in Black Pied Holstein cows according to genotypes for indel polymorphism in *APOB* gene (haplotype cholesterol deficit HCD) (*M*±SEM, Leningrad Region, 2017)

Year of birth	Status for HCD	Number of animals	Milk yield, kg	Fat, kg	Protein, kg
		Lactation 1			
2012	HCD^+	3	8750±440	342.6±28.6	281.7±20.4
	HCD^-	22	8894±256	333.7±10.1	280.8±7.8
2013	HCD^+	8	9471±261[a]	353.8±7.8	292.0±8.5[c]
	HCD^-	73	8252±142[b]	340.0±5.9	272.1±4.5[d]
2014	HCD^+	10	8780±378	346.5±10.0	268.9±9.1
	HCD^-	101	8646±126	347.0±5.4	271.0±4.1
		Lactation 2			
2012	HCD^+	3	10339±787	390.6±76.9	330.8±43.8
	HCD^-	22	9596±385	371.9±16.4	300.1±11.3
2013	HCD^+	6	10872±346[e]	428.7±20.1[g]	339.7±12.2[i]
	HCD^-	49	9480±233[f]	384.7±9.0[h]	299.9±6.9[j]

N o t e. Statistically significantly differences between HCD^+ and HCD^- are marked with letters: a, b at $p < 0.01$; c, d at $p < 0.05$; e, f at $p < 0.001$; g, h at $p < 0.05$; i, j at $p < 0.01$.

2. Dispersion analysis of breeding value estimates of Black Pied Holstein cows heterozygous for haplotype cholesterol deficit (HCD) (Leningrad Region, 2017)

Production trait	HCD effect	p-value
Milk	4.076	0.0442
Fat	6.617	0.0105
Protein	1.905	0.1680

Average EBV for milk, fat, and protein production for HCD carriers were 1100.8; 42.5 and 28.3 kg, respectively, with 1030.5; 39.3 and 27.1 kg for HCD^- animals. That is, EBV was higher in the first group for milk production by 6.8 %, for fat yield by 8.1 %, and for protein yield by 4.8 %. Dispersion analysis (Table 2) illustrates stable positive effect of HCD carriage on productive performance.

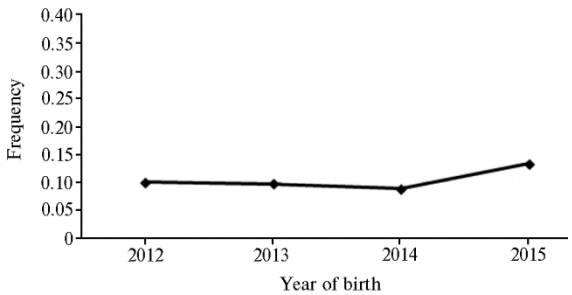

Fig. 3. Frequency of cholesterol deficit mutation (HCD) carriers among Black Pied Holstein calves of different years of birth (Leningrad Region, 2017).

Among the tested animals of different years of birth, frequency of HCD mutations was nearly 10 % (Fig. 3) with a trend towards growth in offspring born in 2015, which highlights the need to constantly control the herd in pedigree farming units to reduce the number of HCD carriers.

We did not find any valid differences between HCD^+ and HCD^- cows in the blood cholesterol level (3.04±0.31 and 3.33±0.12 μmol/l, respectively) and blood triglycerides (0.197±0.01 and 0.170±0.01 μmol/l). For both groups, these metabolites were within the reference concentrations, i.e. 1.5-4.5 μmol/l for cholesterol and 0.05-0.3 μmol/l for triglycerides [24]. Milk yield for 305 days after the last completed lactation did not vary between groups and was 10302±791 and 10191±453 kg, respectively.

For better assessment of the influence of selection for milk production on the spread of HCD carriers, we have analyzed the linkage disequilibrium (LD) between SNP in *APOB* gene regions at distance of nearly 1000 bp from the gene in both directions. Average distance between SNPs on chip in the studied

region was nearly 55 kbp. Estimates had shown absence of haploblocks in this region of genomes of the HCD^+ sire descendants in the Holstein herd population of Leningrad region. Mean LD value was low ($R^2 = 0.077 \pm 0.008$).

It should be noted that according to pedigree records, Holstein sires of different origin (Netherlands, USA, Canada, and Russia) were used for breeding at the studied farms. Some of them have a confirmed status of latent HCD carriers. Literature sources contain insufficient information about the link between the recessive mutations and productive performance of milk-type cattle. In fact, however, the sires carrying recessive mutations often serve as enhancers, and wide spreading of the genetic defects is promoted by the fact that such defects are usually linked with economic trait genes [25]. Thus, analysis of pedigree records of sons and nephews of Skokie Sensation Ned, the stirps of DUMPS disorder, had shown that heterozygotes have significantly higher genetic potential of milk yield [26]. S. Saleem et al. [27] have noted that qualitative values of Holstein sire sperm remain unchanged despite the HCD status. In previous studies we have found that HCD status does not render significant effect on several reproductive parameters in cows (e.g. the age of the first insemination, first calving, and number of inseminations until conception, duration of service-period and intercalving period) [28].

A number of research papers report [29, 30] that sires and calves, the latent HCD carriers, have lower blood concentration of triglycerides and cholesterol compared to animals lacking mutant allele of *APOB* gene. However, blood concentration of cholesterol (as well as triglyceride concentration) is influenced by such factors as nutrition, physiological state of animals, and diseases of different etiology. Therefore, HCD defect could be diagnosed only by molecular testing. In our research, we did not find valid deviations from the normal values in blood biochemical indicators of HCD carriers, which could be due both to small sample size, as well as to genetically determined compensatory mechanisms in several animals [11, 31].

Tracking of selection and genetic characteristics in breed and determination of the genealogical affiliation of sire are required to control genetic defects [32, 33].

It may be interesting to note that HCD carriers do not reduce productive capacity in cattle herd. Moreover, average estimated breeding value in the studied population for milk, fat, and protein production in HCD carriers was higher than in the intact animals. Dispersion analysis had revealed stable positive effect of HCD carriage on productive performance. Such predominance lacks clear explanation, but could be possibly due to location of *APOB* gene in the genome area responsible for high milk production. At the same time, analysis of the linkage disequilibrium between SNP in this region shows lack of haploblocks in the of Holstein population of the Leningrad region. Accordingly, selection for increased productive capacity does not result in significant growth in frequency of HCD mutation carriers.

Therefore, we did not reveal any differences in blood concentration of cholesterol and triglycerides during the first phase of interlactation period in the studied population of Black Pied Holstein cows depending on the status for recessive HCD (haplotype cholesterol deficit) mutation. Use of cows which are the HCD carriers does not reduce productive performance in the herd. Nevertheless, monitoring of the populations for HCD carriage is necessary since use of heterozygous sires may result in sick and non-viable offspring, and, consequently, may cause economic losses to farming units.

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GENETIC CHARACTERISTICS OF REGIONAL POPULATIONS OF NENETS REINDEER BREED (*Rangifer tarandus*)

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Abstract

Nenets breed is the most numerous indigenous breed of domestic reindeer (*Rangifer tarandus*). Due to their biological versatility, the breed's representatives easily adapt to new pastures. The breeding zone covers the territory from the Kola Peninsula in the west to Taimyr in the east. However, there is still no information on the genetic structure of the Nenets domestic reindeer breed. This paper is the first to present data on STR markers of reindeer of the Nenets breed from the Russian regional populations, the breeding zone of which almost completely covers the current range of the breed. The aim of our work was to characterize the allele pool of domestic reindeer of the Nenets breed, which originate from different regional populations. The sample included 787 specimens from 15 Nenets populations collected in Nenets (KAN, ILB, IND, TAB, HRP, PIL, IZH, SEV) and Yamalo-Nenets Autonomous Districts, the Komi Republic (INT), Murmansk (MUR) and Arkhangelsk (ARH) regions, as well as from the Taimyr municipal district (TUH, DUD). Polymorphism in 14 STR markers, including NVHRT21, NVHRT24, NVHRT76, RT1, RT6, RT7, RT9, RT27, RT30, RT25, RT13, NV03, RT5 and NV73, was studied with the genetic analyzer ABI3130xl. Data processing was performed using software GenAIEx 6.501, GENETIX 4.05 and R package "diveRsity". The Neighbor Net graph based on pairwise values of Nei's genetic distances was created with SplitsTree 4.14.5 software. In the studied populations, the average number of alleles per locus (Na) ranges from 9.71 in INT to 6.07 in DUD. The effective number of alleles (Ne) is minimal in the groups DUD and KAN (Ne 3.63) and the maximum in PIL, IZN, INT, SEV, and MUR (Ne 4.55). The number of informative alleles increases from 4.42 in KAN to 5.57 in INT and TAB. Private alleles are found in the INT, MUR, TAB, and TUH groups. In 14 populations, observed heterozygosity (Ho) varies from 0.604 (DUD) to 0.693 (TAB) and 0.695 (IZM). The allelic richness (Ar) ranges from 5.727 in KAN to 7.070 in INT. A heterozygote deficiency was detected in all populations under study. The analysis of Neighbor Net graph showed that the populations of NAO (except KAN) are clustered together with the groups SEY, INT and ARH, the grazing and migratory areas of which are located in the NAO. The Taimyr groups DUD and TUH are separated from the other populations of the Nenets reindeer. The KAN differs from the other populations of the NAO territory, with F_{st} from 0.058 between KAN and IND to 0.083 between KAN and INT, which is indirectly indicated by the lowest level of genetic diversity (Ho = 0.539; Ar = 5.727). Thus, our present study provides the most complete data on the status of the allele pool and level of biodiversity of the Nenets reindeer breed.

Keywords: Nenets breed, *Rangifer tarandus*, reindeer, STR markers, genetic diversity

Nenets breed is the most numerous breed among four accredited domestic reindeer breeds of in the Russian Federation. Its herding zone covers the territory of Murmansk Region (Kola Peninsula), Nenets Autonomous District (AD), the Komi Republic, Yamalo-Nenets, Khanty-Mansiysk, and Taimyr (Dolgano-Nenets) autonomous district. Regional herding of domestic reindeers has its own characteristics deriving from the difference in the land relief, specificity of food resources, and ethno cultural component.

Reindeer herding on Kola Peninsula is mainly concentrated on the east with dominated plain terrain [1]. Until the middle of XIX, domestic reindeer herding was more a traditional way of life of Saami, than of coast-dwellers. Saami had domesticated forest reindeer species and maintained them in small herds. In the 1880s, first Izhma-Komi families have arrived on Kola Peninsula due to lack of pastures [2]. Colonists had brought between 2.5 and 5 thousand of Nenets reindeers, which number reached 42 thousand by 1909 [1], and had implemented large commercial reindeer husbandry in the region. Izhma reindeers had differed appreciably from the Saami in darker color. Gradually, Izhma and Saami reindeers had mixed with predominant hybrid species in herds. Recently, all population of domestic reindeers had acquired consolidated species composition [3]. Reindeer husbandry in the Komi Republic is concentrated in the Komi-Izhma Region of forest-tundra and tundra. In the XVIII century, Izhma-Komi had started borrowing economic bases of reindeer husbandry from the Nenets and, by beginning of the XIX century, had been actively breeding their own large herds for trade and food requirements [4]. Such progress was due to implementation of a set of innovative approaches, i.e. all year pasturage of large herds, night guarding of herds, increase of the share of females and fawns, new pace of changing of the seasonal pastures [5]. Recently, due to the lack of pastures Izhma-Komi actively use resources of Nenets (during summer) and Khanty-Mansiysk AD (during winter) [1]. Nenets AD remains the leading region of Nenets reindeer husbandry. By geomorphologic properties, such district refers to plain tundra bordering on the coasts of Barents and Kara Seas [1]. Modern Nenets preserve their traditional life style, which is closely related to migrations of reindeer herds during the year [6]. Main landscape of Yamalo-Nenets AD is characterized by arctic moss-and-lichen and low bush tundra merging into forest tundra on the south [1]. Camps of reindeer herdsmen of the Northern Yamal are located in river bottoms on flat tundra plains covered by sparse vegetation [6]. Meanwhile, according to A.A. Uzhakov [7], the past 20 years had witnessed prospering of northern reindeer husbandry and intensive growth of the population, which results in exhaustion of the regional feed resource. The largest in the world tundra area is located in Taimyr Region of the Krasnoyarsk Territory, extending from the Putorana Plateau on the south and Laptev Sea on the north [1]. Noskovskaya and Tukhardskaya tundra are two dominating reindeer husbandry centers on the peninsula. Before the first half of the XX century, indigenous ethnic inhabitants of Taimyr Peninsula, Dolgans and Nganasans, were active in reindeer husbandry [5]. Explosion of wild deer population walking away domestic species was one of the reasons of the industrial crisis in the 1950s [1]. For this reason, Dolgans and Nganasans had almost completely switched over to wild reindeer [5]. Recently, domestic reindeer industry in Taimyr is slowly revived [8].

Several authors highlight several ecotypes inside the Nenets breed, division into which is based on phenotype traits (fawning term, constitutional peculiarities, live body weight, and color) [9]. Nevertheless, until present days studies of biodiversity and genetic structure of Nenets breed in all its diversity have not

been conducted.

Microsatellites, or STR markers, are successfully used in studies of reindeers and its counterparts for settlement of various issues, of which for establishment of its genetic structure [10] and biodiversity extent [11], as well as for studying of reindeer differentiation from mainland of the Eurasian area and arctic islands [12]. Besides, the efficacy of STR markers for identification of the genetic relations inside single specie as exampled by the domestic cattle breeds [13] and between the regional populations inside single specie as exampled by sheep [14] was demonstrated. However, information on genetic structure of Nenets breed of domestic reindeer is missing until present days.

Present paper introduces details of STR marker based molecular and genetic studies of Nenets reindeer breed from regional populations, breeding area of which almost completely covers contemporary area of such breed.

Our purpose was to characterize allele fund, to study biodiversity of the regional populations of domestic Nenets reindeers, and to establish phylogenic relationship between them.

Techniques. Sample included 787 tissue specimen (ear fragments) taken from domestic reindeers (*Rangifer tarandus*) of Nenets breed in 2017 for further analysis (2018); 15 populations of different geographic origin were studied.

DNA was extracted by columns Nexttec™ (Nexttec Biotechnologie GmbH, Germany). PCR analysis was conducted for 14 microsatellite loci, 9 of which (NVHRT21, NVHRT24, NVHRT76, RT1, RT6, RT7, RT9, RT27 and RT30) were previously successfully tested in genetic studies of reindeer [15]. Loci RT25, RT13, NV03, RT5, and NV73 were used for *R. tarandus* for the first time. A 14 μl reaction mixture contained (per sample) 1.5 μl of 10 % PCR buffer, 1.5 μl of 2 mM dNTPs solution, 0.15 μl of 100 mM $MgCl_2$, 2.54 μl of 10 mM primers, 0.2 μl (1 U) Taq DNA polymerase (Dialat Ltd, Russia), 8 μl bidistilled water, and 1 μl (50-100 ng) matrix DNA. Stock solution of PCR buffer contained 16.6 mM $(NH_4)_2SO_4$, 67.7 mM Tris-HCl (pH 8.8) and 0.1 % Tween 20. The annealing temperature for the primers was chosen based on their melting points. PCRs were carried out by standard protocols (a thermal cycler Mastercycler, Eppendorf, Germany).

Allele sizes were determined using a genetic analyzer ABI3130xl (Applied Biosystems, USA) with Gene Mapper v. 4 software. Matrix for genotypes was created with Microsoft Excel software.

Allele diversity indicators were calculated with GenAIEx 6.501 [16], including average allele number per locus (Na), effective allele number (Ne), informative allele number (with frequency of over 5 %) (Na 5 %), and the number of private alleles. Expected (H_e) and observed (H_o) geterozygosity, inbreeding coefficient (F_{is}) with 95 % confidence interval (CI, $t_{0.05} \times$ SEM), rarified allele diversity (Ar) values were calculated in package R "diveRsity" [17] in the software environment R [18].

To establish genetic relations between the populations, pair genetic distances D_N by M. Nei [19] were calculated with GENETIX 4.05 software [20], and paired F_{st} values [21] were calculated with GenAIEx 6.501 software. Genetic relations between the reindeer groups were visualized by Neighbour Networks based on matrix of pared D_N values uing SplitsTree 4.14.5 software (http://www.splits-tree.org/) [22]. Previously obtained data [15] of microsatellite analysis of wild Taimyr deer genome (WLD, $n = 57$) was used as outgroup for construction of genetic networks.

Samples of wild indiciduals were collected in three geographical regions of Taimyr AD, the Belogolovka Village (BEL, $n = 13$; 72°9′33.13″N, 91°18′92″E), Kongudoyar township (KGR, $n = 19$; 72°7′62.05″N, 91°36′09″E) and Ust-

Avam township (UAV, n = 20; 71°11′40.71″N, 92°82′07″E). Geographical map illustrating the sample collection points was created with package R "maps" [18] and visualized with R "ggplot2" [19] in software environment R.

Results. Characteristics of the studied domestic reindeer are provided in Table 1. Figure 1 shows location of sampling points of domestic and wild deer.

1. Characterization of the samples of domestic Nenets reindeer (*Rangifer tarandus*) used in molecular studies (2017-2018)

Household, sampling points	Symbol	n	Total population (January 1, 2018)	Geographic location, geomorphologic region
APC Indiga		48	7419	Nenets AD, Zapolyarny region, Indiga township; Malozemelskaya tundra
SRO Ilebts	ILB	58	585	Nenets AD, Nelmin-Nos township; Malozemelskaya tundra
SRO Tabseda (Sand Hill)	TAB	96	1801	Nenets AD, Nelmin-Nos township; Malozemelskaya tundra
APC Harp"	HRP	91	12266	Nenets AD, Krasnoe township, Zapolyarny region; western part of Bolshezemelskaya tundra
IAPC Kanin	KAN	31	8955	Nenets AD, Kanin Peninsula; Kanin range, Kanin tundra, White Sea-Mezen massive
APC Izhma olenevod and Co.	IZH	45	27785	Nenets AD; Bolshezemelskaya tundra (central part)
Yamal Branch of All-Russia Research Institute of Veterinary Entomology and Arachnology, deer breeding team No. 2	BRG2	49	1673	Yamalo-Nenets AD; territory, Eastern slope of Ural edge
Family Tribal Community of Indigenous People of the North Ilebts	SEY	47	11150	Yamalo- Nenets AD, Seyaha township; Yamal tundra
APC Put' Ilyicha	PIL	37	15533	Nenets AD; Bolshezemelskaya tundra (central part)
IAPC Tundra	MUR	43	25674 (for 2010)	Murmansk Region, Lovozersky District; north and north-east of Kola Peninsula
PFE Vylko A.V.	ARH	47	607	Arkhangelsk Region, Dolgoshchelye township of Mezensky District; White Sea-Mezen massive
Complex Inta Pripolarnaya LLC	INT	96	9140	Komi Republic, Inta city; Bolshezemelskaya tundra (eastern part)
Severniy LLC	SEV	47	22507	Nenets AD; Bolshezemelskaya tundra (central and eastern parts)
Vasily Palchin's houshold, team No. 10	TUH	15	900 (for 2015).	Taimyr (Dolgano-Nenets) municipal district, Tukhard township; forest tundra
PFE Yarodskiy P.A., team No. 1	DUD	37	1800	Taimyr (Dolgano-Nenets) municipal district, Dudinka city; South-Taimyr low-land area

N o t e. n — number of heads in a group; AO — autonomous district; APC (or IAPC) − Agricultural Production Cooperative (Integrated Agricultural Production Centre), SRO — family and tribal community of indigenous small-numbered people of the north, PFE − Peasant Farm Enterprise.

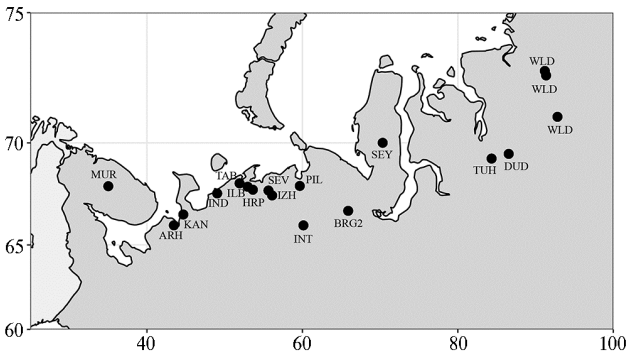

Fig. 1. Sampling of wild (WLD) **and Nenets domestic reindeer (*Rangifer tarandus*)** (2017)**.** See abbreviations in Table 1.

Mean number of alleles per locus in Nenets deer was minimum for DUD (6.071±0.399) and TUH (6.286±0.485), and maximum for INT (9.714±0.529) and HPR (9.286±0.559) (Fig. 2). Mean effective number of alleles was 4.337±0.108 across all populations and varied from 4.858±0.491 for INT to 3.438±0.341 and 3.631±0.385, respectively, for DUD and KAN. INT and TAB groups had precedence over the remaining populations by number of informative alleles (5.571), whereas HRP, DUD and KAN had the minimum numbers (4.643, 4.643, and 4.429, respectively).

Private alleles were not identified in 11 out of 16 studied populations. Possibly, pasturage and migration ways of herds from different households and family-tribal communities have crossed in either way and, thus, gene exchange could not be excluded. Nevertheless, we found private alleles in four groups (INT, MUR, TAB, TUH) (0.286, 0.143, 0.710 and 0.710 alleles per locus), which is mainly due to selection work in these households. Thus, production cooperative Tundra is a tribal reproducer of Nenets domestic reindeer, community Vasiliy Palchin founded in 2001 after reformation of the Tukhard farm, possessed in use the public reindeer population, agricultural complex Inta Pripolarnaya LLC keeps up more strict tribal pedigree records and selection.

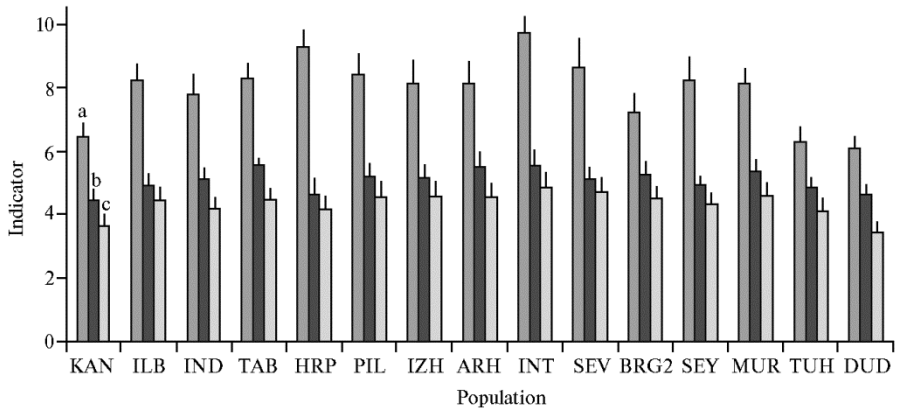

Fig. 2. Allele profiles of studied populations of Nenets reindeer (*Rangifer tarandus*) (2017): a — mean number of alleles per locus, Na; b — number of informative alleles, Na 5 %; c — effective number of alleles per locus, Ne. See abbreviations in Table 1.

Observed heterozygotisity exceeded 0.600 in 14 out of 15 populations with maximum values for TAB (0.693) and IZM (0.695) (Table 2). Allele diversity values varied from 5.727 for KAN to 6.921 in PIL and 7.070 for INT. Positive values of inbreeding coefficient F_{is} confirmed the heterozygote deficit occurred in all studied populations. Note, the KAN group is the least genetically diverse by several parameters (H_o = 0.539; Ar = 5.727) and shows the greatest heterozygote deficit, which could be possibly explained by geographical isolated area of this population (Kanin Peninsula).

2. Genetic diversity of Nenets reindeer (*Rangifer tarandus*) populations (2017) for 14 STR markers

Group	*n*	H_o	H_e	F_{is}	Ar
KAN	31	0.539±0.043	0.673±0.042	0.178 (0.063; 0.294)	5.727±0.381
ILB	58	0.612±0.040	0.735±0.033	0.166 (0.090; 0.242)	6.505±0.400
IND	48	0.617±0.031	0.729±0.028	0.147 (0.077; 0.216)	6.287±0.395
TAB	96	0.693±0.045	0.759±0.019	0.090 (-0.012; 0.191)	6.741±0.342
HRP	91	0.633±0.036	0.723±0.029	0.112 (0.008; 0.215)	6.598±0.443
PIL	37	0.651±0.040	0.744±0.029	0.116 (0.009; 0.223)	6.921±0.482
IZH	45	0.695±0.030	0.748±0.027	0.061 (-0.026; 0.147)	6.622±0.479
ARH	47	0.640±0.043	0.752±0.024	0.148 (0.052; 0.243)	6.750±0.448
INT	96	0.654±0.034	0.762±0.027	0.142 (0.081; 0.202)	7.070±0.433
SEV	47	0.673±0.049	0.760±0.024	0.112 (-0.000; 0.224)	6.791±0.577
BRG2	49	0.650±0.054	0.758±0.020	0.146 (0.014; 0.278)	6.172±0.425
SEY	47	0.637±0.049	0.739±0.026	0.143 (0.042; 0.245)	6.590±0.495
MUR	43	0.629±0.039	0.750±0.028	0.164 (0.087; 0.241)	6.741±0.348
TUH	15	0.657±0.045	0.711±0.038	0.057 (-0.064; 0.178)	6.286±0.485
DUD	37	0.604±0.050	0.670±0.034	0.086 (-0.038; 0.211)	5.317±0.346

N o t e. *n* — number of individuals per sample, year.; H_o — observed heterozygosity, H_e — expected heterozygosity, F_{is} — inbreeding coefficient, Ar — rarified allele diversity. Range variation coefficient F_{is} at confidence interval of 95 % is provided in parenthesis. See abbreviations in Table 1.

Genetic relations of the studied reindeer populations were established based on analysis of genetic network structure (Fig. 3) and F_{st} values. Two con-

ventional clusters were noted oppositely in the network.

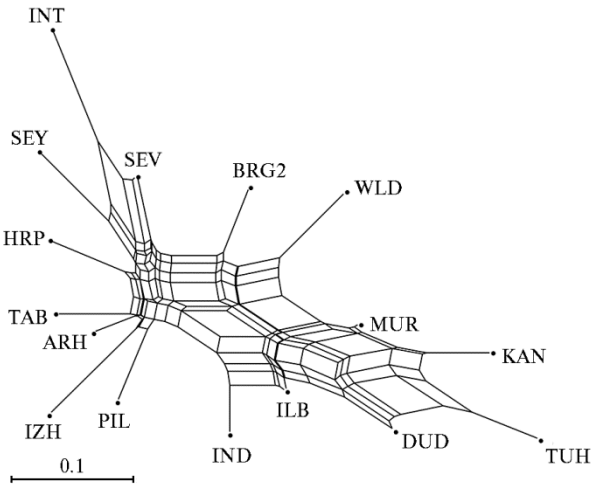

Fig. 3. Genetic relations between 15 populations of Nenets domestic reindeers (*Rangifer tarandus*) (2017) and a group of wild deer (WLD) as **Neighbor Net graphs based on Nei's genetic distance matrix [19].** See abbreviations in Table 1.

The first cluster grouped populations of Nenets AD (except for KAN), and SEY, INT, and ARH, and joining branches IND, ILB and BRG2. Such distribution, probably, reflects the history of certain herds. For instance, Seyakhin herds are isolated from the main massive of the breed since they are located in tundra all year round and do not migrate for wintering to forest tundra as animals from other households of Yamal District. Nevertheless, this group originates from reindeer herds from Nenets AD, which the observed clustering possibly reflects. Besides, herd pasturage and migration territories are often located close to each other (for instance, for ARH from Mezensk Region and for INT from Komi Republic, they are close to such in Nenets AD). Deer from ARH group demonstrate some genetic relationship with populations of Nenets AD, reflected in insignificant F_{st} values (from 0.019 with TAB to 0.030 with ILB). In its turn, INT group forms one of the longest network branches and, according to F_{st} values (0.038 for TAB and 0.055 for ILB), is more clearly differentiated that evidences of the intended selection in this breeding farm. The least F_{st} value for INT was found with deer SEV (F_{st} = 0.020), which also initially originate from the Komi Republic. We should note genetic relationship between IND and ILB groups (F_{st} = 0.014), which, possibly, points out to presence of common originators of herds or close breeder exchange.

The second conventional cluster included groups KAN, DUD, and TUH. DUD and TUH groups (F_{st} = 0.027) are representatives of Taimyr domestic deer, separated from other populations of Nenets reindeers. Herewith, TUH is the most genetically separated group, with F_{st} from 0.053 for IND and BGR2 to 0.087 for INT. Other well differentiated group is KAN (F_{st} from 0.058 for IND to 0.083 for INT), which is bred in Kanin Peninsula. Possibly, KAN consolidation with Taimyr deer is due to their distance from other populations, but not to close relationship between each other.

MUR group formes separate branch close to network centre which is possibly due to its closeness to common ancestor form of breed. Group of wild Taimyr deer (WLD) occupies interim position, as per insignificant difference in F_{st} values (from 0.031 to 0.053).

Reindeer (*Rangifer tarandus*) widely spread in arctic and subarctic zones of Asia, Europe, and North America is an important element of tundra, forest-tundra, and forest ecosystems. In this regard, studying of the genetic diversity and differentiation of groups of such type of hoofed mammals is of interest for many researchers [25-30]. The mean values of the expected heterozygosity found in six caribou populations inhabiting Canadian Alberta and British Columbia provinces varied from 0.740 to 0.790 [25]. For populations inhabiting different

Quebec ecosystems, the expected and observed geterozygosity values were 0.630-0.778 and 0.620-0.770, respectively [26]. Variability for He was within 0.620-0.860, and for Ho within 0.740-0.860 in four deer herds inhabiting Alaska [27, 28]. He (0.649-0.761) and Ho (0.374-0.530) were calculated for groups of deer selected at eight points of the Great Khingan Mountains of Inner Mongolia [29]. M.A. Cronin at al. [30] had studied genetic diversity of domestic reindeer populations using samples from Alaska (H_e = 0.352-0.472; H_o = 0.325-0.456), Northway (H_e = 0.432; H_o = 0.364), and from three points of Magadan Region (H_e = 0.415-0.477; H_o = 0.375-0.402). In our research we have obtained commensurable values of expected and observed heterozygosity, the H_e = 0.670-0.762; Ho = 0.539-0.695. A Russian deer group in research of M.A. Cronin et al. [30] possibly belongs to domestic population inhabiting small common area or represented by genetic relatives, which, possibly, resulted in lower genetic diversity parameters as compared to those we found out. Heterozygote deficit was also recorded for deer groups from China, which significantly exceeded F_{is} values established in our research paper, i.e. 0.320-0.532 [29] against 0.057-0.178. Allele diversity values calculated in our research is quiet close to those obtained by K.H. Mager et al. [28]: Ar = 5.32-7.07 and Ar = 5.70-7.21, respectively.

Possibility to differentiate territorial populations inside *R. tarandus* species based on microsatellites was also studied in sufficient details; however the findings significantly varied. Thus, F_{st} between the caribou populations varied from 0.025 (maximum value between the groups inhabiting one side of Peace river) to 0.044 (minimum value between the groups inhabiting different sides of Peace river) [25]. Values F_{st} among eight reindeer groups inhabiting one and the same archipelago ranged from 0.006 to 0.075 [29]. Three caribou ecotypes (forest, plain, and mountain types) inhabit Quebec province, with F_{st} values among them from 0.087 to 0.172, and F_{st} inside forest ecotype from 0.016 to 0.097 [26]. F_{st} values between Nenets reindeer populations in our study vary from 0.014 to 0.087, which, possibly, evidences on the lack of clearly manifested ecotypes unlike data obtained by M.A. Courtois et al. [26].

Comparison of geographic location of the breeding points of certain Nenets deer herds with genetic network structure had shown that Taimyr deer are differentiated from deer inhabiting Nenets and Yamalo-Nenets Autonomous Districts, whereas clastirezation was not manifested within the districts. K.H. Mager et al. [28] had shown that several herds of domestic reindeer in Alaska were clearly differentiated from each other (F_{st} = 0.23), whilst the others were not (F_{st} is near zero 0). We have observed the same in populations of Nenets breed (maximum value F_{st} = 0.087).

Interesting, the private alleles, as we have already noted, are identified only in groups INT, MUR, TAB, and TUH. We may assume that it was mainly the result of selection work in the households (similar studies have not previously been conducted).

Thus, polymorphism analysis for 14 STR markers had shown that genetic diversity in the studied Nenets reindeer populations within the Russian Federation insignificantly differs from the similar values in its North American and European conspecifics. Probably, it reflects certain genetic stability in these deer groups. Nevertheless, these findings do not allow us to draw conclusion on existence of several ecotypes inside the Nenets reindeer breed. This is also evidenced by some conditionality of population intrabreed clustering. Possible reasons could be specific biology properties, lack of strict pedigree records, and uncontrolled forced redistribution of deer. Besides, escaping of domestic individuals to tundra and living with wild deer may provide gene transfer which shall be accounted in

interpretation of molecular analysis data.

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SELECTION OF PREPARENTAL LINES OF PLYMOUTH ROCK CHICKEN USING MARKER GENES *K* AND *k*

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Abstract

Chicken lines with marker genes of sex-linked economically important traits are of a particular breeding interest. Here we report on the first results on the creation of an autosex maternal parental form of dual purpose Plymouth Rock breed by sequential selection of chickens from experimental Russian breeding lines. The paternal and maternal pre-parental Plymouth Rock lines were selected for productivity indices and for growth rate of wing feathers. Genotypes with slow (line X4) and fast (line X3) feathering rates were maintained via phenotypic evaluation at 1 day of age and culling individuals with fast and slow feathering rate, respectively; heterozygous males and their progeny were also culled. Chicks with slow feathering rate feature poor development of tectrix and remex, the tectrix being longer or equal to the remex; chicks with fast feathering rate feature the well-developed tectrix which is shorter than the remex. The quantitative PCR technique (real-time polymerase chain reaction) was also used to identify individuals with homo- and heterozygous sex-linked *K* and *k* alleles; the analyses were performed using feather pulp samples. Live bodyweight at 5 weeks of age increases significantly ($p \leq 0.001$) in the selected individuals of generation F_5 compared to F_1, i.e in X3, the index is 13.6 % higher in males and 15.4 % higher in females; and in X4, it is 15.2 and 14.2 % higher ($p \leq 0.001$), respectively. Breast muscle score is improved by 7.3 and 5.0 % in males and by 6.2 and 5.0 % ($p \leq 0.001$) in females of X3 and X4 lines, respectively; leg muscle scores is 7.5 and 5.3 % higher in males and 10.5 and 2.5 % higher ($p \leq 0.001$) in females. The progress in the reproductive performance also occurs: egg production during 52 weeks of age was improved by 4.1 eggs per hen (3.39 %) in X3 line and by 4.7 eggs (3.7 %, $p \leq 0.001$) in X4 line; percentage of eggs suitable for incubation was 0.6 and 1.0 % higher, and hatch was 0.5 and 2.2 % higher in X3 and X4 lines, respectively. These results in better chick output per hen (by 3.9 and 7.0 % in X3 and X4 lines). Thus the selection improved the most of the productivity indices in every successive generation; the resulting paternal (X3) and maternal (X4) lines differed from the initial purebred chicken in the productive performance and carried marker genes of slow (X4) and fast (X3) feathering rate. Their crossing brings to a maternal form which is autosex for *K* and *k* genes with sexing accuracy 99.6 %.

Keywords: Plymouth Rock breed, breeding, chicken lines, feathering type, genotype, marker genes, feathering genes, *K* and *k* alleles, sexing, productivity

The challenge of planning and identification of chicken sex in commercial egg and meat poultry farming remains acute [1]. Study of sex genes allows practitioners to implement breeding programs [2, 3]. Routine sex determination methods in poultry are sexing by cloacae and fecal steroids, laparoscopy, and karyotyping. However, such procedures are not reliable, expensive, take long time;

and some of them are painful and even dangerous for poultry.

It is known that sex genes in poultry are located in sex chromosomes [4-6], one Z and one W chromosome in females, and two Z chromosomes in males [7, 8]. Recently, DNA technologies for sex identification have been developed [9, 10]. Genes marking autosexed 1 day old chicks are successfully used in selection of egg-laying and meet-type chickens [11, 12]. DNA markers also allow identification of chromosome regions controlling main properties and critical traits, genetic polymorphisms [13-15]. Study of such loci and their functional activity [16] attributes to perspectives of more effective targeted breeding for economically significant traits [14, 15, 17-19].

Presence of autosexing markers significantly facilitates separation of 1 day old chickens of both sexes. Selection by coat color (color sex) and by growth of tectrix and remex (feather sex) is possible [17, 20-22]. Feathering is under the same genetic control as sexual differentiation. Slow feathering is determined by *K* allele (dominant), fast feathering by *k* allele (recessive). Crossing of fast feathering heterozygous male chickens with slow feathering female chickens give offspring with slow feathering males and fast feathering females. When autosexing method is used, accuracy of separation of 1 day old resultant broiler hybrids and chickens of the female parental line into males and females is significantly increased with double reduction of spent time. Besides, sex determination accuracy is not decreased with growth of chickens. Evidently, creation of specialized chicken lines which are the carriers of marker genes of sex-linked qualitative traits is of special interest.

Here we report our first results on the creation of an autosex maternal parental form of Plymouth Rock breed based on the experimental Russian breeding lines accounting for homo- and heterozygous state of sex-linked *K* and *k* alleles. This form would be further used to create the cross of meat-type chicken with autosex maternal parental form.

Purpose of the research was to assess and to select poultry of paternal and maternal lines by the growth rate of wing feathers and by productive capacity to create cross of meat-type chicken with autosex maternal parental form.

Techniques. Birds of Plymouth Rock breed (experimental paternal line of maternal parental form X3 and experimental maternal line of maternal parental form X4) were used in the study under farming conditions (Selection and Genetic Center Smena, Moscow Region, 2014-2018). Birds were kept in floor pens. Feeding and keeping conditions were as per recommendations (Methodological guidelines for feeding of agricultural poultry. Sergiev Posad, 2015) and technological reglament (Methodological guidelines for technical designing of poultry farms RD-APK 1.10.0504-13. Moscow, 2013).

For selection, 10-15 nestles (13 females and one male in each) were formed in each line, and at least 364 descendants were assessed in each gneration (F_1-F_5). Selection group of X3 line consisted of *kk* enhancers homozygous for fast feathering gene and neutral individuals accounting for valuable economical traits (live body weight, chest and leg weights, feed conversion, egg-laying performance). In X4 line, *KK* individuals homozygous for slow feathering gene, were selected for egg-laying, yield of hatching eggs, their weight, hatchability, live body weight of poultry, chest and leg muscle scores, and feed conversion).

Productive capacity in nestles was accounted individually. Live body weight, egg-laying capacity, egg weight, puberty, and chest and leg muscle scores were determined by commonly accepted methodologies [23]. Individual caps and standard wing band set were used to control pedigree origin at incubation.

Feathering type was visually established in 1 day old chickens separated by sex using Japanese method (by the presence and form of sex bump), at slow growth rate of wind the tectrix feathers are longer than or the same as remex ones,

at fast growth rate the tectrix are shorter than remex and are well developed.

Molecule typing of homo- and heterozygotes by *K* and *k* alleles was done in CJSC Sintol (Moscow) with the use of qualitative real-time PCR (qPCR), relevant primers and amplifying modes [24]. Feather pulp samples were collected to extract DNA [24]. Multiplex qPCR was conducted using an ANK-32M device (Institute of Analytic Instrument Engineering RAS, Russia) with sequencing of amplification products using a genetic analyzer Nanophore 05 (Institute of Analytic Instrument Engineering RAS, Russia) according to the manufacturer's protocols [24].

Software Statistica 10.0 (StatSoft, Inc., USA) and Microsoft Excel were used for statistical processing. Results were presented as means (*M*) and standard errors of means (±SEM). Statistical significance of the compared indicators was determined by *t*-Student criteria. Statistically significant values were at $p \leq 0.05$. Values m_r (standard error of mean correlation coefficient of the analyzed traits by line) and t_r (significance of correlation significance) were calculated based on the following formulae:

$$m_r = \frac{1-r^2}{\sqrt{n}},\ t_r = \frac{r}{m_r} = \frac{r\sqrt{n}}{1-r^2},$$

where *n* is the number of birds in a sample; the values were considered statistically significant at $p \leq 0.05$.

Results. Genotype determining slow feathering was maintained by rigorous selection for phenotype and by exclusion of fast feathering 1 day old chickens. Homozygosity of males in line X4 for slow feathering was verified by assessment of its manifestation in males, selected to form nestles, by estimation of the offspring until reproduction of initial lines. Both heterozygous males and their offspring were excluded. Genotypes with fast feathering was similarly maintained; homozygosity of males in line X3 was controlled by assessment of males destined for nestles by quality of offspring until reproduction of initial lines, without further use of heterozygous males and their offspring.

1. Dynamics of proportion (%) of low and fast feathering 1 day old Plymouth Rock chickens in experimental maternal parental form during selection (F_1-F_5) (Selection and Genetic Center Smena, Moscow Region, 2014-2018)

Generation	Feathering at age of 1 day	Line X3		Line X4	
		males	females	males	females
F_1	Slow	88.9	99.1	92.4	81.9
	Fast	11.1	0.9	7.6	18.1
F_2	Slow	77.2	82.7	99.3	84.9
	Fast	22.8	17.3	0.7	15.1
F_3	Slow	66.6	59.2	100	99.5
	Fast	33.4	40.8	0	0.5
F_4	Slow	31.3	26.9	100	99.9
	Fast	68.7	73.1	0	0.1
F_5	Slow	0	0	100	100
	Fast	100	100	0	0

N o t e . X3 — paternal line of maternal parental form, X4 — maternal line of maternal parental form.

For consolidation of the lines X3 and X4 population by feathering for a number of years, males in nestles were assessed by feathering rate of 1 day old offspring chickens (in line X3 the total number was from 2319 in F_1 to 10880 in F_5; in line X4 the number was from 2466 to 14916, respectively) (Table 1). These results are coherent with other known reports [21, 25-27]. Inside lines X3 and X4, homogeneity by wing feathering rate was reached in different times, but finally, in 2018 feathering rate in all poultry of X3 line was fast, and of X4 slow (see Table 1). The autosex poultry of maternal parental form (for *K* and *k*) with sexing accuracy of 99.6 % was produced from their crossing.

Assessment of productive performance of poultry from the experimental

lines had shown (Table 2) that live body weight of 5 week old chickens of maternal parental form of X3 line was higher than in maternal parental form of X4 line: in males by 6.21 and 4.79 %, in females by 7.09 and 8.28 % ($p \leq 0.001$) in F_1 and F_5, respectively. Body weight of F_5 chickens aged 5 weeks was higher than in F_1, in line X3 by 13.6 and 15.4 % ($p \leq 0.001$) (for males and females), in line X4 by 15.2 and 14.2 % ($p \leq 0.001$). Chest muscle score in F_5 chickens had increased in line X3 for males by 7.3 %, in line X4 by 5.0 %, for females by 6.2 and 5.0 % ($p \leq 0.001$). The same trend was noted in leg muscle score: increase in males by 7.5 and 5.3 %, and in females by 10.5 and 2.5 % ($p \leq 0.001$) in lines X3 and X4, respectively. The total number of chickens estimated for body weight, leg and chest muscle scores in lines X3 and X4 were 2210 and 2370 for F_1, and 10700 and 14810 for F_5 (see Table 2).

2. Improvement of productive performance of Plymouth Rock experimental lines of maternal parental form during selection (F_1-F_5) (Selection and Genetic Centre Smena, Moscow Region, years 2014-2018)

Trait	Sex	Line X3		Line X4	
		F_1	F_5	F_1	F_5
Body weight of 5 week old chicks, kg	Males	1.54±0.004	1.75±0.004	1.45±0.005	1.67±0.005
	Females	1.36±0.003	1.57±0.004	1.27±0.003	1.45±0.004
Chest muscle score, points	Males	4.10	4.40	4.00	4.2
	Females	4.05	4.30	4.00	4.2
Leg muscle score, points	Males	2.00	2.15	1.90	2.00
	Females	1.90	2.10	2.00	2.05
Chicken survivability, %		96.8	97.0	97.0	97.1
Egg-laying performance per initial laying bird, units:					
for 30 weeks		17.10±0.038	19.70±0.375	19.80±0.377	22.00±0.321
for 52 weeks		121.00±1.750	125.10±1.235	126.40±1.730	131.10±1.234
Egg weight of 30-week old females, g		56.90±0.171	57.20±0.176	56.50±1.174	56.90±0.177
Puberty, days		183.40±0.415	184.00±0.396	183.10±0.418	183.70±0.397
Yield of hatching eggs, %		91.5	92.1	91.8	92.8
Egg fertility, %		89.8	91.7	91.6	93.0
Hatching rate, %		74.5	75.0	76.3	78.5
Chickens per layer		82.5	86.4	88.5	95.5
Female survivability, %		96.8	97.0	97.0	97.1

N o t e . X3 — paternal line of maternal parental form, X4 — maternal line of maternal parental form.

Reproductive properties have been improved during 5 years of selection in lines X3 and X4. The egg-laying capacity during 52 weeks of life had increased by 4.1 (3.39 %) and 4.7 eggs (3.7 %) ($p \leq 0.001$); yield of hatching eggs by 0.6 and 1.0 %, hatching rate by 0.5 and 2.2 %, which had increased hatching rate per one layer by 3.9 and 7.0 % (the sample size of $n = 1040$). Annual selection effect in lines X3 and X4 for body weight on day 35 comprised 2.7 % (42 g) and 3.0 % (44 g) in males, 3.1 % (42 g) and 2.8 % (36 g) in females; for chest muscle score 1.46 and 1.0 % (males), 1.23 and 1.00 % (females); for egg-laying performance 0.68 and 0.74 % (lines X3 and X4). Thus, the majority of chicken economic traits became better from generation to generation upon intensive selection. Birds of these lines, being carriers of relevant marker genes of slow (line X4) and fast (line X3) feathering rates, differed from the initial genetic material by productive properties and could be used for production of cross lines with improved productive properties. Crossing of these lines had resulted in maternal parental autosexed form for genes of slow (*K*) and fast (*k*) feathering rates with 99.6 % sexing accuracy.

Correlation coefficients between the body weight of 35 day old chicken and chest muscle score were high, positive and slightly differed both between males and females, and between the lines or generations (Table 3). Positive and valid correlation was preserved between the live body weight and leg muscle score, but its value was slightly lower. High positive and valid relationship was noted between the live body weight in chickens aged 35 days and width of chest, length of

thigh s. These results are in line with regularities described for a relationship of the body weight with other indicators at early age [28].

3. Correlations between body weight and other meat yield indicators in 35 day aged Plymouth Rock chickens in the experimental lines of maternal parental form during selection (F_1-F_5) (Selection and Genetic Centre Smena, Moscow Region, years 2014-2018)

Line	Sex	F_1		F_5	
		$r \pm m_r$	t_r	$r \pm m_r$	t_r
		Chest muscle score			
X3	Males	0.689±0.007	98.37	0.692±0.012	57.67
	Females	0.611±0.008	76.43	0.616±0.011	56.07
X4	Males	0.669±0.007	100.53	0.685±0.010	68.50
	Females	0.701±0.007	126.64	0.707±0.009	78.59
		Leg muscle score			
X3	Males	0.329±0.012	27.65	0.337±0.010	33.70
	Females	0.344±0.011	30.60	0.349±0.010	34.96
X4	Males	0.509±0.009	56.99	0.495±0.013	38.08
	Females	0.471±0.009	52.37	0.483±0.007	69.03
		Chest width			
X3	Males	0.678±0.014	48.43	0.682±0.012	56.83
	Females	0.692±0.010	69.27	0.699±0.013	53.78
X4	Males	0.665±0.016	41.56	0.672±0.015	44.80
	Females	0.659±0.012	54.91	0.656±0.011	59.77
		Thigh length			
X3	Males	0.452±0.022	20.55	0.480±0.024	20.00
	Females	0.427±0.029	17.77	0.433±0.025	17.32
X4	Males	0.464±0.021	22.09	0.461±0.020	23.05
	Females	0.478±0.029	16.50	0.485±0.027	17.97

Note. X3 — paternal line of maternal parental form, X4 — maternal line of maternal parental form.

Thus, we have improved the majority of economic traits due to selection in paternal (X3) and maternal (X4) lines of maternal parental form in generations F_1-F_5, in these, only birds preserving marker alleles of slow (allele *K*, line X4) and fast (allele *k*, line X3) feathering rates were selected. Their crossing resulted in autosex (for alleles *K* and *k*) maternal parental form with sexing accuracy of 99.6 %.

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EFFECTS OF DIFFERENT LEVELS OF PROTECTED L-CARNITINE ON MILK PRODUCTION, METABOLISM, AND REPRODUCTIVE PARAMETERS OF HIGH-PERFORMANCE DAIRY COWS

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Abstract

Amino acid and vitamin provision is known to be crucially vital in the nutrition of highly productive dairy cattle. Currently, important role of carnitine in carbohydrate, fat and protein metabolism has been established, including the transport of long-chain fatty acids through the mitochondrial membrane, excretion of potentially toxic metabolites from cells, regulation of gluconeogenesis, and synthesis of growth hormone. However, there are no recommendations on dietary L-carnitine dosage for newly calved cows with different milk yields. Moreover, data on the effect of dietary carnitine are often contradictory. In this paper, we for the first time compare effective postpartum doses of Carnipass™ and determine optimal amount of protected of L-carnitine (45 mg per 1 kg of produced milk). The aim of our research was to study the effect of different doses of dietary L-carnitine in a protected form on milk productivity, quality, intensity and peculiarities of physiological and biochemical processes in cows during early post calving period. Experiments, including physiological ones, were carried out on four groups of Holstein black-motley cows (8 cows per each, Dubrovitsy unit of Klenovo-Chegodaevo experimental farm, Moscow Province, 2014). Farm tests (Agrofirma Detchinskaya, Maloyaroslavetsky Redion, Kaluga Province) was conducted in 2015-2016. All animals ate a basic ration. The cows of experimental groups II, III and IV additionally received dietary Carnipass™ (Lohmann Animal Health GmbH & Co. KG, Germany), 5.5, 8.3 and 11.1 g equal to 1.0, 1.5 and 2.0 g of L-carnitine, respectively, 21 days before calving and 30, 45 and 60 mg of L-carnitine per 1 kg of produced milk for 120 days after calving. The average daily milk yield in cows of groups II, III and IV was 29.5, 31.6 and 31.4 kg, or by 0.9, 3.0 ($p < 0.05$) and 2.8 kg ($p < 0.05$) higher compared to the control group. When recalculated for milk with 4 % fat content, this index was 3.7, 10.7 ($p < 0.01$) and 10.0 % higher ($p < 0.01$). The somatic cell counts in the milk of experimental cows were from 217.5×10^3 to 42.0×10^3 per 1 cm^3, which is $50.0\text{-}74.5\times10^3/cm^3$ lower than in the control group. The use of L-carnitine at different doses allows reduction of costs per 1 kg of 4 %-fat milk by 3.8-10.8 % feed energy units, 3.6-10.5 % digestible protein, and 3.8-10.7 % concentrated feed. Dietary L-carnitine (45 and 60 mg/kg of milk) increases digestibility of dry matter by 3.06-2.71 %, of proteins by 3.79 ($p < 0.05$) and 3.90 % ($p < 0.05$), respectively, of fat by 0.55-0.06 %, of cellulose by 2.13-1.49 %, and of nitrogen-free extractive substances by 3.41-2.66 %. In cows fed with L-carnitine, blood protein reserves increase due both to albumins and globulins, and protein index is 5.4, 17.0 ($p < 0.05$) and 15.1 % ($p < 0.05$) higher. Activity of transamination enzymes, alanine aminotransferase and aspartate aminotransferase, also rises. The blood concentration of urea decreases by 30.1-35.5 %, which also indicates better nitrogen metabolism. Increase in glucose content by 4.7-9.8 %, and decrease in the amount of bilirubin and cholesterol by 1.4-4.9 and 3.9-8.8 %, respectively, may indicate better function of the liver. At a dosage of 45 mg/kg L-carnitine improves parameters of non-specific immunity. Phagocytic index, bactericidal and lysozyme activity increase by 0.85 units ($p < 0.01$), 6.91 % ($p < 0.001$) and 5.43 % ($p < 0.001$), respectively. In commercial farm tests (Detchinskaya Agro-Firm, Maloyaroslavetskii Region, Kaluga Province, 2015-2016) L-carnitine in a dose of 45 mg/kg has increased milk yield by 11.6 % ($p < 0.05$), as recalculated for 4 %-fat milk, with a decrease in costs per unit of milk production. This is in line with our experimental data. Insemination index is also 0.5 units less, and the service period is 24 days shorter. The profit due to use of protected dietary L-carnitine (45 mg/kg) was 3808 rubles per cow.

Keywords: high producing dairy cows, feed additives, L-carnitine, digestion, digestibility, metabolism, immunity, milk production, reproduction

Along with optimization of feeding [1] and improvement of diets [2] it is reasonable to use methods promoting improved functioning of gastrointestinal tract, liver, and other systems contributing to more effective metabolism in highly productive cows [3] to use their productivity potential [4].

It is known that one of the most important components of biologically complete nutrition of animals and humans is amino acid supply [5, 6]. Important role of methylation in genome functioning [7], immune processes, stress responses and physical loads [8], body detoxication [9] due to lipotrotic-hepatoprotective action of methyl-containing compounds [10, 11] had been proven. Acute deficit of methyl-containing metabolites in highly productive dairy cattle is due to their insufficient level in the feed [12, 13]. Even when diets are enriched with high-protective additives (soybean, rape, and sunflower cakes), the diets require additional sources of methyl-containing compounds in form of methionine, the first limiting amino acid for ruminants [14, 15], as well as choline, betaine, vitamins and mineral substances participating in synthesis of vital compounds for transmethylation [16].

Recently, particular attention is paid to carnitine, which is synthesized in animal body at sufficient supply of lysine, methyonine, choline, vitamins C, B_3, B_9, B_{12}, B_{15}, cobalt, and iron [17, 18]. Carnitine plays important role in carbohydrate and protein metabolism [19, 20]. Carnitine participates in transport of long-chain fatty acids through mitochondrial membranes [21], in removing potentially toxic metabolites from cells, in regulation of gluconeogenesis [22], in synthesis of growth hormone [23, 24]. L-carnitine is used in medicine to maintain cardio-vascular function [25], to strengthen heart muscle and control blood cholesterol level, to render neuroprotective effect, to improve immunity, to prevent atherosclerosis and heart attack, to promote body restoration after loads and stresses [26], to regenerate tissues, to grow muscle tissue more intensively (anabolic action) [27, 28]. L-carnitine inhibits accumulation of arachidonic acid in phospholipids of thrombocytes [29], participates in body detoxication [30], is widely used for treatment of kidney and liver diseases [31], acute infectious diseases, during pregnancy and lactation [32, 33], and for improvement of sperm production in agricultural animals [34, 35].

Post-calving period in highly productive cows is associated with significant reconstruction and acceleration of metabolic processes [36, 37]. Herewith, tissue lipoproteins are used to ensure milk synthesis, which results in a reduction of body weigh in newly-calved cows, incidence of ketosis, hepatosis and other diseases [38], significant milk deficit, decrease in productive use, and deterioration of reproductive function [7].

Although outcomes of numerous researches evidence on the effective use of protected L-carnitine in diets of cattle, recommendations on L-carnitine dosages for newly-calved cows with dissimilar productive capacity are missing. Besides, information on the effect of additionally supplied carnitine are often contradictory [39] and require further detailed studying of physiological and productive action of various dosages of protected L-carnitine in cattle feeding.

Present paper introduces different dosages of protective L-carnitine (in form of Carnipass™) in diets of newly-calved highly productive cows in terms of its influence on digestibility and use of nutritive feed substances, ruminal metabolism, morphological, biochemical, and immunological blood parameters, milk yield and milk quality values. Finally, the optimal dosage of protected L-carnitine (45 mg per 1 kg of produced milk) was established and recommended for use.

Our purpose was to study the effect of various dosages of protected L-

carnitine on milk yield, qualitative milk values, intensity and character of physiological and biochemical processes in newly-calved highly productive cows during days in milk (DIM).

Techniques. For studies (Dubrovitsy farm, research farm Klenovo-Chegodaevo, Dubrovitsy Village, Moscow Region, 2014), four groups of purebreed Black Pied Holstein cows (*Bos taurus taurus*) of the 2nd and 3rd lactations (8 animals per group) were formed accounting for number of calvings, body weight, and milk yield during previous lactation period. Experimental animals in group I (control) and tested groups II-IV got main diet prepared according to the standards [40]. In addition to feed, 21 days in advance to calving the animals from groups II, III and IV got 5.5; 8.3 and 11.1 g of Carnipass™ (Lohmann Animal Health GmbH & Co. KG, Germany) with 18 % of active substance, or 1.0; 1.5 and 2.0 g of protected L-carnitine as per active substance. After calving, cows got 30; 45 and 60 mg of protected L-carnitine per 1 kg of produced milk within 120 days.

Feed consumption was determined by the difference between the supplied quantity and the residual quantity. Milk samples were taken from each cow each ten days. Each average daily sample for analysis was proportional to the produced milk (according to the State Standard GOST 13928-84). Quantitative composition of protein, fat, milk sugar, Ca, P in samples was assessed with an analyzer Bentley 150 (Bentley Instruments, Inc., USA) according to GOST 5867-90, GOST 25179-90, GOST 3626-73, GOST 3625-84, and GOST 3624-92. Milk fatness was determined butyrometrically by Gerber method, protein by formol titration, dry substance by calculation, number of somatic cells according to GOST 23453-90. Productive performance of each cow during the experiment, as well as in average by a group of animals was estimated both as volume of milk of natural fatness and as that re-calculated in terms of 4 %-fat milk. Feed use (energetic feed units, digested protein and concentrates) were determined based on actual consumption related to 1 kg of 4%-fat milk.

Physiological digestion trial to study the digestibility of nutritional substances was conducted in animals of I, III and IV groups 90 days after calving (3 cows with average productive capacity were taken from each group). During the digestion trial the cows were in individual standing stalls equipped by feedboxes and fecal and urine collection facilities. Accounting period took 5 days. Average daily samples of feed, their residues, fecal masses and urine were collected and analyzed by common zootechnical methods [41].

At the end of test, concentration of total protein and its fractions, concentration of glucose, urea, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, alkaline phosphatase, cholesterol, calcium, and phosphorus was determined 3 hours after feeding in blood samples taken from the animals of each group ($n = 3$) with the use of an automatic biochemical analyzer Chem Well (Awareness Technology, Inc., USA). Bacterial activity was assessed by photo nephelometric method, lysozyme activity by V.I. Mutovin [42], phagocyte activity by the ability of blood cells to ingest and digest.

To study ruminal metabolism, ruminal digesta was taken from highly productive cows 3 hours after feeding with further determination of pH, content of volatile fatty acids (VFA), ammonium nitrogen, mass fractions of protozoon and bacteria. Acidity was determined with a pH-meter Aquilon-410 (Aquilon Corp., Russia), total number volatile fatty acids by steam distillation with a Markham apparatus, ammonium nitrogen by Conway's micro-diffusion method, total nitrogen by Kjeldahl method after trichloracetic acid-induced precipitation [43]. Biomass of protozoon and bacteria was determined by differentiated centrifugation.

In pursuance of testing, two groups of Red Pied cows, 25 cows each,

were formed (LLC Agrofirm Detchinskoye, Maloyaroslavetskiy District, Kaluga Region, 2015-2016). Cows in the tested group were fed with protected carnitine 21 days in advance of calving (dosage 8.3 $g \cdot animal^{-1} \cdot day^{-1}$) and on day 120 after calving (45 mg per 1 kg of produced milk) with combined feed in addition to the main balanced common diet. Animals in control group were not supplied with carnitine.

Milk yield, qualitative milk properties, and feed costs were assessed by the above described methods. Animals were weighted during morning hours before feeding in different physiological periods: before launching, 3-5 days before calving, and 5 days, 1, 2, 3, 4 months after calving. Insemination index and service period was established. Based on zootechnical records, economic feasibility of supply of L-carnitine in alimentation of highly productive dairy cattle was estimated during testing.

Data was biometrically processed by *t*-Student criteria. Average mean (*M*), standard error of mean (±SEM), and statistical significance (p) values were calculated. Test results were considered highly statistically significant at $p < 0.001$, and statistically significant at $p < 0.01$ and $p < 0.05$ (44).

Results. Consumption of nutritional substances and energy by cows in tested groups getting different dosages of protected L-carnitine was comparatively equal and ensured high milk yields (Table 1).

In adding protected L-carnitine to the diet (30; 45, and 60 mg/kg of produced milk), average daily milk yields in cows of groups II, III, and IV was 29.5; 31.6, and 31.4 kg, i.e. exceeded the control by 0.9; 3.0 ($p < 0.05$) and 2.8 kg ($p < 0.05$). In terms of 4 %-fat milk, the difference was 3.7; 10.7 ($p < 0.01$) and 10.0 % ($p < 0.01$), respectively. Milk fat, protein, and lactose levels in daily milk of cows of tested groups were 3.6-10.7; 3.4-11.0, and 4.0-10.7 % higher as compared to control. Number of somatic cells in milk produced from cows in tested groups averaged 217.5-242.0 ths/cm^3 and was lower than in the control by 50.0-74.5 ths/cm^3, not exceeding the norms for the highest grade. Content of dry substance, fat, protein, lactose, calcium, and phosphorus in milk of cows in the control and tested groups was relatively equal. Use of different dosages of protected L-carnitine promoted lowering of feed costs: by 3.8-10.8 % for energy feed units, by 3.6-10.5 % for digested protein, by 3.8-10.7 % (per production of 1 kg of 4 %-fat milk) for concentrates as compared to control.

1. Milk yield and quality in Black Pied Holstein cows receiving dietary Carnipass™ during the first 4 months of lactation (*M*±SEM, Dubrovitsy Farm, Experimental Farm Klenovo-Chegodaevo, 2014)

Indicator	Group			
	I (control) ($n = 8$)	II ($n = 8$)	III ($n = 8$)	IV ($n = 8$)
Quantitative indicators				
Natural milk, kg	28.60±0.63	29.50±0.81	31.60±0.72*	31.40±0.73*
Fat in milk, %	4.20±0.19	4.22±0.21	4.21±0.17	4.21±0.17
4 % milk, kg	30±0.47	31.1±0.53	33.2±0.65**	33.0±0.64**
Total volume of natural milk, kg	3432	3540	3792	3768
Total volume of 4%-fat milk, kg	3600	3732	3984	3960
Milk quality indicators				
Dry substance, %	14.12±0.21	14.16±0.24	14.19±0.27	14.20±0.31
Protein, %	3.30±0.36	3.31±0.27	3.32±0.19	3.31±0.34
Lactose, %	4.82±0.03	4.82±0.02	4.82±0.03	4.82±0.02
Somatic cells, ths/cm^3	292.0±70.5	242.0±67.7	217.5±65.4	225.0±64.6
Calcium, %	0.163±0.01	0.162±0.01	0.163±0.01	0.163±0.01
Phosphorus, %	0.950±0.004	0.960±0.005	0.950±0.004	0.950±0.004

N o t e. See description of groups in *Techniques* section. Test period was 120 days.
*, ** Differences from control are statistically significant at $p < 0.05$ and $p < 0.01$, respectively.

Upon feeding of highly productive newly-calved cows with dietary pro-

tected L-carnitine at 45 and 60 mg/kg milk dosages, dry substance digestibility increased by 3.06-2.71 %, protein by 3.90-3.79 %, fat by 0.55-0.06 %, fibre by 2.13-1.49 %, nitrogen free extractive substances by 3.41-2.66 % as compared to control (Table 2). Carnitine promoted better use of nitrogen by 3.1-3.3 g as compared to control and ensured valid differences in protein digestibility ($p < 0.05$).

2. Feed nutrient digestibility coefficients (%) in Black Pied Holstein cows receiving dietary Carnipass™ (*M*±SEM, Dubrovitsy Farm, Experimental Farm Klenovo-Chegodaevo, 2014)

Indicator	Group		
	I (control, $n = 3$)	III ($n = 3$)	IV ($n = 3$)
Dry substance	72.20±1.24	75.30±1.18	75.00±1.19
Organic substance	74.60±1.16	77.60±1.19	77.20±1.23
Protein	70.10±1.06	74.10±0.12*	73.90±1.14*
Fat	70.30±1.09	70.90±1.27	70.40±1.23
Fibre	61.80±1.21	64.90±1.21	63.30±1.35
Nitrogen free extractives	74.50±1.14	77.90±1.34	77.10±1.37

N o t e. See description of groups in *Techniques* section. Test period was 120 days.
* Differences from control are statistically significant at $p < 0.05$.

In general, ruminal pH was within 6.65-6.79. In groups II, III and IV, concentration of ammonia was 17.56; 18.92 and 18.98 mg% (0.37; 1.36 and 1.79 mg% higher than in control), of volatile fat acids 9.34; 11.22 and 11.27 μmol/100 ml (0.21; 2.09 and 2.14 % higher than in control). Growth in levels of ruminal metabolites was due to acceleration of microbial processes in forestomach and growth in the number of protozoon in rumen (by 6.7-36.6 %), and of bacteria (by 9.1-31.8 %) as compared to the control values. Such significant changes in microbial processes in the intestinal tract promoting better feed digestion were, possibly, due to partial availability of L-carnitine active substance not fully protected in the form used.

3. Biochemical blood indicaors and non-specific immunity in Black Pied Holstein cows receiving dietary Carnipass™ (*M*±SEM, Dubrovitsy Farm, Experimental Farm Klenovo-Chegodaevo, 2014)

Indicator	Group			
	I (control) ($n = 3$)	II ($n = 3$)	III ($n = 3$)	IV ($n = 3$)
Total protein, g/l	82.70±5.86	83.80±4.92	86.10±6.17	85.90±5.98
Albumins, g/l	28.60±1.47	30.70±1.54	32.80±1.76	32.60±1.81
Globulins, g/l	54.10±3.24	53.10±4.23	53.30±4.89	53.30±5.14
Albumin/globulin ratio	0.53±0.08	0.57±0.10	0.62±0.09*	0.61±0.11*
Urea, μmol/l	4.80±0.98	3.70±0.65	3.60±0.72	3.60±0.76
ALT, IU/l	20.10±3.16	21.70±2.87	22.40±3.47	22.20±3.54
AST, IU/l	85.10±5.43	89.30±6.07	91.60±6.75	91.30±6.72
Glucose, μmol/l	3.20±0.23	3.30±0.18	3.50±0.12	3.40±0.15
Bilirubin, μmol/l	4.70±0.19	4.60±0.15	4.50±0.12	4.50±0.09
Alkaline phosphatase, IU/l	98.40±18.34	96.50±16.67	83.70±17.92	84.01±19.17
Cholesterol, μmol/l	4.80±0.21	4.60±0.32	4.30±0.16	4.40±0.18
Calcium, μmol/l	2.40±0.13	2.50±0.28	2.80±0.31	2.70±0.26
Phosphorus, μmol/l	1.50±0.15	1.50±0.19	1.60±0.18	1.60±0.18
Bacterial activity, %	78.40±1.12	79.10±0.11	85.30±0.64***	85.20±0.78***
Lysozyme activity, %	35.20±2.34	35.90±2.65**	40.60±2.86**	40.50±2.92**
Phagocyte index	3.70±0.12	3.90±0.16**	4.60±0.11**	4.50±0.15**
Phagocyte number	2.90±0.11	3.20±0.19	3.50±0.17	3.50±0.13
Phagocyte activity, %	66.20±4.24	66.90±4.87	68.30±4.52	68.40±4.63

N o t e. See description of groups in *Techniques* section. A/G — albumin to globulin ratio, ALT — alanine aminotransferase, AST — aspartate aminotransferase,
*, **, *** Differences from control are statistically significant at $p < 0.05$, $p < 0.01$ и $p < 0.001$, respectively.

Better digestibility of feed nutrients under the influence of carnitine promoted better metabolism of body substances and rendered positive effect on protein, carbohydrate and fat and mineral metabolism (Table 3). Improvement of nitrogen metabolism in animals supplied with L-carnitine was evidenced by growth of the total blood protein (by 1.5-4.6 % compared to control) and pro-

tein index (by 9.4-17.0 %), in addition to increased activity of transamination ferments (by 8.1-11.5 % for ALT and by 4.9-7.6 % for AST). Urea concentration decreased by 30.1-35.5 %, the least value noted in blood of cows from group III with 45 mg of L-carnitine/kg milk.

Increase in glucose by 4.7; 9.8 and 8.2 % as compared to control in blood of animals from groups II, III, and IV could evidence on improvement of the energy supply of cows getting carnitine. Alkaline phosphatase concentration decreased by 2.5; 8.6, and 8.3 %. Total blood bilirubin was 1.4; 4.7, and 4.9 % lower, cholesterol was 3.9; 8.8 and 8.2 % lower, which may evidence of acceleration of lipid metabolism and liver functions. Non-specific immunity values (phagocyte, bacterial, and lysozyme activity) increased by 2.08; 6.91, and 5.43 % in cows fed with carnitine (45 mg/kg of milk).

Comparison of biochemical and immunological indicators, evidencing on improvement of metabolic processes, shows the dietary protected L-carnitine in a dosage of 45 mg/kg of produced milk to be optimal. Farm test has shown that administration of L-carnitine in the said dosage had promoted increase of the average daily milk yield in absolute value and as per 4 %-fat milk by 10.6 and 11.6 %, respectively, compared to the control ($p < 0.05$) (Table 4). High milk yield with the use of the said dosage of protected L-carnitine are coherent with results obtained in out experiment. At that, we did not reveal significant differences in content of fat, protein, lactose in milk of the experimental animals.

4. Productive performance and milk composition in Black Pied Holstein cows receiving dietary Carnipass™ in farm testing (*M*±SEM, Agrofirm Detchinskoye LLC, Maloyaroslavetsky District, Kaluga Region, 2015-2016)

Indicator	Group	
	I (control) ($n = 25$)	II ($n = 25$)
Average daily milk yield, kg	30.30±0.58	33.50±0.69*
Average daily milk yield, % to control	100.0	110.6
Fat content in milk, %	4.08±0.19	4.12±0.21
Fat content in milk, % to control	100.0	101.0
Average daily yield as per 4 %-fat milk, kg	30.90±0.53	34.50±0.65*
Average daily yield as per 4 %-fat milk, % to control	100.0	111.6
Total yield of 4 %-fat milk , kg	3708	4140
Total yield of 4 %-fat milk, % to control	100.0	111.6
Protein, %	3.24±0.23	3.30±0.31
Protein, % to control	100.0	101.9
Lactose, %	4.74±0.02	4.76±0.02
Lactose, % to control	100.0	100.4

N o t e. See description of groups in *Techniques* section.
* Differences from control are statistically significant at $p < 0.05$.

Used energetic feed units, digested protein and concentrates per unit of production in animals getting carnitine were 12.2, 11.8, and 11.4 % lower than in control.

During the 1st month after calving, control and experimental groups showed maximum loss of body weight (16.9 and 11.6 kg) (Table 5). During the 2nd months of lactation, loss of body weight in cows receiving carnitine decreased to the least extent than in the control ($p < 0.05$). During days in milk, a 29.9 kg decrease from the initial weight was noted in control group, whereas it was lower in the experimental group (19.9 kg, difference of 2.9 %, $p < 0.05$). Average daily live weight gain of 63 g in the experimental group as compared to control occurred on month 3 of lactation, which generally evidences of improved use of feed nutrients and energy when dietary L-carnitine was fed.

When protected L-carnitine (45 mg/kg of milk) was added to the diet, number of pregnant cows increased by 16 % for two sexual cycles, which was also reflected in better insemination index which decreased by 0.5 units and short-

er service period (by 24 days). During farm testing, surplus from milk sale was 3808 rubles per cow upon use of Carnipass (dosage of L-carnitine was 45 mg of active substance per 1 kg of produced milk).

5. Body weight and its change after calving in Black Pied Holstein cows receiving dietary Carnipass™ in farm testing (*M*±SEM, Agrofirm Detchinskoye LLC, Maloyaroslavetsky District, Kaluga Region, years 2015-2016)

Показатель	Group	
	I (control) (n = 25)	II (n = 25)
Body weight, kg:		
before launch	596.0±24.7	601.0±24.8
before calving	676.0±25.9	683.0±26.1
after calving		
in 5 days	575.0±22.6	581.0±23.9
in 1 month	558.1±22.8	569.4±23.1
in 2 months	546.4±22.5	561.1±22.6
in 3 months	545.1±21.9	563.0±23.5
in 4 months	548.5±22.5	574.2±23.4
Change of the body weigh after calving, kg:		
for 1 month	–16.9±1.5	–11.6±1.2
for 2 months	–11.7±1.3	–8.3±1.2
for 3 months	–1.3±0.7	+1.9±0.8
for 4 months	+3.4±0.8	+11.2±1.1

N o t e. See description of groups in *Techniques* section.

Our findings are in line with other data. Thus, the same milk yields were obtained in Red Pied Holstein cows upon use of dietary Carnipass™ (15 g · animal^{-1} · day^{-1}, CJSC Rus, Timashevsky District, Krasnodar Territory) [45, 46]. D.W. LaCount et al. [31] had established that upon addition of carnitine (nearly 6 g · animal^{-1} · day^{-1}) to the diet with 3 % raw fat, its blood concentration and content in liver tissues in cows had increased with improved lipid digestibility. V.N. Romanov et al. [47] report on positive physiological and productive effects of carnitine on calves (15 g · animal^{-1} · day^{-1}): improved metabolic processes in fore-stomach, increased digestibility of nutrients and retention of nitrogen in general contributed to more intensive growth in animals. Yu.P. Fomichev et al. [48] report that complex use of biologically active substances, the L-carnitine (Carnipass™), choline chloride and dehydroquercitine (Ecostimul-2), during interlactation period and at beginning of lactation not only enabled to prevent ketosis in highly productive cows, but also rendered positive effect on their milk yield and survivability. It is known that carnitine plays an important role in energy metabolism. Upon studying of the effect of additionally fed carnitine it was shown that it is accessible for ruminants even in non-protected form. Carnitine also ensures protection against toxic action of ammonium, which is observed at consumption of non-protein nitrogen or feed rich in soluble nitrogen [6].

Thus, use of protected carnitine in diets of highly productive cows during the most important periods of their physiological cycle, the interlactation phase and new-calving, promotes intensification of metabolism by improvement of microbial processes in fore-stomach and an increase in feed digestibility. Carnitine intensifies protective body functions, improves nitrogen metabolism, renders positive effect on protein, carbohydrate and fat, as well as mineral metabolism, and promotes growth of milk yield and reproductive values. The dosage of protected dietary L-carnitine of 45 mg of active substance per 1 kg of produced milk we deemed optimal for newly-calved cows.

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REPRODUCTIVE STATUS AND BLOOD BIOCHEMICAL PARAMETERS OF HOLSTEIN COWS WITH DIFFERENT MILK PRODUCTIVITIES IN CONNECTION WITH THE DYNAMICS OF LIPID METABOLISM DURING THE POSTPARTUM PERIOD

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Abstract

In the early period of lactation, the energy needs of high-yielding dairy cows sharply increase, which leads to a negative energy balance. For it compensation, the organism spends its internal resources, mainly lipid reserves. Normalization of cholesterol production at the beginning of the postpartum period is obviously associated with an increase in the animal reproductive ability. However, it remains unclear what role in the regulation of the reproductive function can play a change in the blood level of triglycerides. Here, we compared for the first time the duration of the calving to conception interval in Holstein cows with medium (MP) and high milk productivity (HP) depending on the increase or decrease in the blood concentration of triglycerides from the end of month 1 to the end of month 2 of lactation. Furthermore, we found a connection between the calving to conception interval, the serum content of triglycerides, and some other indexes of metabolism. Cows (*Bos taurus taurus*) of the Holstein breed of the 1st calving with milk yield of 6336±160 kg per 305-day lactation (n = 19; EKH Klyonovo-Chegodaevo, settlement Klyonovskoe, Moscow) and of the 2nd-3rd calving with milk yield of 10007±420 kg per 305-day lactation (n = 14; PZ Prinevskoe, Vsevolozhsk Region, Leningrad Province) were used in the experiments. In 3-4 and 7-8 weeks after calving, the blood samples were collected to assay the concentrations of triglycerides, total cholesterol, total protein and its fractions, urea, creatinine, glucose as well as the activity of aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2). All cows were divided into 2 groups. Group I consisted of animals in which the blood level of triglycerides decreased by the end of the 2nd month of lactation (MP: n = 10, HP: n = 8). Group II consisted of animals in which no such decrease was observed (MP: n = 9, HP: n = 6). Twelve months after calving, the average values for the calving to conception interval and milk yield per 100-day lactation were determined in all groups. In cows with MP between the 3rd-4th and 7th-8th weeks of lactation, the blood triglycerides concentration decreased 1.2 times ($p < 0.001$) in group I and increased 1.1 times ($p < 0.01$) in group II. The total blood cholesterol content grew significantly (1.2-1.3 times) in animals of both groups. Meanwhile, the calving to conception interval was 1.6 times shorter in group I than in group II (86±12 vs. 140±21 days, $p < 0.05$). In cows with HP, by the end of the 2nd month of lactation, the concentration of triglycerides decreased 1.6 times ($p < 0.01$) in group I and increased 2 times ($p < 0.001$) in group II. The content of total cholesterol rose 1.2-1.5 times in the blood of animals of both groups. The calving to conception interval in group I was 1.5 times shorter than in group II, but this reduction was not reliable. In addition, in these animals, the milk yield per 100-day lactation in group I was 534 kg higher than in group II ($p < 0.05$). Correlation analysis revealed a negative relationship between the duration of the calving to conception interval and the triglyceride content in the blood of cows with HP at the end of the 1st month of lactation (r = 0.56 with $p < 0.05$). At the end of the month 2, a positive relationship between these indicators in cows with MP and HP

was observed ($r = 0.60$ with $p < 0.01$ and $r = 0.56$ with $p < 0.05$, respectively). In animals with MP, the duration of the calving to conception interval was also associated with biochemical parameters, which correlated with serum triglyceride concentrations. Thus, in cows of the Holstein breed, the dynamics of lipid metabolism in the middle of the first trimester of lactation was characterized by an increase in the total cholesterol content, whereas the blood level of triglycerides varied in different individuals differently. The decrease in the concentration of triglycerides from the end of month 1 to the end of month 2 of lactation, obviously, causes improving the reproductive function and leads to a reduction in the duration of the calving to conception interval, regardless of the milk productivity of animals. Concurrently, in animals with the high milk productivity, such a decrease is more pronounced and may be related to an increase in the milk yield.

Keywords: Holstein breed, cows, triglycerides, metabolism, reproductive ability, milk productivity

Low reproductive capacity in milk-type cows is a serious problem for contemporary cattle breeding [1, 2]. Weakened fertility (subfertility) in animals is the outcome of various reproduction disorders, mainly, elongation of post-calving anestrus, ovarian dysfunction, reduction of oocyte and embryonic viability, as well as deterioration of reproductive health due to decrease of immunity in general [1, 3]. Such disorders are mainly typical for Holstein cows and result in longstanding inter-calving interval, which significantly exceeds 400 days [4, 5]. At that, term of economic use of Holstein cows comprises in average no more than three lactations due to high culling of animals in herd [6].

It is known that reproductive function in cows with high genetic potential of milk production heavily depends on the intensity and trend of metabolic processes controlled by metabolic hormones [1, 7, 8]. At early period of lactation, energy needs drastically increase in animals, which is accompanied by gradual body adaptation to new metabolic state. Lack of nutritive substances could not be promptly replenished by high consumption of feed, especially in terms of low appetite in such cows [9]. Negative energy balance is formed, for compensation of which the body uses its internal, mainly lipid, resources [10, 11]. As a result of mobilization of fat depots, free fatty acids grow in the blood and further acidification results in increased number of ketone bodies, first of all, β-hydroxibutyrate [12-14]. Concentration of free fatty acids and β-hydroxibutyrate in blood points at negative energy balance in high yielding cows in post-calving period [15]. Besides, increase in concentration of such metabolites may negatively influence reproductive function and deteriorate reproductive health of animals due to total reduction of immunity [7, 13, 14].

Lipids including triglycerides, cholesterol, and phospholipids, as well as their derivatives, provide energy and play significant role in functioning of endocrine system and several intracellular signal ways [10].

Blood lipid level of Holstein cows varies, which, probably, becomes a consequence of body adaptation to new metabolic state. Reduction of cholesterol concentration was found immediately before calving and its gradual growth by the end of the 1st to 2nd month of lactation [16, 17]. Besides, concentration of triglycerides in the blood during post-calving period was lower than during the interlactation period. At that, growth of cholesterol concentration in blood in milk-type cows in post-calving period is related to earlier restoration of the sexual cycle and further decrease of the interval from calving to conception [18, 19].

Previously we had shown that upon injection of bovine placenta extract to Black Pied cows before calving, concentration of cholesterol in their blood grows in the first 3 weeks after calving, lutein ovarian activity is intensified in 2 months after calving, and then decreased in the next service period [20]. It means that regulation of cholesterol production at beginning of post-calving period evidently relates to the improved reproductive capacity of animals. Meanwhile, it is still unclear what role in regulation of reproductive function in cows may be played by change of concentration of blood triglyceride, the other component of

lipid metabolism.

Present paper introduces comparison between duration of the service period in Holstein cows with average and high milk production values (accordingly, 6336±160 and 10007±420 kg for 305 days of lactation) depending on the nature of changes in concentration of triglycerides in blood serum from the end of 1st until termination of the 2nd month of lactation. It was shown that reduction and increase in triglyceride concentration are especially expressed in animals with high milk production values. At that, duration of service period is associated with changes in concentration of triglycerides, as well as with changes of other metabolic values correlating with such concentration.

Purpose of research paper was to study relations between the reproductive function and protein and carbohydrate metabolism with changes in lipid metabolism during the post-calving period in cows with different milk yield values.

Techniques. Research was conducted in the Experimental Farm Klyonovo-Chegodaevo (Klyonovskoe Settlement, Moscow) and Production Farm Prinevskoye (Vsevolozhsk District, Leningrad Region) in 2016-2017. Cows (*Bos taurus taurus*) of Black Pied Holstein breed of the 1st calving with average milk yield (6336±160 kg for 305 days of lactation, Experimental Farm Klyonovo-Chegodaevo) and 2nd-3rd calving with high milk yield (10007±420 kg for 305 days of lactation, Production Farm Prinevskoye) were used. Animals were managed in loose housing conditions. Their diet was in line with zootechnical standards. All tests were conducted according to principles enclosed in Helsinki Declaration (World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects, 1964-2013), and best laboratory practices (National Standard of the Russian Federation GOST R 53434-2009).

A total of 19 cows with mean milk productivity (MP) and 14 cows with high milk productivity (HP) with restored sexual cycle were selected for research that was confirmed by normal manifestation of estrus after day 45 of lactation. Blood was collected from tail vein of animals in 3-4 and 7-8 weeks after calving (April-July) with the use of vacuum system Apexlab (Hebei Xinle Sci&Tech Co., Ltd, China) and delivered to the laboratory within 1-2 hours. Serum obtained by 15 min centrifuging at 2500 g was frozen and stored at –20 °C. Concentration of triglycerides, total cholesterol, total protein and its fractions, urea, creatinine, glucose, as well as activity of aspartate aminotransferase (AST, EC 2.6.1.2) and alanine aminotransferase (ALT, EC 2.6.1.2) enzymes was determined in blood serum samples with a biochemical analyzers ChemWell (Awareness Technology, USA), ACCENT 200 (PZ CORMAY S.A., Poland) by using reagents of Analyticon Biotechnology AG (Germany) and PZ CORMAY S.A. (Poland).

In 12 months after calving, mean values for service period and milk yield for 100 days of lactation were identified based on analysis of zootechnical and pedigree records in all groups of cows.

Obtained data was processed by one-way ANOVA test or two-way repeated measures ANOVA test with software SigmaStat (Systat Software, Inc., USA). Mean (*M*) and standard error of mean (±SEM) values are provided in table below. Statistical significance was measured by Tukey's test. Correlation coefficients (*r*) were calculated by Pearson's method and their statistical significance was assessed by SigmaStat.

Results. According to the nature of change in concentration of triglycerides in blood between weeks 3-4 and 7-8 of lactation, all animals were divided into two groups. This lactation period was chosen due to the fact that luteal activity of cow ovarium was increased during the aforesaid time interval [20]. Group I included species, triglyceride concentration in blood of which had decreased by the end of the 2nd month of lactation ($n = 10$ and $n = 8$, the cows with

mean and high milk productivity, respectively). No such decrease was noted in animals of group II (n = 9 and n = 6). Blood triglyceride concentration decreased 1.2 times in cows with mean productivity between weeks 3-4 and 7-8 of lactation ($p < 0.001$) in group I and increased 1.1 times ($p < 0.01$) in group II (Table 1). Consequently, by the end of month 2 such value was higher in cows with positive triglyceride concentration changes ($p < 0.001$). By weeks 7-8 of lactation, significant (1.2-1.3-fold) increase in the total blood cholesterol and 1.8-2.1-fold decrease of De Ritis ratio (AST/ALT) were in both groups, which may evidence of shifting metabolism towards anabolism. Besides, an increase in albumins (1.1-fold, $p < 0.01$), urea (1.4-fold, $p<0.01$), and ALT activity (1.7-fold, $p < 0.01$) occurred in group I. These results denote improvement of protein-synthesizing liver function caused by more intensive urea cycle and glucose-alanine cycle in animals with negative changes in blood concentration of triglycerides.

1. Blood biochemical indicators at the end of the 1st and 2nd months of lactation in Holstein cows of mean productivity depending on blood triglyceride concentration (M±SEM, n = 19; Experimental Farm Klyonovo-Chegodaevo, settlement Klyonovskoe, Moscow, 2016-2017)

Indicators	Time after calving			
	group I (n = 10)		group II (n = 9)	
	3-4 weeks	7-8 weeks	3-4 weeks	7-8 weeks
Triglycerides, μmol/l	0.206±0.006	0.175±0.007**[a]	0.189±0.006	0.213±0.007*[b]
Total cholesterol, μmol/l	3.89±0.19	4.90±0.14**	4.34±0.20	5.17±0.21*
Total protein, g/l	86.8±1.7	87.8±2.1	88.3±2.9	91.6±2.5
Albumins, g/l	24.6±0.8[c]	27.2±0.6*	27.5±1.1[d]	28.5±0.8
Globulins, g/l	62.2±1.6	60.6±2.4	60.7±2.4	63.0±2.5
Urea, μmol/l	4.90±0.43	6.91±0.43*	5.24±0.39	6.00±0.55
Creatinine, μmol/l	82.0±5.5	79.1±3.8	79.7±7.6	75.7±4.2
Glucose, μmol/l	2.08±0.36	1.79±0.25	1.62±0.21	2.02±0.17
AST, IU/l	70.1±3.4	62.2±4.6	87.7±14.9	66.7±4.6
ALT, IU/l	10.0±1.1	17.2±1.6*	11.9±1.6	15.2±0.8
AST/ALT	7.99±1.03	3.87±0.50*	8.29±1.44	4.55±0.50*

N o t e. Mean productivity — 6336±160 kg for 305 days of lactation. Animals were divided into groups by changes in blood triglyceride concentration. AST — aspartate aminotransferase, ALT — alanine aminotransferase.
[a, b, c, d] Differences between the groups are statistically significant at $p < 0.001$ and $p < 0.05$, respectively.
*, ** Differences between time intervals for one group are statistically significant at $p < 0.01$ and $p < 0.001$, respectively.

2. Blood biochemical indicators at the end of the 1st and 2nd months of lactation in Holstein cows of high productivity depending on blood triglyceride concentration (M±SEM, n = 14; CJSC "Pedigree Plant "Prinyevskoe", Vsevolozhsk District, Leningrad Region, years 2016-2017)

Indicators	Time after calving			
	group I (n = 8)		group II (n = 6)	
	3-4 weeks	7-8 weeks	3-4 weeks	7-8 weeks
Triglycerides, μmol/l	0.158±0.015[a]	0.096±0.013**[c]	0.082±0.015[b]	0.162±0.014***[d]
Total cholesterol, μmol/l	3.51±0.21	5.21±0.22***	3.80±0.11	4.64±0.24*
Total protein, g/l	72.3±1.2	76.0±0.7*	74.1±3.2	72.5±2.7
Albumins, g/l	34.4±0.9	35.8±1.2	32.7±1.8	32.6±0.9
Globulins, g/l	37.9±1.4	40.2±1.4	41.4±2.4	39.9±2.5
Urea, μmol/l	3.56±0.24	5.95±0.45***	3.97±0.37	5.36±0.33*
Creatinine, μmol/l	101.0±6.8	83.2±1.6	93.0±5.4	91.6±6.5
Glucose, μmol/l	2.89±0.17	3.10±0.09	2.95±0.14	2.82±0.18
AST, IU/l	96.3±5.1	85.2±1.8	81.2±5.4	82.2±4.4
ALT, IU/l	16.8±2.6	26.5±2.3*	18.8±2.2	21.0±4.4
AST/ALT	6.98±2.21	3.48±0.47	4.95±1.15	6.73±3.12

N o t e. High milk yield — 10007±420 kg for 305 days of lactation. Animals were divided into groups by changes in blood triglyceride concentration. AST — aspartate aminotransferase, ALT — alanine aminotransferase.
[a, b; c, d] Differences between the groups are statistically significant at $p < 0.01$ and $p < 0.01$.
*, **, *** Differences between time intervals for one group are statistically significant at $p < 0.05$; $p < 0.01$ and $p < 0.001$, respectively.

Changes in blood triglyceride concentration in cows with high productivity were more pronounced (Tables 2). By the end of the 1st month of lactation,

triglyceride concentration was 1.9 times higher ($p < 0.01$) in group I than in group II. By the end of the 2nd month it decreased 1.6 times ($p < 0.01$) in group I and increased 2 times ($p < 0.001$) in group II, due to which the values in group II were higher than in group I ($p < 0.01$). Trend of changes in other metabolism indicators between the 3-4th and 7-8th weeks of lactation also had a number of specificities. Both animal groups were characterized by significant increase in total blood cholesterol (1.2-1.5-fold) and urea (1.4-1.7-fold). At the same time, double decrease of De Ritis ratio in group I was insignificant due to high variability of such indicator, whereas slight increase thereof was in group II. Accordingly, shift of metabolic processes towards anabolism had not yet occurred in most cows with high productivity by the end of the 2nd month of lactation. Increase of blood ALT activity (1,6-fold, $p < 0.05$), as well as total protein (1.1-fold, $p < 0,05$) found in group I was mainly due to globulins.

The time from calving to conception in cows with mean productivity to a greater extent depended on the nature of changes in blood triglyceride level (Table 3). Service period of such animals in group with negative changes in triglyceride concentration was 1.6 times shorter than in group with positive changes ($p < 0.05$). Service period in cows with high productivity in group I was also 1.5 times shorter than in group II, however such decrease was insignificant due to high variability of the indicators. Besides, milk yield in such animals for 100 days of lactation in group I was higher than in group II ($p < 0.05$), whereas milk yield in cows with mean productivity slightly increased in group I.

3. Reproduction indicators and milk yield in Holstein cows of different productivity depending on changes in blood triglyceride concentration from the end of the 1st until the end of the 2nd month of lactation (M±SEM, $n = 33$)

Indicators	Milk productivity			
	mean		high	
	group I ($n = 10$)	group II ($n = 9$)	group I ($n = 8$)	group II ($n = 6$)
Service period, days	86±12[a]	140±21[b]	136±23	199±32
Milk yield for 100 days of lactation, kg	2452±115	2303±167	4342±155[c]	3808±118[d]

Note. Mean and high milk productivity values are 10007±420 and 6336±160 kg for 305 days of lactation. Animals were divided into groups by changes in blood triglyceride concentration.
[a, b; c, d] Differences between the groups are statistically significant at $p < 0.05$ and $p < 0.05$, respectively.

4. Correlation coefficients (r) between service period, blood triglyceride concentration and biochemical idicators at the end of the 1st and 2nd month of lactation in Holstein cows of different milk productivity

Pair of compared indicators	Time after calving			
	animals with MP ($n = 19$)		animals with HP ($n = 14$)	
	3-4 weeks	7-8 weeks	3-4 weeks	7-8 weeks
Service period—triglyceride concentration	0,25	0,60**	–0,56*	0,54*
Service period—cholesterol concentration	0.44	0.60**	0.42	0.12
Service period—total protein	0.26	0.50*	–0.13	0.23
Service period—glucose level	0.22	0.57*	0.18	–0.18
Service period—AST activity	–0.13	–0.53*	0.42	0.11
Service period—AST/ALT	–0.52*	–0.37	0.19	0.28
Triglyceride concentration—cholesterol concentration	–0.08	0.47*	–0.50	–0.19
Triglyceride concentration—glucose level	0.39	0.53*	0.13	0.05
Triglyceride concentration—AST activity	–0.55*	–0.11	0.19	–0.19

Примечание. Mean (MP) and high (HP) milk productivity are 10007±420 and 6336±160 kg for 305 days of lactation. AST — aspartate aminotransferase, ALT — alanine aminotransferase.
*, ** Statistical significance of r values ($p < 0.05$ and $p < 0.01$, respectively).

Correlation analysis revealed negative relationship between the service period and blood triglycerides in cows with high productivity ($p < 0.05$) at the end of the 1st month of lactation, and positive relationship between such values in cows with mean ($p < 0.01$) and high milk productivity ($p < 0.05$) at the end of 2nd month (Table 4). In 2 months after calving, service period in animals with

high milk productivity was also positively related to concentration of cholesterol ($p < 0.01$), total protein ($p < 0.05$) and glucose ($p < 0.05$) and negatively related to AST activity ($p < 0.05$). Besides, at the end of the 1st month of lactation the calving to conception interval correlated with De Ritis ratio ($p < 0.05$). It should be noted that service period was associated with the biochemical indicators related with blood level of triglycerides (only in case of total protein correlation coefficient $r = 0.39$ is insignificant). At the same time, dependence between the service period and cholesterol concentration or protein carbohydrate metabolism, which, in its turn, did not correlate with triglyceride concentration, was not found in cows with high milk productivity. Such results denote that association of service period with these blood indicators may be secondary and determined by their relationship with triglyceride concentration arising under the effect of total central regulator(s), for instance, one or several metabolic hormones [7, 8].

After calving, blood triglyceride level in cows is determined by two main factors: intensive hepatic accumulation during negative energy balance [21] and increased need of udder in milk fat synthesis [22]. Triglycerides enter into the blood as part of lipoproteins of very low density, formation of which requires cholesterol [10]. In our research, total cholesterol concentration increased by the end of the 2nd month of lactation in all cows, whereas triglyceride concentration only in group II. Besides, such concentration to a greater extent depended on the cholesterol synthesis in animals with mean milk productivity, which is confirmed by correlation between both indicators of lipid metabolism ($p < 0.05$) by the end of the 2nd month of lactation (see Table 4). Decrease of triglyceride concentration was especially manifested in group I with high productivity, in which milk yield for the first 100 days of lactation was increasing more significantly than in cows with mean productivity. Evidently, reduction of blood triglycerides in such animals mainly depends on intensity of synthesis of milk fat involved triglycerides. In general, blood triglycerides were also lower in animals with high milk yields. Lack of correlation between the milk yield for 100 days of lactation and blood concentration of triglycerides may evidence on nonlinear relationship between these indicators.

High genetic potential of milk productivity based on lactation dominant is considered as a factor negatively influencing reproductive performance of cows [1, 3]. In addition, the results we report here show that among animals from a herd with the same milk production, some individuals may have a higher milk yield, which may lead to a decrease in the concentration of blood triglycerides, and this decrease may be associated with a decrease in service period of this animal.

Service period in cows to a greater extent depends on early embryonic mortality, which, in its turn, depends on quality of oocytes and embryos [23, 24]. Changes in blood concentration of different metabolites cause relevant changes in follicle liquid and reproductive tract [25]. It means that metabolic status of mother cows impacts the micro environment where oocytes and embryos are developed. It is of particular importance for oocytes since growth of surrounding follicles from primordial to pre-ovulation stage takes nearly 180 days [26].

It is shown that concentrations of triglycerides in the blood of cows and in the liquid of dominant follicles correlate [27]. Although triglycerides supply important energy source, their excessive accumulation by oocytes and embryos deteriorates function of mitochondria and increases the risk of oxidation stress [10]. Thence, lower triglyceride concentration by the end of the 2nd month of lactation in group I could be related with quality preservation of oocytes (and/or embryos), which could result in a decrease of embryonic mortality and shorter service period. Nevertheless, increased total cholesterol during this period of lactation does not associate with higher reproductive function.

Previously we have shown that service period in Black Pied cows is related to parameters of protein-carbohydrate metabolism at the end of days in milk [28]. Therefore, in this research we have determined these biochemical parameters and their association with service period and blood triglycerides in Holstein cows. It was found that concentration of triglycerides is not the only factor associated with duration of the service period. Firstly, increased blood ALT activity characteristic of cows from group I regardless of milk yield at the end of the 2nd month of lactation is similar to that found on days 70-90 after calving in Black Pied cows with shorter service period [28]. Secondly, duration of the service period in animals with mean productivity is associated with concentrations of total protein and glucose, AST activity and De Ritis ratio, while associations of these parameters with protein-carbohydrate metabolism depende on correlation of the later with triglyceride concentration.

In present research, the service period in animals with high milk productivity was much longer than in animals with mean productivity, regardless of lower concentration of triglycerides in their blood. This is in line with the concept on determining rope of cow's genotype in reproductive function [29]. Besides, additional reduction of triglycerides in animals with high productivity, evidently, positively influences their reproductive performance and results in service period shorter than typical for the genotype.

Thus, lipid metabolism in the middle of the 1st trimester of lactation in Holstein cows is characterized by rising of total blood cholesterol in all tested animals, whereas triglyceride concentration varies among individuals in different ways. Reduction in triglycerides concentration from the end of the 1st until the end of the 2nd month of lactation, evidently, contributes to improvement of the reproductive function and results in shorted service period regardless of animal milk productivity. Herewith, such reduction is more expressed in animals with high milk production and could be related with an increase in milk yields.

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POSTNATAL CHANGES IN MINK (*Mustela vision*) MINERAL METABOLISM ASSESSED BY MICRO- AND MACROELEMENTS IN BLOOD AND FUR

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Abstract

Mineral deficiency remains relevant in fur farming. That is why researchers are still developing methods to test whether mineral supply of animals is sufficient. Fur, unlike blood which composition strongly depends on many factors, is convenient biomaterial to control mineral levels in animal body. In this paper, we revealed a relationship between the blood and hair mineral composition and studied for the first time whether these parameters reliably reflect mineral welfare of the standard male minks fed with commonly used diets. Standard male minks of Saltykovskii breeding farm (Moscow Province) were grouped by age. Male minks, due to sexual dimorphism, are twice as large as females and all changes in their body manifest more quickly and reliably. Blood and hair were sampled from healthy standard male minks during postnatal ontogenesis, i.e. in 30-day-old animals and in 90-day-old animals, additionally, hair samples were taken from male minks aged 2 months, 7 months and 12 months. Contents of macro- and microelements were measured by atomic emission and mass spectrometry using an optical emission spectrometer Optima 2000^{TM} DV and ELAN 9000 ICP-MS mass spectrometer (Hitachi, AIC, Inc, Japan). The skin development during ontogenesis was controlled histologically. Hair condition was studied by electronic scanning microscopy (Hitachi-S-520, Hitachi, AIC, Inc, Japan). It was found that reference contents of macro- and microelements in blood and hairs of standard male minks are characteristic of each phase of postnatal ontogenesis. In blood, the concentrations (mmol/l) averaged 1.6-4.2 for calcium, (2.7-9.9)×10^{-5} for cobalt, (0.23-1.00)×10^{-3} for chromium, (0.53-2.10)×10^{-2} for copper, 5.9-11.3 for iron, (1.5-6.3)×10^{-3} for iodine, 29-44 for potassium, 0.64-1.23 for magnesium, (0.35-2.70)×10^{-2} for manganese, 82-103 for sodium, 4.8-21 for phosphorus, (1.8-6.3)×10–3 for selenium, and 0.24-0.89 for zinc. The blood levels of all these elements were maximal in one-month and three-month old minks and minimal at sexual maturity and body maturation of the animals. In hairs, the average contents (mmol/kg) were 4.5-8.5 for calcium, (0.92-2.70)×10^{-4} for cobalt, (2.3-7.9)×10^{-3} for chromium, (0.20-0.73)×10^{-1} for copper, (1.02-4.10)×10^{-1} for iron, (1.58-7.20)×10^{-3} for iodine, 0.44-1.70 for potassium, 0.70-1.92 for magnesium, (0.38-1.70)×10^{-2} for manganese, 9.1-39 for sodium, 0.97-2.40 for phosphorus, (2.0-4.6)×10^{-3} for selenium, and 0.28-0.46 for zinc. Accumulation of the most important elements in the mink hair was maximal during 30 days of life and minimal in 7-month old animals. Mineral compositions of blood and hair of standard male minks correlate and depend on the animals' age. The strong and moderate (positive and negative) correlations are found for 8 elements, Ca, Mg, Na, P, Co, Cr, Se and I. For all 13 elements studied, there are reliable correlations between their levels in animal hairs and in the diets. Apparently, the mineral composition of hairs can be used as a test of dietary balance of mineral elements for each age of the standard mink.

Keywords: *Mustela vision*, mink, postnatal ontogenesis, mineral metabolism, mineral composition of blood, mineral composition of hair

Mineral substances enter animal body mainly with feed and are involved in numerous metabolic processes at different levels [1-5]. We would consider only some of main mineral elements (sodium, phosphorus, calcium, magnesium, iodine, potassium, chrome, cuprum, iron, cobalt, zinc, selenium, manganese) re-

quired for formation and maintenance of the pelage and skin structure and function in fur animals, which is important both for deepening of the basic knowledge about their mineral metabolism, as well as for practical work.

Various pathological processes occur in animal if mineral substances in feed are misbalanced or if their metabolism is disturbed. We would analyze only those that are accompanied by changes in dermal and hair coat. Interesting, many adverse environmental factors may not render significant effect on vital processes since animal body has physiological and biological mechanisms ensuring homeostasis provided the presence of all necessary bioactive and mineral components [6-10]. Growth, development, pelt size and quality are directly dependent on strict adherence to the feeding and housing conditions [11, 12], as metabolic processes, homeostasis, and functions of antioxidant system are influenced by these conditions [13-16]. Essential mineral substances which ensure metabolic reactions and state of skin and hair coat corresponding to animal age and season are needed in specific quantities. Comparison of the concentrations of mineral elements in blood, organs and tissues, and in feed mixtures reflects animal body supply with minerals [6, 7, 15, 17]. Unfortunately, number of publications on the topic with regard to fur animals is quiet small, although the issue on the root causes of mineral deficiencies and reliable methods for identification thereof is most acute in the global fur farming practice [6, 11, 18-20]. It could be assumed that the content of mineral components in the blood provides a more informative description of the state of mineral metabolism in animals, but this indicator is rather labile, depends on many factors and changes even daily. Therefore, more convenient and reliable estimates of mineral status are required to learn more about the character and relationship of metabolic processes in different organs and tissues. Such control is also important to ensure welfare and productive performance of the animals [19, 21, 22].

Changes in the mineral content during growth of fur animal and their hair coat reflect longstanding changes in mineral metabolism in the body, and, thus, hair could be conveniently used as a biomaterial for analysis and also, its removal would not cause stress in animal [19-22].

In present paper we have analyzed age-related dynamics of the quantitative changes in 13 most important micro- and macroelements and have assessed to what extent mineral composition of animal blood and hair coat objectively reflects whether mineral nutrition is enough. Our findings show that these indicators in standard minks are significantly interrelated with eight elements (sodium, phosphorus, calcium, magnesium, iodine, chrome, selenium, and cobalt) out of 13 studied and depend on the age of animals, whereas mineral concentration in hair coat and diet correlates for all 13 elements. Accordingly, mineral content of hair for each pubertal group of minks could be assessed as indicator indirectly characterizing quantity of mineral substances in their feed.

Purpose of our research was to compare age-related dynamics of morphological and biochemical indicators reflecting the state of mineral metabolism, upon analysis of blood, hair coat and skin samples in physiologically healthy minks during different periods of postnatal ontogenesis.

Techniques. Experiments were carried out in 2006-2014 (pedigree farm Saltykovskii, Balashikha District, Moscow Province) on physiologically healthy standard male minks. Groups of 5 animals each were formed for each age based on pair-analogues principle. Experimental animals were kept in sheds and fed depending on the age, size, and season [15, 16]. Feeding was of meat-and-fish type as recommended by Afanas'ev All-Russia Research Institute of Fur Animal and Rabbit Breeding (Moscow Province); mineral composition of the diets was analyzed according to the recommendations [16]. Physiological state of animals

was assessed by commonly accepted clinical methods (by body weight, quality of fur coat and blood morphological and biochemical parameters, i.e. hemoglobin, total protein, proteins of blood plasma and their fractions) [23].

Whole blood and hair were analyzed at transitional phase (age of 30 days), at natural alimentation phase (age of 90 days), and in animals aged 7 and 12 months. Additionally, hair samples were taken from animals aged 60 days; skin samples were analyzed at the same age. Blood was collected on empty stomach from the end of tail or finger by single-use syringes, then placed in sterile vials and kept at 0-4 °C for 3-5 days the most or at –18 °C in single-use polypropylene vials with air-proof covers. After slaughter, a 2 cm^2 skin cutout was taken from scrapings of ramp part of pelt (hair was pulled out) for histological study; hair samples from 1-month-old mink were collected from the whole pelt. Hairs for mineral and histological analysis were carefully decontaminated by washing in soap solution, triple rinse in distilled water, and drying on filter paper. Afterwards, hairs were passed through ethylic alcohol in ascending concentrations (50, 75, and 96 %), defatted by acetone (Chemme, Russia), rinsed in distilled (deionized) water and dried at of 60 °C. Until analysis, hair samples were kept in paper bags.

Upon studying the mineral composition of blood, hair, and diets [16], mineralization and decomposition of biomaterial was done according to applied methodologies [6, 16]. During the experiments, only biomaterial of animals with normal fur formation was used. Blood, hair, and diet samples were analyzed for 13 elements (Fe, I, K, Ca, Co, Mg, Mn, Cu, Na, Se, P, Cr, and Zn) by inductively coupled plasma mass spectrometry (a quadrupole mass-spectrometer ELAN 9000 ICP-MS, PerkinElmer, Inc., USA) and by atomic emission spectrometry (an Optima 2000^{TM} DV, PerkinEImer, Inc., USA). Manipulations (insertion of samples, measurements, and statistical processing of outcomes) were fully automated by WinLab32 software (PerkinElmer, Inc, USA) in OS Windows 2000.

In histological control of hair coat, guiding hair cuticle (bed and grain) was examined (an electronic scanning microscope Hitachi-S-520, Hitachi, AIC, Inc., Japan). After defattening, hair samples dried on filter paper were glued on copper plate with polystyrene, samples were 10 min vacuumed (at 4 mPa), followed by gold sputtering by an Eiko lB-3 Ion Coater (Eiko Engineering Co., Ltd., Japan). Morphology of hair cuticles was studied by scanning microscope Hitachi-S-520 (Hitachi, AIC, Inc., Japan; ×15-3000 magnification, 4 μm resolution), the results were documented [24].

Once collected, skin samples were placed for 1 day in 10 % formalin, followed by dehydration and defatting by G.A. Merkulov [25]. Longitudinal and transversal sections were prepared using microtome with freezing chamber (TOC-1, Russia), staining with hematoxylin and eosin and celloidin embedding were according to common methods. Preparations were viewed under microscope (BIOLAM, LOMO, Russia) at 10×10 magnification without filter and documented with SONY camera (Japan; 20 s hold, 0.2 diaphragm).

Five repeated measurements of sample (5 analytic repeats) were done at analysis of each element. Tables 1 and 2 contain means (M) and standard error of means (±SEM) (defined subject to GOST 8.207). Correlations were analyzed by t-Student criterion. Correlations deemed to be weak at $r < 0.3$ and $r > –0.3$, medium at $0.3 < r < 0.69$ and $–0.69 < r <– 0.3$, and high at $r > 0.69$ and $r < –0.69$. Medium and high correlations were statistically significant at $p < 0.001$, $p < 0.01$, and $p < 0.05$.

Results. Only mink males were used in the experiment since due to sex dimorphism they are twice bigger than females, and all changes in their body are more apparent and could be detected earlier. Hair sampling at ages of 30 days, 60 days, 90 days, 7 and 12 months was due to the fact that these periods

correspond to main morphophysiological stages in formation of organs, tissues, body systems, and hair coat [26].

Age-related changes in the mineral content of the blood in standard mink males. Mineral substances in the blood are the body resources used for growth and life activities. Blood levels of the mineral substances optimal for metabolism is maintained due to water-salt metabolism regulation mechanisms based on response generated in nervous center of hypothalamus in response to signals from vessel and tissue receptors [3, 27]. Homeostasis of chemical elements is maintained due to mineral substance release from a labile bound form and deposition in relevant organs (bone tissue, liver, muscles, spleen, skin, subcutaneous fibre, etc.) or by regulation of absorption at digestion and by excretion.

Neurohumoral regulation mechanisms of water-salt metabolism are imperfect in immature mink body at early postnatal and transitional phases. At this age, animals rapidly accumulate and loose water with mineral substances [7, 28, 29]. According to our studies (Table 1), blood concentrations of Na (97±7 μmol/l) and K (34±2 μmol/l), involved in intracellular metabolism, regulation of osmotic pressure, and protein synthesis [3, 28, 30] were higher during more intensive growth of standard minks (transitional phase, 1 month age). Besides, concentration of the most important intracellular macroelement magnesium (1.23±0.07 μmol/l, see Table 1), a cofactor of oxidation phosphorilation enzyme, which is also involved in protein biosynthesis, carbohydrate and nucleic acid metabolism [3, 30], is maximum in the blood of 1 month aged minks (as compared to other ages). Evidently, this level of magnesium in 1-month old animals is required for intensive metabolism during intensive growth. Further formation and maturing of organs and tissues were accompanied by decrease in blood magnesium, whereas content of potassium and sodium was high (see Table 1) and slightly changed with age.

1. Concentration (μmol/l) **of macro- and microelements in blood of standard mink males (*Mustela vison*) of different age** (*M*±SEM, pedigree farm Saltykovskii, Balashikha District, Moscow Region)

Element	Age, months			
	1 (n = 25)	3 (n = 25)[a]	7 (n = 25)[b]	12 (n = 25)[c]
Ca	3,5±0,4	4.2±0.5$^{\#}$	1.6±0.2***,***	2.0±0.3$^{\#}$,***,**
Co	(9.9±0.5)×10^{-5}	(4.5±0.2)×10^{-5}***	(2.7±0.2)×10^{-5}***,***	(8.6±0.4)×10^{-5}***,***,$^{\#}$
Cr	(3.9±0.4)×10^{-4}	(10.0±1.0)×10^{-4}***	(2.3±0.2)×10^{-4}***,**	(3.7±0.3)×10^{-4}***,***,$^{\#}$
Cu	(12.3±0.7)×10^{-3}	(21.0±1.0)×10^{-3}***	(8.3±0.6)×10^{-3}*,$^{\#}$	(5.3±0.3)×10^{-3}***,***,***
Fe	7.9±0.5	11.3±0.7***	7.8±0.5***, $^{\#}$	5.9±0.4**,***,**
I	(3.9±0.4)×10^{-3}	(6.3±0.5)×10^{-3}**	(2.1±0.2)×10^{-3}***,***	(1.5±0.2)×10^{-3}*,***,***
K	34±2	36±2$^{\#}$	44±3*,*	29±2***,*,$^{\#}$
Mg	1.23±0.07	0.64±0.04***	0.67±0.04$^{\#}$,***	0.70±0.03$^{\#}$,$^{\#}$,***
Mn	(11.4±0.7)×10^{-3}	(9.3±0.6)×10^{-3}*	(3.5±0.2)×10^{-3}***,***	(27.0±2.0)×10^{-3}***,***,***
Na	91±7	82±7$^{\#}$	103±7*,$^{\#}$	94±7$^{\#}$,$^{\#}$,$^{\#}$
P	21±2	11±1***	18±2**,$^{\#}$	4.8±0.5***,***,***
Se	(4.8±0.5)×10^{-3}	(6.3±0.6)×10^{-3}*	(2.8±0.3)×10^{-3}***,**	(1.8±0.2)×10^{-3}*,***,***
Zn	0.24±0.02	0.89±0.06***	0.58±0.04***,***	0.44±0.03*,***,***

N o t e. [a] — difference between 3 months and 1 month; [b] — between 7 months and 1 months, 7 month and 3 month; [c] — between 12 months and 1 month, 12 months and 3 months, 12 months and 7 months.
*, **, *** Differences are statistically significant at $p < 0.05$; $p < 0.01$ and $p < 0.001$, respectively; $^{\#}$ — unreliable differences.

Body weight increases with linear growth of minks. In this, mineral substances serve as construction material [29, 31] which is required for intensive development of skeleton, muscles and teeth [20] terminated by transition to definitive diet [32]. Our research showed that blood concentration of Ca in minks aged 3 months, and concentration of P and Mg at age of 1 month were maximum. Higher Ca (3.5-4.2 μmol/l), P (11-21 μmol/l) and Mg (0.64-1.23 μmol/l) values at age of less than 3 months are, evidently, also explained by the fact that these elements as a part of muscle tissue are required for more intensive development of young animals. The fact that blood levels of Mg, Ca,

Na and K ensuring functioning of muscle and nervous tissues at transitional phase and by beginning of natural nutrition was high is possibly related to more intensive formation and development of these functional systems during early ontogenesis [33]. By 3 months blood concentration of zinc also increased (from 0.24 to 0.89 μmol/l) stimulating (along with macroelements) formation of bones [21, 34], and of copper (from 0.0123 to 0.021 μmol/l) which is required for osteogenesis and activity of osteoblasts. During the intensive growth, the need for such elements is very high [21, 29, 34].

Coordination of metabolic processes is due to participation of the central nervous system [27] via control of synthesis and penetration of hormones, containing macro- and microelements, into the blood. In our research, upon active growth all mineral elements (except for manganese) (see Table 1) increased in whole blood until 3 months of age as compared to other age groups. Our results are coherent with the fact that intensive protein metabolism (mostly its anabolic phase) is characteristic of this period, with positive balance of nitrogen due to Na- and K-containing enzymes.

Upon formation of organs, tissues, and functional systems, the intensive metabolism needs compounds containing cobalt, the essential element catalyzing transamination and synthesis of amino acids for structural, transport, receptor proteins, hormones, enzymes, etc., required for growing body of minks aged from 1 to 3 months [14, 18, 37]. Transaminases, the aspartate aminotransferase (AST) and alanine aminotransferase (ALT), are especially active in minks during growth and development period [36]. We believe that total protein in blood serum during intensified growth and development of minks increases sufficiently not only due to Co reaching $(0.045\text{-}0.099)\times10^{-3}$ μmol/l, but also due to Cu (0,0123-0,021 μmol/l), since hematopoietic processes and metabolism of many proteins require these elements [18, 37].

The need for metabolic energy changes during postnatal development. This is mainly influenced by animal age, metric parameters (body weight and size), seasonality and physiological state (sexual behavior, pregnancy, parturition, etc.). Earlier development of minks (from birth to 3 months) is accompanied by great energy consumption and, possibly, the need for Cu, Fe, Mn, and Zn, which are related to enzymes of oxidation metabolism [29]. Apparently, this explains significant blood levels of cooper, manganese, and iron, as components of enzymes performing tissue respiration, redox reactions, and also as factors of stimulation of hematopoiesis [29]. Our research showed that blood levels of zinc, chrome, iodine, and selenium required for 3-month aged typical minks significantly exceed the values in 1-month aged animals and is maximum compared to other age groups. We explain this by improvement of digestion, immune, endocrine, and central nervous systems. By the 3 month age, standard young minks reach the sizes of adult animal, which results in the need for iodine, the microelement in charge for main types of metabolism and homeostasis, as well as for selenium which, being part of selenium-proteins, increases immunoreactivity [26, 27, 29]. Enzymes including zinc and chrome are important for metabolism of fatty acids and fat utilization [22, 29]. It should be noted that the need for fat in early phases of minks' postnatal ontogenesis is especially high since fat serves the source of linolic acid required for structure formation and functioning of organs and tissues [22]. Also, Fe-, Cu- and Zn-binding oxidoreductases participate in carbohydrate and fat metabolism closely related to protein metabolism. The same elements play an important role in synthesis of glycoproteins and mucopolysaccharides required for formation of connective tissue. All these explain the highest blood concentration of Fe, Cu, and Zn in 3-month aged young minks.

Hormonal function of testicles is intensified at pubescence phase of males (7-8 months), which is accompanied by growth of blood concentration of sex hormones (progesterone, estradiol, and testosterone) [38]. Our research shows that blood level of manganese, iodine, and zinc in 7-month aged minks is low compared to 1 and 3-month aged minks. By this age, growth decelerates, metabolic pathways mainly become rather stable, all organs and tissues, as well as winter hair coat, have fully completed their formation. Apparently, the need for such elements decreases. At the same time, the need in iodine for function of thyroid hormones which ensure timely pubescence of young animals, in manganese as an activator of many enzymes on which function reproductive organs depend, and in zinc involved in production of male sex hormone testosterone and maintenance of reproductive function is associated with intensive establishment of reproductive system [16, 19, 38].

In minks, metabolism decreases during autumn and winter [16, 18] because of its seasonal nature in fur animals and dependence on duration of the light day [35].

At 7-12-month aged minks, intensity of digestion, metabolism and energy exchange, load on circulatory and respiratory systems and kidneys decreases due to termination of formation of organs and regulation mechanisms [18, 19]. However, blood concentration of copper, $(8.3\pm0.6)\times10^{-3}$ μmol/l, and zinc, 0.58±0.04 μmol/l, remains high to support functioning of skin and hair coat (formation of skin and hair pigments, synthesis of keratin and collagen) [16], while levels of other elements were significantly lowered or became minimal as in case of Ca, Co, Cr (see Table 1). Fur shedding occurs during the maturity phase (12-month age). Apparently, mink body does not any more need such amounts of minerals as during intensive growth, and concentration of all micro- and macroelements (except for Zn, Co, and Mg) becomes minimal or slightly differs from indicators during pubescence period (7-month age) (see Table 1).

Thus, reference blood concentrations of mineral elements are stable at each age of standard minks. Correlation analysis shows that mineral content of blood and diets used for each age of typical minks [16, 39] are interrelated. We have got the following correlation coefficients (r): K — 0.19^a, Na — 0.78^c, I — -0.48^b, Ca — 0.59^b, Mg — 0.83^c, Cu — -0.26^a, Zn — 0.65^b, Co — 0.21^a, Mn — -0.17^a, Fe — -0.62^b, Cr — -0.55^b, Se — 0.82^c, P — 0.56^b; at $r < 0.3$ and $r > -0.3$ the correlation is weak (a), at $0.3 < r < 0.69$ and $-0.69 < r < -0.3$ it is medium (b), and at $r > 0.69$ and $r < -0.69$ high (c); correlation coefficients marked by b and c letters are statistically significant ($p < 0.05$; $p < 0.01$ or $p < 0.001$). Although, medium and high correlations were generally detected for nine elements (P, Mg, Na, Ca, Fe, Cr, Zn, I, and Se), with close positive and negative correlations observed only for several elements (Mg, Na, and Se). Thus, we believe that blood mineral composition suits to assess whether the diet provides animal mineral needs.

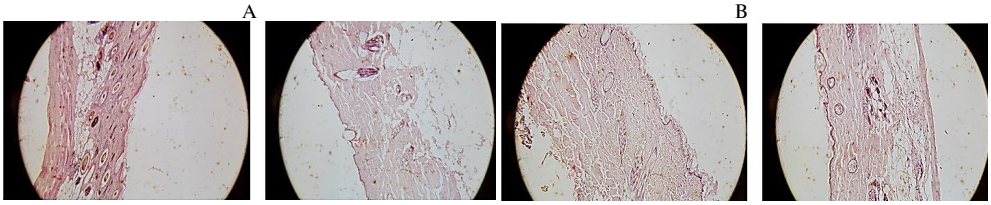


Fig. 1. Skin of a standard mink (*Mustela vison*) male: A — longitudinal sections, 1-month age (left) and 3-month age (right); B — transversal sections, age 7-month age (left) and 12-month age (right). H&E staining, ×100 magnification.

Age-related changes of mineral composition of hair coat

in standard minks. Morphostructure of skin and its derivatives shows (Fig. 1-3) that used feeding and housing provided skin and hair coat formation during postnatal ontogenesis without pathologies.

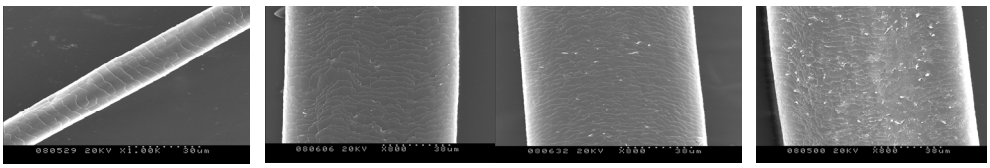
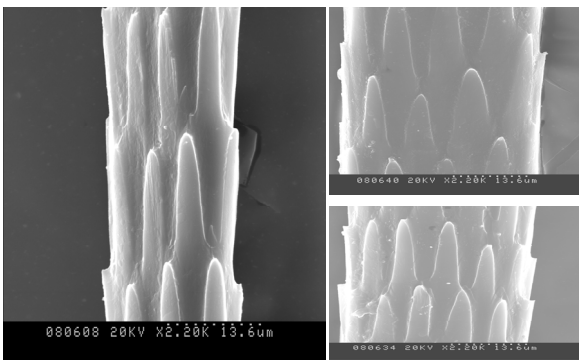


Fig. 2. Histological structure of grain in directional hair of standard mink (*Mustela vison*) males aged 1 month, 3 months, 7 months and 12 months (from the left to right). Scanning electron microscopy (Hitachi-S-520, Hitachi, AIC, Inc., Japan; ×800 magnification).



Fug. 3. Histological structure of bed of directional hair in standard mink (*Mustela vison*) males aged 3 months (left), **7 and 12 months** (right up and right down). Scanning electron microscopy (Hitachi-S-520, Hitachi, AIC, Inc., Japan; ×2200 magnification).

We had established mineral composition of hair coat for each age group of standard minks (Table 2).

2. Concentration (μmol/l) **of macro- and microelements in hair coat of standard mink (*Mustela vison*) males of different age** (*M*±SEM, pedigree farm Saltykovskii, Balashikha District, Moscow Region)

Element	Age, months				
	1 (n = 25)	2 (n = 25[a]	3 (n = 25)[b]	7 (n = 25)[c]	12 (n = 25)[d]
Ca	8.5±0.8	7.8±0.8[#]	7.0±0.5[#],[#]	7.0±1.0[#],[#],[#]	4.5±0.5[#],*,**,**
Co	(2.7±0.4)×10^{-4}	(2.4±0.2)×10^{-4}[#]	(1.01±0.12)×10^{-4}***,***	(1.19±0.12)×10^{-4}[#],*,**	(0.92±0.05)×10^{-4}*,***,***,[#]
Cr	(7.9±0.6)×10^{-3}	(5.0±0.4)×10^{-3}***	(2.3±0.2)×10^{-3}***,***	(3.8±0.4)×10^{-3}**,***,*	(2.5±0.2)×10^{-3}**,[#],***,***
Cu	0.073±0.05	0.042±0.03***	0.023±0.002***,***	0.031±0.004*,***,***	0.020±0.002**,[#],***,***
Fe	0.41±0.04	0.30±0.02***	0.102±0.008***,***	0.104±0.008[#],***,***	0.148±0.009**,***,***,***
I	(7.2±0.5)×10^{-3}	(2.7±0.2)×10^{-3}***	(1.6±0.1)×10^{-3}***,***	(25.2±0.2)×10^{-3}**,[#],***	(7.1±0.6)×10^{-3}***,***,***,[#]
K	1.7±0.3	1.03±0.18*	0.87±0.05[#],**	0.74±0.13[#],**,[#]	0.44±0.08*,***,**,***
Mg	1.92±0.17	1.46±0.08*	0.77±0.03***,***	0.70±0.03[#],***,***	0.88±0.08*,[#],***,***
Mn	(17.0±1.0)×10^{-3}	(10.4±0.7)×10^{-3}***	(6.9±0.5)×10^{-3}***,***	(3.8±0.4)×10^{-3}***,***,***	(5.3±0.5)×10^{-3}*,***,***,*
Na	39±3	32±2*	28±3[#],**	9.1±0.4***,***,***	12±2[#],*,***,***
P	2.4±0.4	2.4±0.4[#]	2.3±0.4[#],[#]	0.97±0.17**,**,**	1.1±0.2[#],*,*,*
Se	(22.2±1.3)×10^{-4}	(46.0±5.0)×10^{-4}***	(24.0±3.0)×10^{-4}***,[#]	(20.0±3.0)×10^{-4}[#],[#],***	(20.1±1.3)×10^{-4}[#],[#],***,[#]
Zn	0.369±0.015	0.35±0.03[#]	0.369±0.015[#],[#]	0.46±0.03*,*,*	0.277±0.015***,***,**,**

N o t e . [a] — differences between 2 months and 1 month; [b] — between 3 months and 2 months, 3 months and 1 month; [c] — between 7 months and 3 months, 7 months and 2 months, 7 months and 1 month; [d]— between 12 months and 7 months, 12 months and 3 months, 12 months and 2 months, 12 months and 1 month.

*, **, *** Differences are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; [#] — unreliable differences.

Thus, levels of most macro- and microelements in hair were maximum in minks aged 1, 2, and 3 months, when primary and secondary hair coat is formed (see Fig. 1). Our histological study shows highly developed fat tissue in derma (see Fig. 1, light field), which, due to morphological immaturity of skin, serves as depot of required elements, including minerals. Hair follicles (bulbar partы) are immersed in fat tissue. In 1 month old animals, histostructure of bed cuticle and grain of directional hair, which has yet been formed during embryonal ontogenesis, differed from other age groups (see Fig. 2, 3) with no defects of scales. Starting from month 3, cuticle structure was slightly changing with ageing, with no defects in scale layer too (see Fig. 2, 3). Morphological and histological studies showed great changes in skin and hair coat by 3 months (see Fig.

1). Because of intensive growth of the secondary hair coat, shedding of the primary coat and the beginning of winter hair coat formation, derma becomes thicken, depth of follicles of the secondary and winter hair coats changes (see Fig. 1). The main dermal part is reticulate structures where hair coat formation processes actively occur. Herewith, fat tissue decreases in the skin with observed dense network of blood vessels transporting nutrition substance, including minerals, to cells, cellular components of derma become numerous. These morpho-physiological changes are accompanied by a decrease in the number of all elements (except zinc) in hair coat (differences are statistically insignificant for sodium and phosphorus in 2- and 3-month aged animals).

In minks aged 7 months, hair coat was matured and skin was transferred to rest state. Depth of epidermis, derma, size of hair follicles and their depth during the period of termination of growth and maturation of winter hair coat decreased, whereas number of hairs per bundle increased reaching the maximum at complete maturity of winter hair coat. At that, depth of derma became minimal, it had well developed papillary layer (see Fig. 1).

The mineral state changed with morphophysiolgical formation of hair coat, i.e. chrome, cuprum, iodine, and zinc increased and manganese, sodium, and phosphorus decreased, with insignificant changes for other elements. Physiological maturation in minks terminates at age of 12 months, which coincides with spring shedding. At that, the number of hair follicle per bundle and width of the secondary and primary follicles increase, and derma becomes loose and thick (see Fig. 1). Change of dermal function during spring shedding was accompanied by changing mineral state of hair: Mg, I, Fe, and Mn concentrations grew, Zn, Ca, Cr, Co, K, and Cu significantly decreased, and other elements changed insignificantly (see Table 2). Correlation analysis revealed positive and negative high and medium correlations between the mineral composition of blood and hair coat (r_1) for eight elements (Na, P, Ca, Mg, I, Cr, Se, and Co). Mineral composition of the diets and hair in minks was interrelated whatever the age. At that, for 13 studied micro- and macroelements correlations (r_2) were close and medium (positive and negative):

	K	Na	I	Ca	Mg	Cu	Zn	Co	Mn	Fe	Cr	Se	P
r_1	0.19^a	-0.88^c	-0.55^b	0.67^b	0.93^c	0.21^a	0.20^a	0.92^c	-0.11^a	-0.17^a	-0.58^b	0.78^c	0.67^b
r_2	$0.83^в$	0.85^c	0.74^c	0.97^c	0.98^c	0.77^c	0.83^c	0.83^c	0.72^c	0.92^c	0.72^c	0.62^b	0.76^c

N o t e. Correlation coefficients (r) are provided for blood and hair coat concentrations (r_1) and for hair coat and diet concentrations (r_2). At $r < 0.3$ and $r > -0.3$ correlations are weak (a), at $0.3 < r < 0.69$ and $-0.69 < r < -0.3$ they are medium (b), and at $r > 0.69$ and $r < -0.69$ they are strong (c). Correlation coefficients marked by b and c letters are statistically significant ($p < 0.05$; $p < 0.01$ or $p < 0.001$).

It is known that distribution of mineral elements over organs and tissues of animals is strictly determined and depends on their age and type, and deviation of these indicators from the normal values evidences on disturbance of the mineral metabolism [7, 16, 39]. Usually, defects of hair coat (dull hair, changed pigmentation, hair breakage, loss, etc.), keratosis, dermatitis, wetting evidence on disturbances of mineral metabolism [6, 16-18]. Our research had shown the importance of control over the mineral balance of the mink's hair coat and diets.

Therefore, our findings provide certain levels of K, Ca, Mg, Na, P, Co, Cu, Cr, Fe, I, Mn, Se, and Z in hair coat and whole blood, which are characteristic of each age of typical minks. Content of all macro- and micro elements in whole blood is maximum in 1 and 3 month aged animals, while minimum in 7- and 12-month aged animals. One-month old animals show the highest levels of elements in hair coat, with minimum in 7-month aged animals. There are correlations between blood and hair mineral compositions for Na, I, Ca, Mg, Co, Cr, Se and P. In minks mineral compositions of hair and diets correlate for

all 13 studied micro- and macroelements. We believe that this could be an indicator to assess feed mineral balance to provide physiological needs of animals during different age-related periods.

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RESISTANCE OF BOVINE OOCYTES TO AGE-RELATED CHANGES AFTER EXPOSURE TO LUTEOTROPIC FACTORS DURING THE SECOND PHASE OF CULTURE TILL THE METAPHASE II STAGE

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Abstract

After the completion of the first division of meiosis in oocytes of various mammalian species, including cattle, the aging processes are acutely activated, which adversely affects the quality of mature ova and their competence to embryonic development after fertilization. Endogenous progesterone (P4) is known to play an important role in maintaining the viability of bovine oocytes, with its production by surrounding cumulus cells increasing significantly in the course of the final maturation period of female gametes from metaphase I to metaphase II (MII). However, effects of P4 and its two main stimulators, prolactin (PRL) and luteinizing hormone (LH), during maturation of oocytes on their resistance to age-related transformations are still poorly studied. We performed for the first time a comparative investigation of abnormal changes of MII chromosomes and apoptotic degeneration of oocytes, ripened by exposure to P4, PRL, and LH in the absence and in the presence of granulosa cells, during the subsequent prolonged culture of the ova. The aim of the present work was to study effects of luteotropic factors, P4, PRL and LH, during the second phase of in vitro maturation of bovine oocytes on the resistance of these latter to age-related changes. Oocytes surrounded by cumulus matured for the first 12 hours in the medium TCM 199 containing 10 % fetal bovine serum (FBS), 10 μg/ml follicle-stimulating hormone (FSH) and 10 μg/ml LH. Then the oocytes were transferred to a new medium, i.e. TCM 199 containing 10 % FBS (control) or the same medium supplemented with 50 ng/ml P4, 50 ng/ml PRL or 5 μg/ml LH, and cultured for the next 12 h in the presence and in the absence of granulosa cells. After 24 h of maturation in the two-phase system, the oocytes were transferred to an aging medium (TCM 199 containing 10 % FBS) and further cultured for 24 h. In media collected after oocyte aging, the P4 content was determined by enzyme immunoassay. The state of the nuclear material in oocytes was assessed using cytogenetic analysis, the presence of apoptosis in oocytes was determined by the TUNEL method. The rate of oocytes being at the MII stage of meiosis after 24 h of aging was similar in all groups and amounted 78.2-88.4 %. In the absence of granulosa cells, the effect of P4 on oocytes during the second phase of maturation led to a subsequent decrease in the frequency of destructive changes in MII chromosomes, from 67.1±2.0 (control) to 51.2±2.9 % ($p < 0.01$), whereas the introduction of these cells into the culture system eliminated the positive effect of the hormone ($p < 0.001$). On the contrary, a similar effect of PRL, reducing the rate of aging ova with the abnormal morphology of MII chromosomes, from 67.7±1.6 (control) to 46.5±5.0 % ($p < 0.001$), we detected only in the presence of granulosa cells. In addition, after ripening in the system not containing granulosa cells, the frequency of the apoptotic degeneration is lowest in the group of aging oocytes exposed to P4 (17.6 % vs. 23.5-25.2 %, $p < 0.05$). In the presence of granulosa cells, this anti-apoptotic effect of P4 is less pronounced. Meanwhile, there is no difference between the compared groups in the content of P4 in the aging medium. The results of the study suggest that effects of P4 and PRL on bovine oocytes during the second phase of maturation may increase their resistance to subsequent age-related changes associated with a decline in the quality. Thus, these hormones can be used to optimize the

maturation conditions of cattle oocytes in the two-phase system.

Keywords: cattle, oocyte, two-phase system of in vitro maturation, age-related changes of oocytes, progesterone, prolactin, luteinizing hormone, granulosa cells

Auxiliary reproductive technologies are one way to maintain stock reproduction in dairy farming. In vitro embryo transfer to recipient animals is widely used in animal breeding practice. Oocyte in vitro maturation used for production of native, cloned or genetically modified embryo is a technology underlying this method [1]. It is known that quality of matured oocytes determining their ability for further development is a key limiting factor at getting of embryo suitable for transfer [2]. Culture systems render critical effect on quality of oocytes, making them the subjects of specific treatments [3, 4].

Regardless of improvement of the methods of in vitro maturation of oocytes in cattle, quality of embryos got in vitro remains lower than those naturally developed, which results in higher frequency of their abnormalities and the offspring with low viability [5]. In standard practice, modernization of culture systems is mainly aimed at modeling conditions inside of ovarian follicles [6-8]. However, applied approaches usually solely account for changes occurring in functional state of oocytes during their maturity. That being said, ageing processes, which negatively influence the quality of matured oocytes and their competence to embryo development after conception, are sharply activated in vivo and in vitro after the first meiotic division in oocytes of different mammal species, including cows [9, 10]. Besides, somatic cumulus cells surrounding oocytes are subjected to apoptotic degeneration at termination of maturation of female gametes and may accelerate negative changes in the later due to ageing [10, 11].

Ageing of matured oocytes, which is called post-ovulatory, is initiated very fast at molecular level [9, 12]. This process, evidently, also occurs during conception, which should be accounted for at modernization of in vitro maturation (IVM) systems for oocytes. Any delay in conception (or artificial activation) may cause low viability of embryos, and, in case of offspring birth, may lead to weakening of its fertility and shorter life [13, 14]. Creation of a culture system promoting increased oocyte resistance to age-related changes may be a way to improve embryo quality in vitro.

With ageing, molecular changes of oocytes are accompanied by morpho-functional changes, which are observed significantly later and studied to a great extent [9, 14]. We have established that such changes in cattle involve destructive transformation of metaphase chromosomes, as well as apoptotic degeneration of oocytes [10, 15]. Pituitary prolactin hormone (PRL) fulfilling luteotropic function in mammal females [16], may slow down such age-related changes and maintain potential to development of cow oocytes matured in vitro during their further prolonged culture [15]. Identified IVM effect could be possibly related to progesterone (P4) production by cumulus cells surrounding ageing oocytes.

It is known that endogenous P4 plays an important role in acquisition of bovine oocytes of the ability to further develop, whereas its production of cells significantly grows during the final stage of maturation of female gametes from metaphase I to metaphase II (MII) [17, 18]. F. Nuttinck et al. [19] found 2-time growth of P4 secretion by cumulus cells associated with matured bovine oocytes 24 hours after their in vitro fertilization, which evidences on possible positive role of such ovarian steroid hormone in preservation of oocyte quality after maturing. The issue on influence of IVM and P4 during maturation of oocytes on their further resistance to ageing transformations remains open. Moreover, information on the role of luteinizing hormone (LH), one more luteotropic factor, in regulation of oocyte ageing is also absent.

The research of regulatory influence of luteotropic factor on anti-ageing resistance of oocytes during final maturation stage is of the greatest interest, since main molecular cytoplasmic transformations occur in this particular period, due to which oocytes acquire competence to further embryo development [2]. Previously, we had developed a two-phase system of in vitro maturation of cattle oocytes and had shown that it could be used as an alternative for the commonly accepted IVM protocol upon getting embryos at blastocyst stage [20].

In present paper we had for the first time conducted comparative research of abnormal changes in chromosomes at stage MII, as well as apoptotic degeneration of oocytes matured under P4, PRL and LH effect at absence and in the presence of granulosa cells during further prolonged culturing of oocytes.

Purpose of present paper is to study the influence of luteotropic factors (progesterone, prolactin, and luteinizing hormone) during the second phase of in vitro maturation on resistance of bovine oocytes to age-related changes.

Techniques. Oocyte-cumulus complexes (OCC) of cows and adult heifers were extracted from antral follicles of 2-8 mm in diameter. Ovaries without any pathology got after slaughter and delivered to the laboratory in 3-4 hours in physiological solution at 30-35 °C were used. Except for specially agreed cases, Sigma-Aldrich (USA) reagents were used.

Prior to extraction of oocytes, ovaries were repeatedly washed in sterile physiological solution with antibiotics (100 IU/ml penicillin and 50 μg/ml streptomycin) and placed in Petri dish with manipulation medium TC-199 containing 5 % fetal bovine serum (FBS), 10 μg/ml heparin, 0.2 mM sodium pyruvate and 50 μg/ml gentamicin. Follicle walls were cut by sterile blade; released oocyte-cumulus cells were collected from manipulation medium and washed 3 times by fresh manipulation medium. Oocytes were collected as described [20]. Oocytes were extracted and collected at 37 °C with the use of a stereomicroscope SMZ (Nikon Corporation, Japan) and heating table MATS-OZ (Tokai Hit, Japan).

OCC (30-35 per 500 μl medium) were cultured in 4-well plates at 38.5 °C, 90 % moisture and 5 % CO_2 in the air. Two-phase culture system was used for getting the matured oocytes. During the first 12 hours, oocytes matured in medium TC-199 with 10 % FBS, 1 mM sodium pyruvate, 50 μg/ml gentamicin, 10 μg/ml pig follicle stimulating hormone (FSH) and 10 μg/ml sheep LH. Afterwards, OCC were transferred to fresh medium and incubated during the next 12 hours in the presence or at absence of monolayer granulosa cell culture. At the second stage of two-phase culture, TC-199 medium containing 10 % FBS, 1 mM sodium pyruvate, 50 μg/ml gentamicin (control) was used, or the same medium added with P4 (50 μg/ml), bovine PRL (50 μg/ml) (Endocrinology Research Center RAS, Moscow) or sheep LH (5 μg/ml) was used (as per our preliminary research, such concentrations of hormones cause the least frequency of chromosome abnormalities in matured cow oocytes).

Extraction and preparation of granulosa cells was done subject to our previously described methodology [20]. Cells washed from blood and follicle liquids (1×10^6/ml) were pre-cultured in 4-well plates in 500 μl TC-199 medium with 10 % FBS, 1 mM sodium pyruvate and 50 μg/ml gentamicin. In 12 hours 250 μl of the medium was replaced by fresh medium with one of the studied hormones in the above concentrations. Afterwards, OCC were placed in wells for co-culture with granulosa cells during 12 hours.

In 24 hours of maturing in two-phase system, OCC were placed in ageing medium (TC-199 containing 10 % serum and 50 μg/ml gentamicin) and additionally cultured during 24 hours. At the end of culture, mediums were collected and kept at of –20 °C. Progesterone concentration in media conditioned

by OCC and granulosa cells was determined by immune-enzyme analysis (EIA) using microplate reader Uniplan (Pikon, Russia) and commercial reagent kits (Innovative Society Immunotech, Russia) at sensitivity 0.4 μmol/l. All analysis were conducted in two replicates, variation coefficient between measurements had not exceeded 13 %.

In 24 hours of prolonged culture, oocytes were released from cumulus cells by OCC incubation in 0.1 % hyaluronidase (in fresh ageing medium) during 1 min at 37 °C and further disaggregation by pipetting (130 μm micropipette hole in diameter). Isolated oocytes were used for cytogenetic analysis or identification of apoptosis by immune fluorescent method.

Oocyte nuclear material was prepared as described [10] and examined at magnification ×1000 (microscope Axio Imager.M2 (Carl Zeiss, Germany). Meiosis stages were determined by morphological criteria [21]. Destructive chromosome changes at MII was assessed as decondensation (loss of clear morphological contours, increase in volume of chromosomes, uneven morphological contours), partial agglomeration, and formation of dense lumpy structures [10].

For analysis of apoptosis, oocytes were fixed in 4 % paraformaldehyde solution in sodium-phosphate buffer (pH 7.2) for 1 hour at room temperature. After fixation, oocytes were incubated during 1 hour in 0.1 % sodium citrate containing 0.5 % Triton X-100. Sings of nuclear material apoptosis in oocytes was assessed by TUNEL method with In Situ Cell Death Detection Kit, fluorescein (Roche Diagnostics, Switzerland) subject to manufacturer's instructions. Afterwards, oocytes were stained for 20 min with DAPI solution (1 μg/ml) to localize chromosomes, transferred on slide SuperFrostPlus (Thermo Scientific, USA) and embedded into Vectashield medium (Vector Laboratories, United Kingdom). Microscope Axio Imager.M2 equipped with fluorescent attachment and ZEN 2 pro software (Carl Zeiss, Germany) were used for photo documentation and assessment of preparations (magnification ×400). Apoptosis rate was determined by a share of TUNEL-positive oocytes (green stain of MII chromosomes) of the total number of oocytes at stage MII.

Oocyte culture tests were conducted in 4-5 independent repeats in each group, there were at least 75 OCC. Data were processed by one-way ANOVA and two-way ANOVA with SigmaStat software (Systat Software, Inc., USA). Results are presented as means (*M*) and standard error of means (±SEM). Tukey's test was used to assess statistical significance between the compared mean values.

Results. Previously we had developed two-phase system for in vitro maturation of cattle oocytes allowing us to improve in vitro quality of late morulas/blastocysts [20]. Such improvement may be due to increase in resistance of matured oocytes to age-related changes, reducing their ability to embryonic development after fertilization [14]. Besides, maturation of oocytes in two-phase system resulted in optimization of estradiol-17β profile in culture medium, whereas P4 concentration by the end of the second phase of maturation remained insufficiently high [20]. At the same time, role of endogenous P4 as positive regulator of oocyte viability, evidently, grows at final stage of oocyte maturation [17, 18].

In this paper, we added luteotropic factors (P4 and two potential stimulators of its production by cumulus cells, PRL and LH) to the medium to normalize P4 level at the second stage of oocyte maturing. To identify possible ways of hormone influence on oocyte competence to embryo development under long culturing, we studied age-related changes in cow oocytes maturing in the two-phase system.

Cytogenetic analysis did not reveal the effect of luteotropic factors on termination of nuclear maturation of oocytes or on meiosis blockage during fur-

ther prolonged culture (Fig. 1, A). Portion of oocytes at stage MII of meiosis in 24 hours of ageing (Fig. 2) was similar in all groups and reached 78.2-88.4 % (see Fig. 1, A). Nevertheless, we revealed influence of P4 and PRL on further state of MII chromosomes during oocyte maturation (see Fig. 1, B).

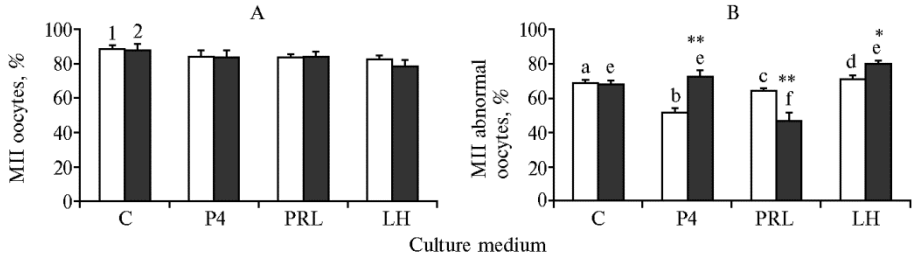

Fig. 1. Chromosomes of cow oocytes surrounded by cumulus cells after 12-hour maturing under the effect of luteotropic factors at absence (1) **and in presence** (2) **of granulosa cells and further 24-hour ageing:** A — nuclear maturation of oocytes, B — abnormalities in chromosomes at metaphase II. C — control, P4 — progesterone, PRL — prolactin, LH — luteinizing hormone. Vertical sections show standard error of means (±SEM, $n = 4$, independent tests).

a, b; b, c; b, d; e, f Differences between the compared groups are statistically significant at $p < 0.01$, $p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively.

*, ** Differences between system 1 and system 2 are statistically significant at $p < 0.05$ and $p < 0.001$, respectively.

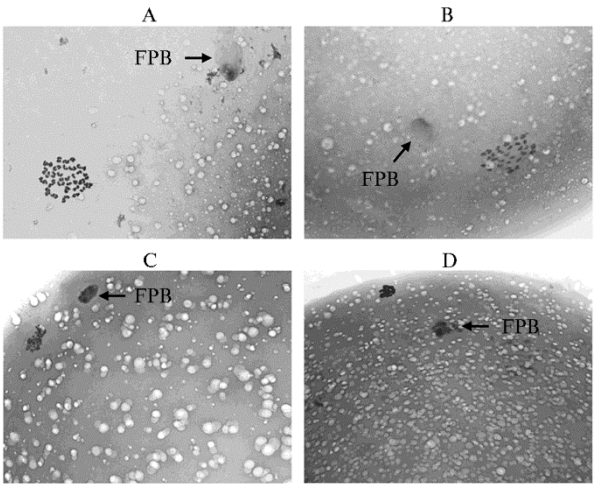

Fig. 2. Representative micro photos of chromosome morphology in cow oocytes at metaphase stage II (cytogenetic preparation): A — without signs of abnormality, B — decondensation, C — decondensation and partial agglomeration, D — dense lumpy structure. Arrows mark first polar bodies (FPB) (magnification ×1000, Axio Imager.M2, Carl Zeiss, Germany).

At absence of granulosa cells, effect of P4 on oocytes during the second phase of maturation resulted in further reduction of frequency of destructive changes of chromosomes during MII from 67.1±2.0 (control) to 51.2±2.9 % ($p < 0.01$), whereas placement of such cells in culture system eliminated positive effect of hormone ($p < 0.001$). Conversely, similar effect of PRL reducing the share of ageing oocytes with abnormal chromosome morphology at stage MII from 67.7±1.6 (control) to 46.5±5.0 % ($p < 0.001$) was found only in presence of granulosa cells. LH did not influence destructive chromosome changes in both systems.

Longstanding effect of P4 on apoptosis in ageing oocytes (Fig. 3, 4) was similar to that on abnormal chromosome transformations at MII. After maturing in the system free from granulosa cells and 24-hour prolonged culture, frequency of apoptotic degeneration was the least in oocytes subjected to P4 (17.6 against 23.5-25.2 % in other groups, $p < 0.05$). Granulosa cells did not suppress anti-apoptotic effect of P4; however in the presence of such cells only oocytes maturing in culture medium with P4 and LH differed significantly (18.3 % against 26.6 %, $p < 0.05$). Neither PRL, nor LH renders longstanding effect on apoptotic degeneration of ageing oocytes.

We did not find any differences in ageing medium concentration of P4 between all compared groups (Fig. 5). Thence, luteotropic factors affecting OCC during the second phase of in vitro maturation did not cause intensification of P4 secretion by cumulus cells surrounding ageing oocytes. Effect of P4 and PRL during maturation of oocytes on their resistance to age-related changes was not related to stimulation of endogenous P4 production in OCC during ageing.

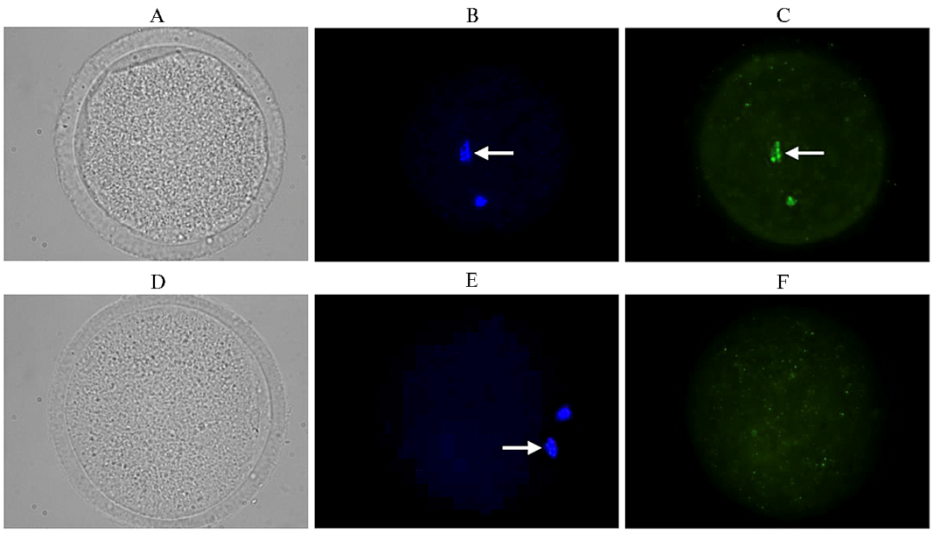

Fig. 3. Representative micro photos of cow oocytes with apoptosis (upper raw) **and without apoptosis** (lower raw)**:** A, D — morphology of ageing oocytes; B, E — DAPI staining of oocyte nuclear material (blue color), arrows mark metaphase II chromosomes; C, F — TUNEL staining of oocyte nuclear material (green color), arrows mark TUNEL-positive MII chromosomes (TUNEL method, magnification ×400, microscope Axio Imager.M2, Carl Zeiss, Germany).

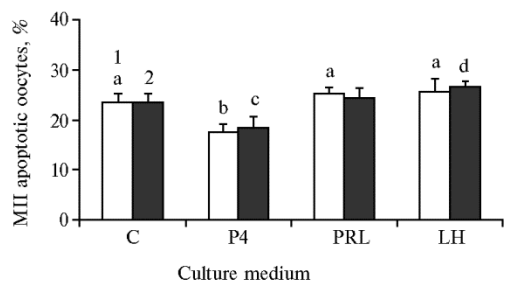

Fig. 4. Effect of luteotropic factors on apoptotic degeneration of cow oocytes surrounded by cumulus cells after 12-hour maturation at absence (1) **and in presence** (2) **of granulosa cells and further 24-hour ageing.** C — control, P4 — progesterone, PRL — prolactin, LH —luteinizing hormone. Vertical sections show standard error of means (±SEM, $n = 5$, independent tests).
[a, b]; [c, d] Differences between the compared groups are statistically significant at $p < 0.05$ and $p < 0.05$, respectively.

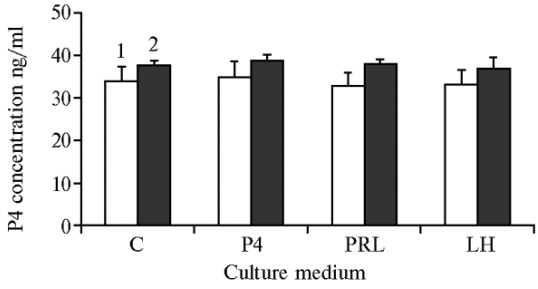

Fig. 5. Effect of luteotropic factors on progesterone (P4) **concentration in ageing medium during maturation of cow oocyte surrounded by cumulus cells at absence** (1) **and in the presence** (2) **of granulosa cells.** C — control, P4 — progesterone, PRL — prolactin, LH — luteinizing hormone. Vertical sections show standard error of means (±SEM, $n = 6$, independent tests).

In present paper we for the first time had studied longstanding effect of P4 and PRL on cow oocytes during the second phase of in vitro maturation. Previously, we had shown that adding of PRL into the commonly accepted one-phase IVM system (i.e. immediately prior to beginning of maturation) does not result in slowing down of destructive chromosome changes in oocytes

at MII stage during their further prolonged culture [22]. Besides, exogenous P4 in one-phase maturation system did not intensify competence of cattle oocytes to embryogenesis after in vitro fertilization [23]. Although LH is commonly considered as a stimulator of oocyte competence to further development and is widely used in routine IVM practice in cattle [1, 24, 25], in our two-phase system LH did not positively influenced the blastocyst yield [20]. In present paper, LH did not also increase the resistance of cow oocytes to age-related changes. I.e. the effects of P4, PRL, and LH on oocytes during the second phase of maturation (i.e. transfer from metaphase I to metaphase II) radically differ from those upon the influence on oocytes blocked at diplotene stage. Our findings agree with data on specific regulation of mice and pig oocyte maturation during MI to MII transformation of chromosomes [26, 27].

Therefore, effect of progesterone and prolactin on cow oocytes at the second phase of maturation in the two-phase in vitro system we proposed results in increase of their resistance to further age-related changes which reduce oocyte quality and, consequently, the yield of full-grade embryos after fertilization. Accordingly, these hormones could be used to optimize cattle oocyte maturation in two-phase in vitro system.

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REPRODUCTIVE QUALITY OF ROOSTERS WITH NORMAL AND MODIFIED GENOME

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Abstract

To date, progress has been made in the production of transgenic poultry: effective gene constructs have been obtained, and efficient systems for recombinant DNA delivery into target cells have been created. However, when breeding a genetically modified poultry, problems may arise with transgenic offspring, in particular its low viability or limited number. This paper is the first to report on histological structure of the testes and the composition of spermatogenic cells in the seminiferous tubules of transgenic roostersand on the quality and fertilizing ability of their semen. The study was carried out on transgenic roosters which were obtained by us in different ways based on chicken (*Gallus gallus* L.) Pervomaiskaya breed and their non-transgenic analogues (vivarium of Ernst Federal Science Center for Animal Husbandry, 2017-2018). group I was transgenic roosters after introduction of the lentiviral vector pWRSV into chicken embryos in vivo (n = 4); group II was transgenic roosters obtained by transplantation of transformed in vitro donor spermatogonia into the testes of sterile recipient roosters (n = 5). The control group included non-transgenic roosters selected as analogues (breed, age). Sperm was collected once every 2-3 days in penicillin vials heated to 30 °C by abdominal massage carried by the same technician. The following semen indicators were investigated: ejaculate volume, sperm motility and concentration in the ejaculate, the head and acrosome area, total spermatozoa length, flagella length. The criteria for evaluation of the fertilizing capacity of semen were the egg fertilization and the hatching of the younger generation. The histological structure of the seminiferous tubules and the composition of spermatogenic cells were also studied. Our experiments show that the quantitative and qualitative indicators of sperm in transgenic roosters decrease compared to the control. The volume of ejaculate, the concentration and motility of spermatozoa were 19, 15 and 1 % lower in group I and 38, 29 and 2 % lower in group II. However, there are no deviations in the safety of the acrosome in transgenic roosters when compared to the control individuals. Histological analysis of testes of the transgenic and non-transgenic roosters also reveals no significant pathological disturbances in the seminiferous tubules. Nevertheless, an insignificant decrease in the number of spermatogenic cells in transgenic individuals occurs when compared to the control ones (up to 19 %). The fertilizing capacity of the transgenic roosters' semen is also lower than that of the control roosters. In group I the differences with the control group for the percentage of chick hatching were 15 %, in the group 2 — 10 % ($p < 0.05$), which may indicate some negative effect of the integration of the transgene on the functional state of the germ cells in the studied genetically modified individuals.

Keywords: roosters, *Gallus gallus* L., transgenesis, gene constructs, transplantation, gene-transformed spermatogonia, acrosome, spermatozoon, semen quality, fertilizing ability, hatching

Purposeful modification of the poultry genome is a promising modern biotechnology, which is considered as an alternative to conventional breeding

[1]. This approach significantly accelerates the production of populations of individuals with the desired and fundamentally new properties, which is impossible by standard breeding methods. However, the techniques used to change the genome of transgenic farm animals are ineffective in creating genetically modified poultry because of the peculiarities of bird's reproduction and development [2]. At the same time, the biology of this phylum gives an opportunity to significantly expand a set of methods for the effective introduction of recombinant DNA into the target cells. Embryos of birds develop ex vivo, which facilitates access to them when performing genetic engineering manipulations. Blastoderm cells [3, 4], primordial germ cells [5, 6], and spermatogonia [7] are considered as promising targets for the introduction of recombinant DNA.

Currently, the methods have been developed for effective directed transfer of recombinant genes into embryonic and somatic chicken cells, i.e. the introduction of recombinant DNA into embryonic cells with the help of lentiviral [8] and retroviral [9] vectors, transplantation of transformed donor primordial germ cells [10, 11] and spermatogonia [12, 13], transformation of primordial germ cells [14] and spermatogonia [15] in vivo. With the use of these approaches, transgenic chickens which produce with egg protein marker proteins [16, 17] and recombinant human proteins [18, 19] were hatched. However, in further breeding of such individuals, the problems with transgenic offspring are possible (reproductive disorders, limited number, and low viability) due to the low fertility of the original parental forms. A number of studies have shown a decrease in the fertilizing ability of the sperm and libido among males of transgenic animals as compared to non-transgenic individuals [20, 21]. Such surveys were virtually not carried out on transgenic poultry. In this regard, it is interesting to study the effect of transgenesis on the functional state of germ cells of genetically modified birds, in particular, roosters.

This paper is the first to report on the histological structure of the testes and the composition of spermatogenic cells in the seminiferous tubules of transgenic roosters obtained using different methodologies. The decrease in the fertilizing ability of the sperm among transgenic roosters as compared to the control is found. For the first time, a decrease in the number of spermatogenic cells in the seminiferous tubules of roosters after transplantation of donor spermatogonia is revealed. At the same time, no significant pathological disorders in the histological structure of the testes were found.

The work objective was to evaluate the reproductive performance of transgenic roosters in comparison with their non-transgenic analogs, namely in the study of qualitative and quantitative indicators of semen, its fertilizing ability, the histostructure of the seminiferous tubules of the testes, and the quantitative composition of spermatogenic cells in them.

Techniques. Transgenic roosters (*Gallus gallus* L.) were derived from Pervomaiskaya breed. Group I was transgenic roosters after the introduction of the lentiviral vector pWRSV into chicken embryos in vivo ($n = 4$); group II was transgenic roosters obtained by transplantation of transformed in vitro donor spermatogonia into the testes of sterile recipients ($n = 5$). The control group ($n = 5$) included non-transgenic roosters aged 8-9 months which were selected as analogs. Transgenic and non-transgenic poultry was kept in single cages (the physiological yard, Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018) and fed with complete feed.

Sperm was collected from roosters once every 2-3 days by abdominal massage. For conditioning sperm collecting, the roosters were prepared within 1-2 weeks. The ejaculate volume was measured with a graduated pipette (up to 1.0 ml). Sperm concentration was calculated in a Goryaev's chamber (Nikon Ni-U

microscope, Nikon Corporation, Japan; magnification ×400). Cell motility was assessed on the warm table at 38-40 °C; the ejaculate was previously diluted 5-fold. The fresh sperm preparations were fixed in methanol for 10 min, and the morphometric analysis (at magnification ×400) was carried out with (Nikon DS-Qi2 digital camera, Nikon Corporation, Japan; 4908×3264 px resolution). The morphometric parameters (area of the head and acrosome, the total length of the sperm, flagella length) were calculated with built-in software NIS-Elements BR 4.30 (Nikon Corporation, Japan). At least 100 sperm cells from each rooster were examined. The state of acrosomes was studied in fixed sperm preparations with the kit for differential staining Diakhim-Diff-Kvik (NPF Abris+, Russia).

The criteria for evaluation of the fertilizing capacity of semen were the egg fertilization and the hatching of the younger generation.

Histological preparations of testes were prepared according to the generally accepted method [22]. Tissue samples were fixed in Bouin's solution (picric acid:acetic acid:formalin in a ratio of 15:1:5) for 48 hours. The preparations were stained with hematoxylin and eosin. For the analysis, only seminiferous tubules with a lumen and a rounded shape (in cross-section) were selected. Spermatogenic cells were identified by morphology [23]. At least 30 seminiferous tubules from each rooster were examined. Images were processed and analyzed with NIS-Elements software (Nikon Corporation, Japan). The diameter of the seminiferous tubules, the number of spermatogenic cells in them, and the cellular composition of the population were determined.

The obtained data were processed statistically with Microsoft Excel program. The arithmetic mean values (*M*) and standard errors of means (±SEM) are presented in the tables. The significance of differences was assessed according to Student's *t*-criterion. Differences were considered statistically significant at $p < 0.05$.

Results. Visual evaluation of semen samples from transgenic roosters and males of the control group did not reveal any significant deviations: the color and smell of the ejaculates met the requirements. However, the differences between the experimental groups in terms of semen quality occur (Table 1).

1. Semen quality of transgenic and non-transgenic Pervomaiskaya roosters (*Gallus gallus* L.) (*M*±SEM, the physiological yard of Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018)

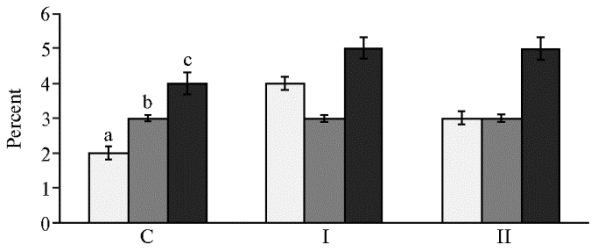
Indicator	Group		
	control	I	II
Ejaculate volume, ml	0.31±0.11	0.25±0.09	0.19±0.08
Sperm concentration, bln/ml	2.98±0.76	2.51±0.65	2.11±0.74
Sperm motility, %	86±6	85±7	84±8
The proportion of sperm with abnormal morphology, %	9±3	12±4	11±3

N o t e. See the description of groups in the section Techniques.

Transgenic roosters have a tendency to decrease the volume of ejaculate and sperm concentration compared to the control, i.e. by 19 and 15 %, respectively, in group I, and by 38 and 29 % in group II. In addition, the sperm of transgenic roosters was less motile, though these differences were less significant and did not exceed 2 %.

Along with the decrease in the ejaculate volume and sperm concentration in the experimental groups, the proportion of sperm with abnormal morphology increased. Differences with the control on this indicator were 33 % in group I and 22 % in group II. The percentage of sperm with abnormal morphology of head, middle part, and flagellum of spermatozoa showes that the most frequent were violations in the flagellum area (Fig.). This indicator was 4 % for the control group and 5 % for the experimental group. In comparison

with this, the share of sperm with abnormal morphology of the head and the middle part in all experimental groups was 1-2 % lower.



The share of sperm with abnormal morphology of the head (a)**, middle part** (b)**, and flagellum** (c) **in the ejaculates of transgenic and non-transgenic Pervomaiskaya roosters (*Gallus gallus* L.):** C — control, I and II — experimental groups (the physiological yard of Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018). See the description of groups in the section Techniques.

Sperm morphometric differences between the experimental groups are also found (Table 2). Among transgenic roosters of group I, the head area increased by 27 % compared to control ($p < 0.01$), of group II by 6 %. In addition, the length of the flagellum and, as a consequence, the total length of a sperm cell decreased slightly (up to 2 %). The acrosomes of transgenic individuals of group I decreased in size by 33 % compared to control, in group II by 25 % ($p < 0.05$). It should be noted, however, that these morphometric changes did not have a significant impact on the integrity of acrosomes: among all roosters, it was almost the same, i.e. 98.9%: for transgenic and 99.0 % for non-transgenic.

2. Semen morphometry of the ejaculates of transgenic and non-transgenic Pervomaiskaya roosters (*Gallus gallus* L.) (*M*±SEM, the physiological yard of Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018)

Group	Area, μm²		Length, μm	
	head	acrosome	total	flagellum
Control	11.9±0.6	1.2±0.1	81±3	68±3
I	15.2±0.7**	0.8±0.1*	79±1	67±2
II	12.7±0.5	0.9±0.1*	80±1	66±3

N o t e. See the description of groups in the section Techniques.

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

The fertilizing ability of the sperm of transgenic roosters was lower compared to the control roosters (Table 3). In the experimental group I, the percentage of fertilized eggs and hatched chickens were respectively 4 and 15 % lower than in the control poultry. In the II experimental group, the differences were 6 and 10 % ($p < 0.05$).

3. Embryo development and chicken hatching efficiency of transgenic and non-transgenic Pervomaiskaya roosters (*Gallus gallus* L.) (*M*±SEM, the physiological yard of Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018)

Group	Eggs put for incubation, pcs.	Among them, pcs. (%)		Eembryos, %	Hatched chickens, %
		not fertilized	fertilized		
Control	50	3 (6)	47 (94)	90	85
I	50	5 (10)	45 (90)	84	70
II	55	7 (12)	48 (88)*	85	75

N o t e. See the description of groups in the section Techniques.

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

Similar data were obtained for transgenic farm animals, in particular, goats and rabbits. Among goats with the human lactoferrin gene *hLF*, there is a decrease in the quality of the semen and its fertilizing capacity (differences up to 13 % compared to the control group) [20, 24]. Among the male rabbits with the gene of the growth hormone of the bull *bGH*, the decrease in libido was observed [21]. At the same time, human lactoferrin transgenic mice had normal reproductive parameters [25]. Among sheep carrying the recombinant gene of

cattle chymosin in the genome the percentage of lambed ewes also corresponded to the regulatory indicators [26].

The decrease in the reproductive qualities of transgenic roosters compared to control was due to the multiple deterioration of semen quality, in particular, the decrease in the ejaculate volume, motility, and sperm concentration (see Table 1). Histological studies have confirmed the decrease in the concentration of sperm in the ejaculates obtained from transgenic roosters. Analysis of the histological structure of the seminiferous tubules showed a reduction in the number of spermatogenic cells in the seminiferous tubules of transgenic roosters compared to the control group (Table 4). These changes were most significant (by 19 %) among transgenic roosters after transplantation of donor spermatogonia (group II). Among transgenic roosters produced by lentiviral vector transduction (group I), the difference with the control group in the number of spermatogenic cells did not exceed 2.5 %.

4. Different types of spermatogenic cells in the seminiferous tubule (pcs.) **of transgenic and non-transgenic Pervomaiskaya roosters (*Gallus gallus* L.)** (*M*±SEM, the physiological yard of Vivarium of Ernst Federal Science Center for Animal Husbandry, 2018)

Group	Total	Sertoli cells	Spermatogonia	Primary and secondary spermatocytes	Spermatids	Spermia
Control	1198±115	18±3	69±5	613±20	218±14	280±17
I	1167±213	15±2	65±8	625±35	205±18	257±25
II	960±88	17±3	58±11	526±31	158±25	201±14*

N o t e. See the description of groups in the section Techniques.
* Differences with control are statistically significant at $p \leq 0.05$.

Comparison of semen quality in roosters upon application of different transgenic methodologies showed that group I was superior to group II (in particular, by 24 and 16 % for the ejaculate volume and sperm concentration, respectively). The semen fertilizing ability was also higher in using the lentiviral vector than after transplantation of transformed donor spermatogonia.

Thus, our investigations have shown that the integration of a transgene affects the functional state of germ cells of genetically modified roosters, with a decrease in the ejaculate volume, sperm concentration and motility, in the number of spermatogenic cells in the seminiferous tubules, and also in fertilizing ability of the semen. Nevertheless, any abnormalities in the integrity of acrosomes of transgenic poultry are not found. The analysis of testes of transgenic roosters and their non-transgenic analogs also did not reveal significant pathological disturbances in the histostructure.

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Dedicated to the blessed memory of Gennady V. Eskin

OPTIMAL MODE TO THAW CRYOPRESERVED SPERM OF HOLSTEIN BULL SIRES

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Abstract

As milk yielding increases, the number of animals with defective sexual cycles and quiet manifestations of hunting grows, and therefore there is a need for semen with high quality. Also, at artificial insemination, after dilution of the native semen, packaging, cryopreservation and thawing, the number of spermatozoa per sperm dose decreases. This also necessitates semen with high quality parameters. One of the key stages of the standard processing of cryopreserved bovine semen prior to artificial insemination is the thawing procedure. Therefore, cryopreservation and thawing procedures should be optimized with regard to the proposed modifications of these methods and the changing reproductive abilities of animals. In our study, we compared the effects of previously recommended thawing protocols and those proposed by us on the safety of spermatozoa of Russian Holstein sires and identified the modes that reliably provide a prolonged positive effect on the quality characteristics of spermatozoa and better preservation of their viability after thawing. The semen of 6-7 year-old Holstein bulls was cryopreserved in polypropylene straws (72 doses) with the use of the IMV Technology equipment (France) and in uncoated pellets (90 doses) on the dry ice plates according to GOST State Standard 26030-2015 and the National Technology for Freezing and Handling Semen of Pedigree Bull Sires. Thus, 162 semen doses 0.25 ml each were analyzed. The sperm motility and movement velocity parameters were assessed immediately and after in vitro post-thawed incubation for 5 to 24 hours at 38 °C. The percentage of motile semen was visually assessed with the Olympus microscope CX41 (Japan) at ×150 magnification. Thereafter, in the same samples the motility (%), the number of the highly motile sperm (%), and the sperm velocity (mcm/sec) were automatically measured with the SFA-500 Sperm Analyzer (Biola Company, Ltd, Russia). The dynamic of thawing temperature up to the final value was measured with the Center 304 thermocouple. It has been ascertained that the effects of different procedures used for thawing straws and pellets are quite steady and approximately the same. However, the best results of the male sex cell survival after the long in-vitro incubation period (for 24 hours at the animal body temperature of 38 °C) are in the samples thawed in a water bath at 38 °C for 10 sec when compared to those for the other methods ($p < 0.001$).

Keywords: Holsteins, sperm, thawing procedure, motility, sperm velocity, straws, uncoated pellets

For more than 70-year history of cryopreservation for male germ cells, the method has been widely used both in research programs on biodiversity conservation [1] and for commercial purposes in the creation of sperm banks of different animal species [2] and human [3]. Such a long history of the issue allows researchers to discuss not only the achievements but also the risks of artificial insemination [4, 5]. It is believed that the increase in milk production has a neg-

ative impact on reproduction; in particular, the decrease in the number of calving from the first insemination is noted [6]. The main reason under consideration is the mismatch of energy consumption [7]. As a result, a number of animals with defective sexual cycles and quiet manifestations of hunting, which complicates the determination of the time of insemination, is increasing. In this regard, the need for semen with high qualitative and quantitative characteristics, capable to stay in the female genital tract for a long time (more than 24 hours) without loss of fertilizing ability arises. At the same time, in artificial insemination after dilution of the native semen, packaging, cryopreservation and thawing, the number of spermatozoa decreases, which also significantly increases the requirements for their quality.

The main structural damage to the sperm of bulls and stallions is associated with freezing and subsequent thawing [8-10] and provoked by the phase transition of water into ice and back [11, 12]. The regimes of thawing temperature and speed are crucial for maintaining the integrity of membranes and fertilizing ability of sperm cells [13, 14]. It is known that the formation of intracellular ice damaging the cell includes emergence of crystallization centers and crystal growth. With gradual and slow cooling, the number of crystallization centers is small, but conditions for their growth are favorable. By increasing the cooling rate, the optimum crystal growth temperature can be avoided without damaging cells. Thawing requires the fastest possible temperature rise to prevent secondary growth of ice crystals inside the cells. A particularly vulnerable point in the process of thawing is significant recrystallization of ice (especially inside the cells) at –40 °C. The formation of the ice centers inside cells can lead to ice crystal growth during thawing [15]. To prevent secondary recrystallization, the object to be thawed must pass the zone of ice crystal secondary growth as quickly as possible. Increasing the thawing rate increases the number of viable cells [16].

The simplest and most effective method of thawing cryopreserved sperm is thawing in a water bath (at 37-40 °C according to different protocols) with stirring to prevent local temperature gradients in the water. The higher the temperature of the water bath, the shorter the pause in the rise of temperature in the thawed object after passing –20 °C [17]. Rapid warming-up can increase the survival of cryopreserved cells by reducing the time of ice crystal re-growth and/or the membrane reorganization. In the experiments by F.I. Ostashko [17], in coated semen doses after thawing in running boiling water, the spermatozoa had motility and fertilizing ability, respectively, 7 and 15 % higher than when thawing in the traditional way.

Structural and metabolic disorders in sperms during freezing and thawing are accompanied by damages to the proximal centrioles, mitochondria, and fibrils. The most expressed changes in the ultrastructure of sperm cells identified in acrosomes and mitochondria, slight structural and metabolic changes in the activity of hyaluronidase, acrosin, aspartate aminotransferase, and respiration occur at temperatures of about 4-6 °C [18, 19].

In the routine practice of breeding enterprises, the use of complex tests based on the determination of ultrastructural damage of sperm is difficult [20-24]. For this purpose, the technologies of automated sperm differentiation by the functional state are proposed, in particular, complex computer analysis of sperm with of exposed microscopy of moving spermatozoa (CASA) [25]. The method for measuring the concentration, total motility, number of actively mobile (fertile) spermatozoa and the average speed of their movement by laser analysis of frequency spectrum of optical density fluctuations at spermatozoa movement through an optical channel [26] is well proved. This method is adapted for practical pur-

poses in Russian breeding enterprises [8, 27].

There is still no consensus on the effects of water bath temperature and time of thawing on the preservation of the functional characteristics of thawed spermatozoa. Many papers are devoted to the problem [28-30], but in most of them, information about the optimal conditions of semen dose thawing is quite contradictory, and these temperatures range from 20 to 75 °C [28, 31-33]. In Russia, in accordance with the national technology, the method of thawing in a water bath at 38 °C for 10 s is widely accepted. In the present work, we for the first time compare different modes of thawing of semen with fixing the end temperature and on this basis chose the optimum, making it possible to maintain prolonged viability of the thawed sperm in 24-hour incubation at 38 °C.

The work objective was to compare different deconservation modes (i.e. existing and proposed by us) for sperm cryopreserved in polypropylene straws and uncoated pellets, and the dynamics of sperm qualitative characteristics (motility, the speed of movement, survivability) during subsequent incubation in vitro at 38 °C.

Techniques. Sperm obtained from Holstein bull sires aged 3-7 years was cryopreserved in polypropylene straws (72 doses) on equipment of IMV company (model IS4, France) and uncoated pellets (90 doses) on the plates of dry ice according to GOST 26030-2015 and the National Technology of Freezing and Use of Semen of Breeding Bull Sires.

When thawing, the sealed tip of straws taken from the water bath was cut off, the temperature of the internal content was measured with a thermocouple Center 304 (Center Technology Corp., Taiwan), the melted semen dose (0.23 ml) was extruded, by pressing the piston on polypropylene tube, into penicillin vial with 0.8 ml of diluent OptiXcell (IMV, France) and heated to 30 °C. The following defrost modes were used: 35 °C, 30 s; 38 °C, 10 s (control); 50 °C, 5 s; 70 °C, 3 s (18 doses per option). When thawing the semen frozen in uncoated pellets, they were quickly taken one by one from the tube with sterile tweezers pre-cooled in liquid nitrogen and placed in penicillin vials with 0.8 ml of OptiXcell diluent or with 2.9 % sodium citrate. In the dry thawing option, the pellets were transferred into clean, dry vials.

Immediately after thawing and 5, 20, and 24 h after incubation in vitro at 38 °C, motility (%) was assessed visually using a microscope (CX41 model, Olympus, Japan; ×150 magnification). The motility (%), pool of fast sperm cells (%) and the average speed of sperm cells (μm/s) were measured in the same samples using a SFA-500 device (NPO Biola, Russia) with a laser analyzer of the frequency spectrum of optical density fluctuations caused by the movement of sperm through an optical channel with special characteristics. Temperature of semen thawing was controlled in dynamics with a thermocouple Center 304 (Center Technology Corp., Taiwan). The percentage was calculated of motile and fast-moving sperm, as well as the number and speed of spermatozoa with straight-forward motion (SFM) at different incubation periods.

The data were processed by the variation statistics methods; the tables show the mean values (M) for each series of the experiment and the standard errors of means (±SEM). The significance of differences was assessed by Student's t-criterion. Differences were considered statistically significant at $p < 0.05$.

Results. Thawing of semen in uncoated pellets was carried out under different protocols (Table 1).

Straws with the semen of one batch were simultaneously thawed in water baths in the specified modes; the temperature inside the straws was measured immediately after thawing. In options, the temperature of the semen was

26.0; 25.3; 26.0; 243 °C (average for all modes 25.0±0.4 °C, the differences statistically insignificant). Therefore, regardless of the mode of deconservation, the final temperature of thawed sperm was almost the same.

1. Modes of thawing of uncoated pellets with cryopreserved sperm of Holstein bulls

Diluent buffer	Diluent temperature, °C	Time, s
In a water bath (40 °C)		
OptiXcell diluent (France)	38	10
Sodium citrate (2.9 %)	38	10
Dry thawing		Up to full thawing
Sodium citrate (2.9 %)	4	12-13
OptiXcell diluent (France)	4	12-13
At room temperature (23 °C)		
OptiXcell diluent (France)	23	Up to full thawing
Sodium citrate 2.9 %	23	Up to full thawing
Dry thawing		Up to full thawing
In the refrigerator (4 °C)		
Dry thawing		Up to full thawing

The compared thawing protocols did not impact significantly the number of sperm cells with SFM (fluctuations of 35-38 %), which was confirmed with SFA-500 analyzer (34.4-36.5 % of cells, the differences are insignificant) (Table 2). This is consistent with the data of other authors for semen of Buffalo [28, 31], cattle [29], and zebu [31]. Similar results were obtained for fast-moving sperm (variations from 23.8 to 24.2 %, the differences are insignificant). The highest speed of sperm movement was in samples thawed at 35 °C, and it decreased during deconservation with increasing temperature of water bath (at the maximum temperature the differences were significant, $p < 0.001$). During thawing of cryopreserved Buffalo sperm, the maximum speed was at + 70 °C [28].

2. Motility and the proportion (%) of mobile spermatozoa in semen of Holstein bulls evaluated by two methods immediately after thawing of 72 doses by different modes (OAO "Reproduction Head Center of Agricultural Animals", the Moscow Region, 2018)

Thawing temperature, °C	Microscopy	SFA-500 analyzer		
	motile spermatozoa, %	motile spermatozoa, %		average rate, μm/s
		slow + fast	fast	
0 h incubation in vitro, 38 °C				
35	35.0±0.90	34.4±0.09*	23.9±0.30	100.7±1.2
38 (control)	38.3±0.53	35.1±0.30	23.7±0.20	97.8±1.03
50	36.6±0.53*	36.5±0.76	24.2±0.40	96.3±0.6
70	35.0±0.90**	34.4±0.70	23.8±0.30	91.3±0.16***
5 h incubation in vitro, 38 °C				
35	30.0±0.90	38.4±0.70	27.8±0.20*	97.8±2.30
38 (control)	30.0±0.93	37.8±0.13	28.7±0.40	102.8±1.70
50	30.0±0.93	34.5±0.66***	26.2±0.20***	102.0±1.70
70	28.3±1.40	38.9±0.20***	27.3±0.20**	96.6±1.36**
20 h incubation in vitro, 38 °C				
35	20.0±0.00***	24.0±0.5**	15.1±0.30***	81.6±0.30
38 (control)	23.3±0.53	26.3±0.56	16.7±0.05	81.6±1.30
50	20.0±0.09***	19.8±0.26***	12.8±0.33***	76.9±1.50*
70	18.3±0.50***	19.8±0.60***	12.5±0.43***	79.1±0.30
24 h incubation in vitro, 38 °C				
35	10.0±1.70***	11.4±1.60***	5.8±0.80***	71.0±0.30***
38 (control)	15.0±0.00	18.4±0.4	11.4±0.46	79.3±1.36
50	8.3±0.53***	10.7±0.7***	4.9±0.6***	62.7±2.70***
70	5.6±0.70***	8.2±0.93***	4.1±0.43***	73.1±0.86***

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

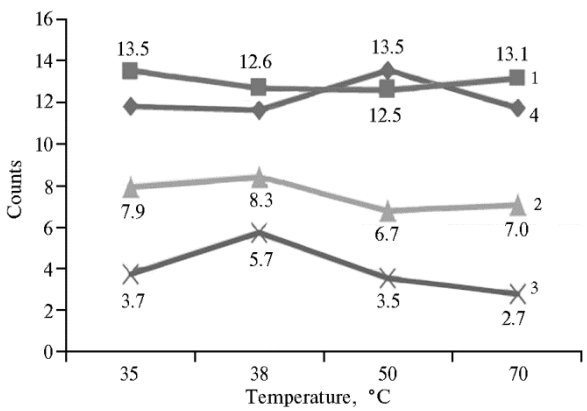
After 5 h incubation in vitro (see Table 2), there was a discrepancy in the results of the motility estimates: visually 28.3-30.0 % of spermatozoa were motile, whereas computer analysis detected more motile gametes (by 4-10 %). In addition, sperm analyzer recorded an increase in motility compared to that immediately after thawing. Similar data were obtained after 2-hour incubation

[29]. Number of such cells was significantly higher (38.9 %) under rapid deconservation (70 °C) and significantly lower when cells were thawed at 50 °C.

Note, the share of fast-moving (fertile) sperm (28.7 %, an average speed of 102.8 μm/s) was significantly higher for the standard thawing technology. The differences between the control and experimental groups were statistically significant in all cases. Probably, this can be explained by the fact that visually sperm cells with SFM are predominantly recorded, whereas a sperm analyzer records all the spermatozoa moving in different trajectories with a speed greater than 25 μm/s. This minimum speed threshold is set to cut off trim cells that do not have their own motility and move with the flow of the fluid [8]. After 20 hours of incubation (see Table 2), the number of spermatozoa with SFM decreased by 10 % on average, with the average speed 20 % lower as compared to that after 5 hours of incubation.

It should be noted that spermatozoa, deconserved by the standard method, preserved more cells with SFM. This trend was observed both in routine microscopy and in the automatic examination (the observed differences are statistically significant). One day after thawing and incubation in vitro at 38 °C (see Table 2), the best preservation (18.1 % of motile sperm) was also characteristic of semen deconserved in a water bath at 38 °C by the standard procedure (the differences with samples in other modes were from 6.7 to 10.0 %, being statistically significant at $p < 0.001$).

The results show that deviations from the conventional method of semen thawing reduce its qualitative characteristics and fertilizing ability. This underscores the need for strict compliance with established regulations. In several publications, it is also reported that the best results are obtained by thawing sperm doses in the range of temperatures from 37 to 40 °C [30, 31]. Strict adherence to the method of sperm thawing can improve the effectiveness of artificial insemination, especially in late ovulation (more than 24 hours after identified estrus; the number of such animals among highly productive individuals is more than 50 %) [27].



Sperm (×10^{6}cell per semen dose) **with a straight-forward movement depending on the temperature of defrosting and different periods of subsequent incubation**: 1 — 5 h, 2 — 20 h, 3 — 24 h, 4 — no incubation (Holstein bulls, OAO Reproduction Head Center of Agricultural Animals, the Moscow region, 2018).

Daily dynamics of cell activity, expressed in absolute values (Fig.), shows that, the number of motile sperms immediately after thawing averaged 33-35 million, and about a third of them are spermatozoa with SFM (when counting sperms with speed below 25 μm/s). After 5 h of incubation in all groups, the number of sperm cells with SFM became 8-12 % higher (except semen thawed at 50 °C). Perhaps, the observed increase is due to the beginning of hyperactivation processes.

It is known that freezing and thawing trigger spontaneous capacitation. It is believed that sperm capacitation is thermally dependent, and the blocking effect is overcome when the sperm returns to a temperature of 37 °C [32]. The key factor here is the partial cryopreservation of the cell membrane, in particular, in the area of the acrosomal cover, resulting in changes in intracellular calcium con-

centration [34]. Even though in cattle sperm motility correlates with fertilizing ability and can be successfully used for its rapid testing, in practice, it is necessary to control the life expectancy of germ cells outside the body.

With increasing incubation period (up to 20 and 24 h), the greatest number of SFM spermatozoa (respectively 8.3 and 5.7 million) remained in samples defrosted in a water bath at 38 °C for 10 s. After 24 h, in samples thawed in a water bath at other temperatures, the number of SFM cells was 2-3 million less (see Fig.). This should be taken into account when inseminating, as in the female genital tract sperm accumulates in a special reservoir of the oviduct, attached to its walls without losing the fertilizing ability during a day, provided its functional integrity is preserved [33].

In experiments with uncoated pellets, the standard conditions of deconservation were changed. According to the approved protocol in Russia, the dose of the semen should be placed in a bottle with 1 ml of 2.9 % sodium citrate, preheated to 38 °C and thawed at 40 °C for 8-10 s to a thin ice rod. We carried out thawing outside the water bath at room temperature of 23 °C, and also used a 40 °C water bath but citrate buffer pre-cooled to 4 °C. Optimal results of pellet thawing were in using a water bath (Table 3). Moreover, the initial indicators of the semen were slightly higher in all variants with chilled citrate. However, after 5 hours of incubation, active sperm was 11.6 % more when defrosting in citrate buffer warmed to 38 °C.

The final thawing temperature determined by thermocouple in the germ cell suspension during thawing in a water bath in both cases was the same and on the average reached about 28 °C with a slight increase in thawing time when using a cooled buffer. Upon defrosting pellets in citrate buffer at room temperature (23 °C), the thawing time increases, the final temperature of the suspension significantly ($p < 0.05$) reduces to 13.9 °C, and the viability of sperm significantly deteriorates compared to that in using water bath (40 °C). Ultimately, this will reduce the effectiveness of artificial insemination.

3. Quality of semen cryopreserved in the uncoated pellets after thawing in a water bath with different diluters and incubation (0 h and 5 h) (Holstein bulls, OAO Reproduction Head Center of Agricultural Animals, the Moscow Region, 2018)

Microscopy		SFA-500 analyzer			Thawing time, s	Semen dose temperature, °C
motile spermatozoa, %		motile spermatozoa, %		average rate, μm/s		
0 ч	5 ч	slow + fast	fast			
Sodium citrate (2.9%)						
Control (1 ml diluent, t = 38 °C, t of defrosting 40 °C)						
36.6±1.60	28.3±1.60	36.0±2.30	21.6±0.90	84.3±2.30	10.0±0.00	28.5±4.20
Group I (1 ml diluent, t = 23 °C, without a water bath)						
33.3±3.30	16.6±4.40*	29.3±1.00*	18.8±1.20	96.6±4.90*	23.3±2.00***	13.9±4.50*
Group II (1 ml diluent, t = 4 °C, t of defrosting 40 °C)						
41.6±1.60*	25.0±2.80	47.3±2.80*	23.1±2.50	73.2±9.90	13.6±1.20	28.3±0.30
OptiXcell synthetic diluent (France)						
Control (1 ml diluent, t = 38 °C, t of defrosting 40 °C)						
31.6±4.40	30.0±5.00	40.5±3.50	23.6±3.10	84.3±6.50	11.3±1.30	28.6±0.80
Group III (1 ml diluent, t = 23 °C, without a water bath)						
35.1±0.10	30.0±2.80	48.8±8.10	28.2±2.30	86.8±5.00	14.6±0.60*	18.3±0.60**
Group IV (1 ml diluent, t = 4 °C, t of defrosting 40 °C)						
36.6±1.60	26.6±3.30	38.0±1.50	22.0±0.20	83.9±3.20	12.0±1.00	29.3±1.30

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

Currently in Russia, breeding enterprises predominantly use imported synthetic diluents for semen. In the current experiment, when replacing the citrate buffer with the OptiXcell synthetic medium, defrosting of uncoated pellets gave ambiguous results that require a further study (see Table 3). As in the citrate buffer variant, immediately after deconservation in a water bath (40 °C), sperm motility was higher in the diluent cooled to 4 °C, but after 5-hour incuba-

tion of active sperm, it was 11.3 % higher in use of initially warm (38 °C) diluent. In this case, the final temperature of the resulting suspension of germ cells and the time of thawing of the sperm did not differ from those for the citrate buffer. Several different patterns emerged at deconservation of uncoated pellets in the synthetic diluent at room temperature, i.e. the thawing was 8.7 s faster, and final temperature of samples increased to 4.4 °C compared to that for citrate buffer. The motility and viability of spermatozoa in the group, where sperm were thawed at room temperature with the OptiXcell synthetic diluent, tended to improve (with not confirmed statistically significant differences). The obtained data indicate the need to study the mechanisms of action of this synthetic diluent during freezing/thawing. It is assumed that the proteins of the seminal plasma bind to the fraction of low density of the egg yolk, which is part of the medium, and this is crucial in the preservation of the cell membrane of sperm [35, 36].

In the experiment with dry thawing (without diluent), a directly proportional relationship was shown between sperm motility and ambient temperature (Table 4). Thus, the best results were obtained at 40 °C (control): 45 % of spermatozoa had SFM immediately after thawing, and 30 % remained viable after 5 h of incubation at 38 °C. With increasing thawing time and decreasing ambient temperature, the proportion of viable spermatozoa decreased: at room temperature after 5 h the survival rate was 21 % compared to 66 % in the first option. With slow deconservation (4 °C), thawing time increased sharply (up to 820 s), which had a detrimental effect on sperm viability: only 6.7 % of the sperm cells retained SFM right after thawing, and only single cells were SFM after 5 h.

4. Quality of semen cryopreserved in uncoated pellets after dry thawing (without diluters) **and incubation** (0 h and 5 h) (Holstein bulls, OAO Reproduction Head Center of Agricultural Animals, the Moscow Region, 2018)

Thawing mode	Motile spermatozoa, %		Thawing time, s	Semen dose temperature, °C
	0 h	5 h		
In a water bath at 40 °C (control)	45.0±2.80	30.0±0.00	56.0±3.05	27.7±8.20
At room temperature 23 °C	23.3±6.00**	5.0±0.00***	222.3±76.00*	12.2±5.14
In the refrigerator at 4 °C	6.7±1.60***	1.7±1.60***	820.0±40.00***	15.2±1.80

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

Table 4 does not contain the data obtained with the SFA-500 analyzer: the characteristics of the device indicate the limits of sperm concentration (5-100 million/ml) for qualitative and quantitative analysis of the sample with an acceptable error; beyond these values, the error increases sharply. In the current experiment, in dry thawing, concentration in a sperm dose increased to 160-190 million/ml, which made the interpretation of the results impossible (additional use of media and buffers to dilute the semen would distort the original effect).

Thus, deconservation of semen packed in straws and uncoated pellets, is applicable worldwide and constantly developing. In the current experiment, the best results with bovine sperm were achieved by thawing for 10 s in a water bath at 38 °C for straws and 40 °C for uncoated pellets. These protocols reliably provide a prolonged positive effect on sperm quality and better preservation of its viability after thawing than other methods ($p < 0.001$). Our results also show that one of the modern modifications of these procedures, namely the use of synthetic yolk-free diluents, requires an in-depth study of the effect of this media on sperm quality of different animal species to clarify the existing protocols of deconservation. The obtained data confirm the well-known fact that even small deviations from the optimal conditions can significantly worsen the properties of sperm and ultimately reduce the effectiveness of artificial insemination. In some cases, the effects of modifications are ambiguous, which indicates a possible complex mechanism of influence of the agents used on the characteristics of

sperm cells and, as a consequence, on artificial insemination.

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CHANGE OF BIOLOGICAL PARAMETERS OF POULTRY SEMEN AT CRYOPRESERVATION

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Abstract

The cryobanks of genetic material are an important element of assisted reproductive technologies. This technology allows more efficient use of genetic material, ensuring to obtain the maximum possible number of offspring for to preserve and restore of rare and endangered species. The most common biomaterial used in programs for the conservation and restoration of the genetic resources of agricultural poultry are spermatozoa. The spermatozoa undergo significant technological treatment during freezing and thawing. Some stages of this cycle are lead to the death of a large part of the cells, damage to their individual organelles or segments. The aim of the research was to study the effect of the freezing and thawing cycle on the biological parameters of spermatozoa in agricultural poultry. The objects of research were adult males of different types of agricultural poultry: roosters *Gallus gallus* (n = 6), quails *Coturnix coturnix* (n = 10), guinea fowls *Numida meleagris* (n = 6), turkeys *Meleagris gallopavo* (n = 3) and geese *Anser anser* (n = 4). Qualitative and quantitative indices of freshly received and frozen semen were studied. These indicators include the percentage of mobile spermatozoa, the proportion of spermatozoa with abnormal morphology, the percentage ratio of spermatozoa with abnormal morphology in the head, middle part and flagellum. Sperm was collected 3 times a week. The ejaculates were diluted with the medium for bird sperm dilution (1:1) followed by equilibration of samples at a temperature of 5 °C for 180 minutes. Before cryopreservation, dimethylacetamide was added to the samples as a cryoprotectant by gradually increasing its concentration to 8 %. The samples were frozen in straws (0.25 ml) with an automatic freezer Cryobath Biofreeze BV-65 (CONSARCTIC, Germany). Sperm quality was assessed using software Zoosperm 1.0 (LLC VideoTesT Goss) and Nikon microscope (Nikon Corporation», Japan) equipped with an image input system. To determine the proportion of viable spermatozoa, a supravital staining of a cell smear with 5 % eosin solution was used. The quality indicators of freshly obtained semen complied with the requirements. It was found that the biological value of spermatozoa decreases during the freeze-thaw cycle in all species of poultry conditioned by a decrease in the activity of germ cells. The sperm of the goose was more cryoresistant than the sperm of roosters, quails and turkeys. In geese, after thawing frozen sperm, the content of sperm cells with a straight-forward movement decreased by 30 %, while in roosters, quails and turkeys up to 40-44 %. During semen equilibration, in all poultry species the proportion of live spermatozoa decreased by 7.0-10.6 %. After the freeze-thaw cycle, the proportion of live spermatozoa in the ejaculate decreased compared to the values found for freshly sperm. In roosters, this indicator decreased by 41.6 % with an increase in the percentage of spermatozoa with an abnormal morphology by 22.0 %. In quails these indicators were 43.8 % and 21.8 % and in guinea fowls — 49.1 % and 28.8 %. The most visible disturbances in the morphology of spermatozoa were noted in the flagellum.

Keywords: *Gallus gallus* L., roosters, *Coturnix coturnix* L., quails, *Numida meleagris* L., guinea fowl, *Meleagris gallopavo* L., turkeys, *Anser anser* L., geese, cryopreservation, spermatozoa, abnormalities, freeze-thaw, equilibration, semen quality

Poultry products are among the main sources of proteins, fats, minerals, and vitamins among food products [1]. The rapid global rate of poultry industry development (from 2012 to 2016, the number of chickens in Russia increased by 14.6 %, in the world by 10.8 %) [2] is driven by its economic efficiency (the poultry is superior to other farm animals in maturity rate and feed conversion rate). The volume of poultry meat production is more than 300 million tons per year but the need for poultry products is only 34 % met. This fact, along with technological effectiveness of the industry, stimulates its development [3, 4].

Limiting factors are the lack of assisted reproductive technologies, including cryopreservation and artificial insemination, the methods to compensate for reproductive losses associated with increased animal productivity [5]. Cryopreservation of biological material is important in practical integration of reproductive technologies [6-8]. However, the use of cryopreserved sperm in the poultry industry is limited, which is largely due to the poor quality of thawed semen. Spermatozoa fertility decreases during freezing and thawing of generative plasma of birds [9, 10]. Spermatozoa of birds have a minimum volume of cytoplasm at a relatively large plasma membrane surface, the only cytoplasmic organoids are mitochondria [11, 12], the nucleus contains very condensed chromatin. In the freezing-thawing cycle, plasma and mitochondrial membranes are damaged mainly, as a result, the integrity of nuclear and mitochondrial DNA is violated, and the activity of spermatozoa is lost [13-15]. In birds, the structure of sperm is significantly different from that of mammals: the flagellum length is 90-100 μm, which is about 8 times longer than the head length [16-18].

Chemical and physical effects during freezing-thawing inevitably lead to changes in the ultrastructure of sperm and affect its biological full-grade. Cryopreservation causes damage to organelles and cell segments. The minor damages in the cells increase during freezing and thawing, and the cells lose their biological quality. Sperm cryoresistance and the ability to resist the damaging effect of ultra-low temperature depend on the states of membranes, their permeability, lipid composition, and fluidity [19, 20]. Due to such changes, the efficiency factor of insemination of thawed sperm is much lower than that of fresh and chilled sperm [21-24].

The cryoresistance of spermatozoa of birds depends on the species, despite the similarity of morphology [25]. The intraspecific variability of cryostasis depending on the linear accessory of males roosters was noted [26]. In the musky drake, sperm is more cryostable than in the Peking duck, sperm of guinea fowl, when compared to a rooster and a turkey, is very sensitive to cryopreservation [27]. The biological value of sperm is influenced by abiotic factors during processing. The main of them is the composition of diluents and cryoprotectors, the freezing and thawing mode [28, 29]. The reaction of sperm to these factors depends on the species characteristics of birds. With the increase in shelf life, the proportion of dead and abnormal sperm increases as well. It also depends on the breed and species characteristics: for example, in the sperm of guinea fowl, the process is more intense than in roosters [30].

In this paper, the biological parameters of sperms in the main species of poultry (roosters, quails, guinea fowls, turkeys, geese) were compared at different technological stages in the freezing-thawing cycle and the data was obtained confirming that in the process of equilibration the content of dead sperm in the generative plasma increases significantly regardless of the bird species.

The work objective was to study the effect of the freezing and thawing cycle on the biological parameters of sperm in different poultry species.

Techniques. Full-grown roosters *Gallus gallus* L. (n = 6), quails *Coturnix*

coturnix L. (n = 10), guinea fowls *Numida meleagris* L. (n = 6), turkeys *Meleagris gallopavo* L. (n = 3) and geese *Anser anser* L. (n = 4) selected for the experiment were kept in individual cages (the physiological yard of Vivarium of Ernst Federal Science Center For Animal Husbandry, 2017-2018). The poultry diet complied with the standards provided for each species.

Sperm was collected 3 times a week using spinal and peritoneal massage. For dilution, storage, and cryopreservation, a synthetic medium was prepared (bidistilled water 100 ml; fructose 1.0 g; glucose 1.0 g; Tris-HCl 0.195 g; disodium phosphate 1.1 g; sodium glutamate 3.0 g). The ejaculates were diluted with the medium for bird sperm dilution (1:1) followed by equilibration of samples at 5 °C for 180 min. Before cryopreservation, dimethylacetamide was added as a cryoprotectant by gradually increasing its concentration to 8 %. The samples were frozen in 0.25 ml straws. An automatic freezer Biofreeze BV-65 (Consarctic Entwicklung und Handels GmbH, Germany) was used.

Sperm quality was assessed using software Zoosperm 1.0 (LLC VideoTesT Goss, Russia) and Nikon microscope (Nikon Corporation», Japan) equipped with an image input system. The proportion and morphology were scored for straightforward moving and non-linearly moving spermatozoa, as well as for motionless cells. To determine the proportion of viable spermatozoa, supravital staining of smears with 5 % eosin solution was used.

The obtained data were processed in the Microsoft Excel software. The mean values (M) and standard errors of means (±SEM) are presented in the tables. The significance of differences was assessed according to Student's t-criterion. Differences were considered statistically significant at $p < 0.05$.

Results. Quality indicators of freshly obtained semen met the established requirements (Table 1). The content of mobile spermatozoa in the ejaculates varied depending on the bird species. The highest rate was in quails (87.5 %), the minimum in geese (64.5%). The average sperm activity in fresh semen of geese was lower than that of roosters, quails, guinea fowls, and turkeys, respectively by 21.4; 23.0; of 17.0 and 17.0 %. The differences between the average sperm motility in geese and other studied species were statistically significant ($p \leq 0.001$). Percent of spermatozoa with abnormal morphology was also higher in geese (from 10.9 to 18.6 %, 14.6±1.2 % on average). In quails, guinea fowls, roosters, and turkeys, it was lower by 1.8; 1.1; 5.4, and 5.0 %, respectively ($p \leq 0.001$). In guinea fowls, spermatozoa with morphological abnormalities were more common than in roosters and quails respectively by 4.3 and 3.9% ($p \leq 0.01$).

1. Quality of fresh semen of different species of poultry

Species	Sample size, n	Motile spermatozoa, %	Spermatozoa with abnormal morphology, %	Live spermatozoa, %
Roosters	6	86.1±6.4	9.2±2.7	89.2±8.1
Quails	10	87.5±3.8	9.6±1.2	92.5±6.1
Guinea fowls	6	82.1±3.5	13.5±2.7*	91.6±5.4
Geese	4	64.5±8.2**	14.6±1.2**	76.5±8.1
Turkeys	3	81.5±3.6	12.8±1.6	87.2±4.2

* The differences between guinea fowls, roosters, and quails are statistically significant at $p \leq 0.01$.

** The differences between geese and other species are statistically significant ($p \leq 0.001$).

The share of live spermatozoa varied from 76.5 to 92.5 % depending on the bird species. During equilibration it decreased. The number of dead spermatozoa in semen samples of roosters' seed after equilibration increased by 7.0 %, quails by 9.5%, guinea fowls by 10.2%, geese by 10.6% and turkeys by 9.8% (Fig. 1). The differences between the live spermatozoa content in all the studied samples were statistically significant at $p \leq 0.001$.

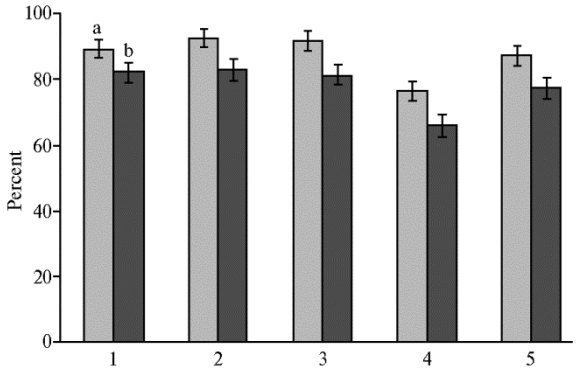

Fig. 1. The proportion of live spermatozoa in the fresh (a) **and equalized** (b) **semen of roosters** (1), **quails** (2), **guinea fowls** (3), **geese** (4), **and turkeys** (5).

After equilibration, a slight decrease in the number of spermatozoa with the straight-forward movement was noted. During further cryopreservation and subsequent thawing, this indicator decreased significantly. After thawing, the share of spermatozoa with the straight-forward movement in roosters decreased by 40 %, in quails by 43 %, in guinea fowls by 41 %, in geese by 30 %, and in turkeys by 44 % (Fig. 2).

The cycle of freezing and thawing adversely affected not only the activity but also the share of live spermatozoa. In roosters, this indicator dropped from 89.2 up to 47.6 %, in quails from 92.5 up to 48.7 %, in guinea fowls from 91.6 up to 45.2 %, in geese from 76.5 up to 38.2 %, in turkeys from 87.2 up to 48.5 %.

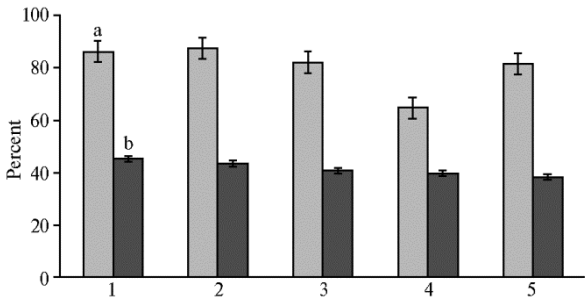

Fig. 2. The proportion of live spermatozoa with the straight-forward movement in the fresh (a) **and frozen-thawed** (b) **semen of roosters** (1), **quails** (2), **guinea fowls** (3), **geese** (4), **and turkeys** (5).

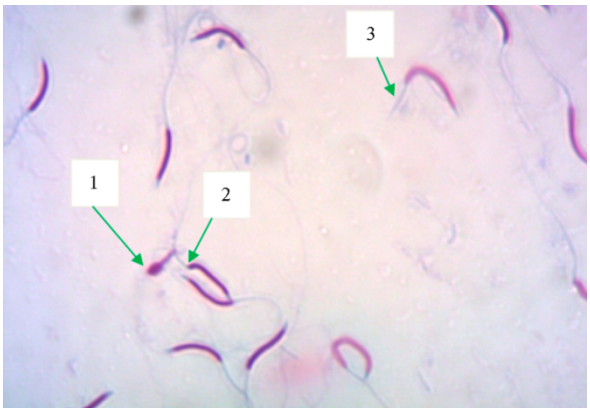

Fig. 3. Pathology of the head (1), **neck** (2), **and flagellum** (3) **in guinea fowls spermatozoa** (eosin staining).

As a result of freezing-thawing, the share of sperm with abnormal morphology increased (Fig. 3). The frequency of abnormalities of different segments changed, and the number of sperm with flagellum pathology increased (Table 2). For example, in the frozen-thawed samples of roosters' sperm in comparison with indicators for the fresh ejaculate, the percentage of sperm with the pathology of heads, middle part and flagellum increased by 0.4, 0.4, and 1.3 %, respectively ($p \leq 0.001$). Other studied species showed a similar trend.

The freezing-thawing cycle significantly changes the biological parameters of the spermatozoa, which are influenced by many factors. Several studies have confirmed the dependence of cryoresistance of poultry sperm on species peculiarities (for example, the cryoresistance of guinea fowl and geese sperm is lower than that of other types) [30, 31]. The sperm structure in these species is common but some properties on which the biological value of sperm depends are species-specific. For example, the differences in the sperm cell subfractions, length, the

number of mitochondria, flagellum fibrous membranes, and metabolic capabilities are species-specific. Therefore, the technology of cryopreservation, successfully used for one species, is unacceptable for others. Spermatozoa of guinea fowl and rooster have many morphological and morphometric similarities but differ in biochemical composition (i.e. in the ratio of cholesterol and phospholipids), which is reflected in the biophysical properties of the membrane. The membrane of the spermatozoa of guinea fowl compared to rooster is rigid, therefore, they are inferior to the latter in cryoresistance. When cryopreserving the sperm of guinea fowl by the technology used for rooster sperm, the fertility rate is only 15 %. The biological value of sperm is influenced by the methods of packing (in granules or packets), and the rates of freezing and thawing.

2. Spermatozoa with abnormal morphology (%) in freshly obtained semen (FOS) and the samples after freezing-thawing (FT) in different poultry species

Species	Abnormal area					
	head		middle part		flagellum	
	FOS	FT	FOS	FT	FOS	FT
Roosters	2.4±0.02	2.8±0.03*	2.6±0.02	3.0±0.03*	4.2±0.03	5.5±0.03*
Quails	2.5±0.01	2.8±0.05*	2.3±0.03	3.1±0.02*	4.8±0.03	5.8±0.03*
Guinea fowls	3.6±0.01	3.9±0.01*	3.7±0.02	4.6±0.03*	6.2±0.08	8.9±0.03*
Geese	4.8±0.02	5.2±0.06*	3.9±0.03	4.2±0.01*	5.9±0.02	7.4±0.02*
Turkeys	4.3±0.02	4.9±0.02*	3.3±0.05	3.9±0.02*	4.6±0.03	5.8±0.04*

* The differences between average values for FOS and FT are statistically significant at $p \leq 0.001$.

The proportion of spermatozoa with abnormal morphology depends on species characteristics, used diluents, and shelf life. Ultrastructural studies confirm a high correlation of sperm activity with ultrastructural lesions in the flagellum and the middle part of the spermatozoa, on which the kinematics of sperm depends [32]. The obtained data of light microscopy on violations of the morphology of the middle part and flagellum, as well as a decrease in activity after freezing-thawing indirectly indicate the accumulation of ultrastructural damages in the middle part and flagella of spermatozoa.

Thus, in cryopreserving semen of different poultry species, the biological value of spermatozoa deteriorates because of a decrease in semen activity. The equilibration of semen before cryopreservation decreases the share of live spermatozoa in all poultry species studied. The proportion of cells with abnormal morphology increases. During freezing and thawing, these indicators increase significantly. In comparison with the fresh sperm, the number of live spermatozoa after freezing-thawing in the ejaculates of roosters decreased by 41.6 %, with a 22.0 % increase in spermatozoa with abnormal morphology. In quails these indicators were 43.8 and 21.8 %, respectively, and in guinea fowls 49.1 and 28.8 %, respectively. The greatest abnormalities in the morphology of sperm occur in flagellum.

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GENETIC POLYMORPHISM OF THE BOVINE VIRAL DIARRHEA VIRUSES IN BIG DAIRY FARMS IN SIBERIA

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Abstract

Bovine viral diarrhea is a widespread disease in the Russian Federation and causes significant economic damage to dairy cattle, especially under intensive commercial farming. The seropositivity of livestock in various Russian regions reaches 65-100 %. The disease is caused by two different virus species, BVDV-1 and BVDV-2, of which the latter is considered more virulent. The persistently infected (PI) animals which are a constant endogenous source of the virus in the herd play a major role in maintaining the stationary trouble of the farms. Short-term sources of the pathogen are transitively infected (TI) animals in which disease proceeds in acute form. Genetic structure of viruses circulating in local livestock populations gives necessary information about the evolution, geography and pathways of the pathogen. However, studies on the genetic polymorphism of viruses in Russia are not enough. This paper is the first to report on a phylogenetic analysis of two types of viral diarrhea virus, isolated from animals of foreign and domestic origin with different clinical manifestations of the disease. These data show the prevalence of BVDV-1b in PI and TI animals, as well as BVDV-2b and BVDV-2c circulation among Siberian dairy complexes (note, BVDV-2b and BVDV-2c in animals of foreign and domestic origin are detected in Russia for the first time). Phylogenetic analysis of viruses circulating among PI and TI of highly productive dairy cattle was based on a comparison of conserved region of viral genome (5′-UTR) using reverse transcriptase PCR (RT-PCR) method. Studies were conducted in five regions of Western and Eastern Siberia: Tyumen, Novosibirsk, Irkutsk regions, the Krasnoyarsk Territory and Northern Kazakhstan on big dairy farms with a population of 800 or more milking cows with an average annual productivity of 7,000-10,000 liters, where at the time of the research vaccination was not carried out or inactivated vaccines were used. The imported livestock was kept mainly in the Tyumen region and the Republic of Kazakhstan. Biomaterial (blood, serum, nasal discharge, lymphoid organs, lungs, vaginal discharges) was collected from clinically healthy persistently infected animals, transitively infected animals with reproductive disorders and respiratory syndrome, as well as aborted fetuses. A total of 479 samples were examined. According to our findings, two BVDV species circulate among the PI and TI animals on the big dairy farms. The phylogenetic analysis reveals seven subtypes of BVDV-1, i.e. 1a (5 %), 1b (35 %), 1c (5 %), 1d (10 %), 1f (20 %), 1i (5 %), 1p (5 %) and two subtypes of BVDV-2, i.e. 2b (10 %) and 2c (5 %). Taking into account the fact that the strategy of livestock breeding is changing in Russia, the number of dairy mega farms" is increasing, which receive animals with different infectious status from many sources. Thence, the study of the genetic polymorphism of the virus is topical. Comparison of data on the origin of animals with the results of phylogenetic analyses can help in determination of the sources and ways of bringing pathogens into a particular region and in identifying and tracking new and highly virulent strains of viruses. This is especially important during the implementation of vaccination programs for animals when the genetic profiles of vaccine strains do not coincide with the profiles of viruses circulating among animals in a particular area.

Keywords: cattle, bovine viral diarrhea, subtypes, BVDV-1, BVDV-2, genome, polymerase chain reaction, 5'-nontranslated region (5'-UTR), nucleotide sequence, molecular epidemiology, phylogenetical analysis

Bovine viral diarrhea (mucosal disease) is widespread throughout the world [1], including Russia where the infection of cattle in different regions reaches 65-100 % [2-6]. The importance of this infection in intensifying dairy and beef farming is increasing. The most economically significant consequences of the diseases are the reproductive disorders and pathologies of the respiratory tract; therefore, seronegative heifers of the mating age and calves up to 6 months of age are more susceptible to infection [7-9]. Permanent adverse epidemiological situation on farms is maintained through the presence of persistently infected (PI) animals, which become a permanent endogenous source of the pathogen. Transiently infected (TI) animals in which the disease proceeds in an acute form can be short-term sources [1].

The disease is caused in cattle by two genetically different types of virus, BVDV-1 and BVDV-2 [10] of cytopathogenic (CP) and non-cytopathogenic (NCP) biotypes [1, 11]. The distribution of species and subtypes of the virus is region-specific and depends on the type of livestock breeding, herd density, productivity, the frequency of introduction of new animals and other factors [12]. The first type of virus is spread all over the world [1], but is found more often in the European countries. The largest number of subtypes (up to 21) was found in cattle in Italy [13] and China [14]. BVDV-2 in cattle was found in the USA [15], Canada [16], Brazil [17], Argentina [18], Uruguay [19], Germany [20], Slovakia [21], Italy [22], South Korea [23], Japan [24] and Mongolia [25]. This type, which is considered more virulent, is divided into six subtypes (2a-2f) [26] and prevails in the USA and Canada (up to 50 % of all isolated strains) [1]. For virus species differentiation, the nucleotide sequencing of genomic RNA is used with the study of a 5′-nontranslated region (5′-UTR) suitable for amplification [28, 29]. In Russia, the works on the phylogenetic analysis of isolates are fragmented, i.e. BVDV-1 was found to be widely distributed among the cattle in the Central region [30] and two antigenically different strains of the virus (1m and 1a) were detected in the population of wood bison and livestock [31].

Previously, the main gender and age groups of animals, most at risk of infection, were identified, which can be used as an indicator and be a source of the pathogen in the herd [4].

In this paper, we conducted for the first time the phylogenetic analysis of two types of viral diarrhea virus (mucosal disease) isolated from animals of foreign and domestic origin with various clinical signs, and revealed the prevalence of BVDV-1b in PI and TI animals and the circulation of BVDV-2b and BVDV-2c at the dairy complexes of Siberia.

The aim of the work is to study the genetic polymorphism of pathogens of viral diarrhea of cattle, circulating among persistently and transiently infected animals, including the imported ones, at large dairy complexes.

Techniques. Seven dairy complexes were surveyed in 2006-2017 in Western and Eastern Siberia (Tyumen, Novosibirsk, Irkutsk regions, the Krasnoyarsk Territory, and Northern Kazakhstan). In each livestock complex the population of dairy black-and-white cows, with average annual milk productivity of 7,000-10,000 liters and above, was 800 heads and more; neither specific prophylaxis of the disease nor vaccination using inactivated strains were performed [4]. Feeding and housing conditions complied with physiological and zootechnical standards.

Biomaterial (blood, serum, nasal discharge, lymphoid organs, lungs, vaginal discharges) was collected from clinically healthy persistently infected animals, transitively infected animals with reproductive disorders and respiratory syndrome, as well as aborted fetuses. Persistent infection was diagnosed only when viral RNA was detected in paired serum samples taken at 30-day intervals. A total of 479 biomaterial samples were analyzed.

For the isolation of viral RNA and reverse transcription, the commercial kits RIBO-sorb and Reverta-L (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia) were used.

Viral RNA in samples of the biomaterial was detected in reverse transcription polymerase chain reaction (RT-PCR) using universal primer pairs: 324 (sense) — 5′-ATGCCC(T/A)TAGTAGGACTAGCA-3′, 326 (antisense) — 5′-TCAACTCCATGTGCCATGTAC-3′ [33], flanking the 5′-UTR region, with subsequent phylogenetic analysis. In 30 μl reaction mixture there were 5 μl cDNA; 1× Taq buffer without Mg^{2+} (Medigen Laboratory LLC, Russia), 0.4 mM dNTP, 3.3 mM $MgCl_2$, 0.15 μm of each primer, 1.5 IU SmartTaq DNA polymerase, water (up to 30 μl). Amplification mode: 5 min at 95 °C (1 cycle); 20 s at 95 °C, 30 s at 54 °C, 40 s at 72 °C (45 cycles); 7 min at 72 °C (1 cycle). PCR products were analyzed electrophoretically in 2 % agarose gel with Tris-borate buffer (pH 8.0) according to the standard methodology.

The nucleotide sequence of the viral genome was determined with the use of BigDye 3.1 kit (Applied Biosystems, USA) by sequencing both DNA strands. The resulting nucleotide sequences were analyzed with BioEdit 7.0.0 (https://bioedit.software.informer.com) and Lasergene 7.1.0 software packages (https://lasergene.software.informer.com). The phylogenetic dendrogram was made using the nearest neighbor method with MEGA v7.0 software (https://www.megasoftware.net/). The topology of the dendrogram was confirmed by the bootstrap analysis method (1000 steps of replication) [13, 34]. The nucleotide sequences of the synthesized fragments were analyzed by alignment with the sequences of other strains BVDV-1 and BVDV-2 from GenBank (NCBI) using the ClustalW software [35].

Results. RNA of the virus was found in 20 (4.17 % of the examined number) samples, including 10 samples from the Tyumen Region, 6 from the Novosibirsk Region, 1 from the Irkutsk Region, 2 from the Krasnoyarsk Territory and 1 from the North Kazakhstan (Table). It was established that two types of the virus circulate among PI and TI animals. The phylogenetic analysis revealed seven subtypes of BVDV-1, i.e. 1a (5 %), 1b (35 %), 1c (5 %), 1d (10 %), 1f (20 %), 1i (5 %), and 1p (5 %). The second type of the virus was found in 15 % of samples, out of them BVDV-2b in 10 %, BVDV-2c in 5 %. The predominant subtype was BVDV-1b (Fig., posted on the website http://www.agrobiology.ru).

The distribution of subtypes had some geographical differences. BVDV-1a was detected only in the Tyumen Region in the internal organs of an aborted fetus. BVDV-1b, additionally to the Tyumen Region, was present in the Novosibirsk Region and the Krasnoyarsk Territory. In the Krasnoyarsk Territory, it was isolated from animals of local breed, in the Novosibirsk Region from heifers imported from Germany and from local cattle, and in the Tyumen Region from animals imported from Holland, USA, Slovenia, and Denmark. PI animals were the source of the virus.

BVDV-1c was found in the Tyumen Region in blood of a calf with respiratory pathology, born by a heifer from Holland, BVDV-1d was present in the Tyumen Region on the farm where the cattle were brought from France, and also in the Novosibirsk Region in calves of local breed with respiratory pathology. BVDV-1f was detected in Northern Kazakhstan in the serum of a calf with respiratory pathology, born by a heifer imported from Germany, in the Irkutsk Region in the serum of a cow of the local breed, in the Krasnoyarsk Region in the serum of a cow of local breed and in the Tyumen region in the serum of a heifer of unknown origin. BVDV-1i was revealed in the Novosibirsk Region in the serum of a calf of the local breed from a troubled farm in respect of respiratory diseases, BVDV1p in the Tyumen Region in the blood of a calf with a simi-

lar pathology, obtained from a heifer imported from Germany. BVDV-2c was found in the Tyumen Region in the internal organs of the aborted fetus from a heifer imported from the USA, BVDV-2b in the Novosibirsk Region in the internal organs of the stillborn calf and aborted fetus of local breed.

Genetic polymorphism of cattle viral diarrhea (mucosal disease) causative agents circulating among persistently infected animals at dairy complexes of Western and Eastern Siberia

Isolate	Type/subtype (5′-UTR)	Region	Source of virus	Origin of animals
N09/16	1b	Novosibirsk Region	Blood serum of a cow	Russia
N08/17	1b		Blood serum of a heifer	Germany
T38/16	1b	Tyumen Region	Blood serum of a PI calf	USA
T11/16	1p		Blood of a calf	Germany
Kz05/15	1f	Northern Kazakhstan	Blood serum of a calf	Germany
N09/15	1i	Novosibirsk Region	Blood serum of a calf	Russia
Ir03/17	1f	Irkutsk Region	Blood serum of a cow	Russia
T13/17	1f	Tyumen Region	Blood serum of a heifer	Unknown
K04/16	1f	Krasnoyarsk Territory	Blood serum of a cow	Russia
K14/16	1b	Krasnoyarsk Territory	Blood serum of a cow	Russia
T11/17	1c	Tyumen Region	Blood serum of a calf	Holland
T15/17	2c		Internal organs of an aborted fetus	USA
T18/17	1a		Internal organs of an aborted fetus	Austria
T01/16	1b		Blood serum of a calf	Slovenia
T30/16	1b		Mesenteric lymph nodes of a calf	Holland
T41/17	1b		Internal organs of a PI heifer	Denmark
T24/16	1d		Lymphoid organs of a calf	France
N19/17	2b	Novosibirsk Region	Internal organs of a stillborn calf	Russia
N12/16	2b		Internal organs of an aborted fetus	
N11/17	1d		Blood serum of a calf	

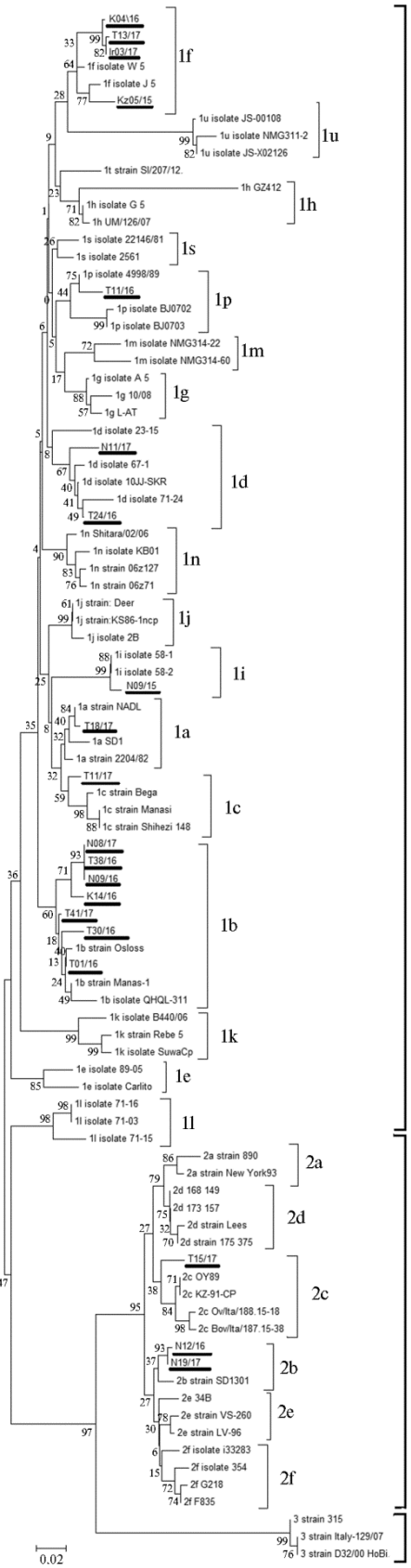
It is known that pestiviruses, due to the structure of their genome, possess significant mutational activity which is expressed in a constant increase in the genotypic and phenotypic diversity of strains, affecting virulence. Their role in the pathology of animals is not fully studied. In addition, viral polymorphism makes it difficult to diagnose diseases and may reduce the effectiveness of vaccination [1, 12]. BVDV-1a and BVDV-1b are the most common; their role has been described for various forms of the disease, and they, along with BVDV-2a, are the components of commercial vaccines worldwide [1]. BVDV-1f was reported to be found in Slovenia [36] and Austria [33] in PI animals with a high frequency. Sporadically, this subtype of the virus was detected in Italy [37] and Turkey [38], but the data on the caused clinical syndromes are not presented.

In Russia, there are no data on the identification of various subtypes of BVDV-2 and their connection with the clinical forms of the disease. Previously, we established the presence of the virus (second type) in animals with various pathologies; however, it was not possible to conduct subtyping [32]. In this paper, for the first time in Russia, the circulation of the virus of subtypes BVDV-2b and BVDV-2c are established.

According to the literature, BVDV-2a and BVDV-2b are recognized as the main etiological agents of the pathology of reproduction and systemic infection and are spread mainly in the countries of North and South America [1, 15, 16].

BVDV-2b was identified in the internal organs of an aborted fetus and a stillborn calf of local breed in the Novosibirsk Region. BVDV-2c is a rare virus subtype detected in Germany in 2013-2014 (North Rhi-ne-Westphalia and Lower Saxony) in seronegative animals during a massive outbreak of cattle viral diarrhea. It caused a decrease in milk productivity in cows, fever, respiratory disease and hemorrhagic enteritis in calves, heifers, and cows [40, 41]. In 2016, its circulation was established among small ruminant animals in Southern Italy [42].

We conducted research in the farms where new animals were imported from various sources, including from abroad. The results showed that the diversi-



Phylogenetic dendrogram of cattle viral diarrhea (mucosal disease) causative agents BVDV-1 and BVDV-2 identified in Siberian farms (2006-2017). Dendrogram is based on sequence analysis of the 5′-nontranslated region (5′-UTR). The ClustalW-based alignment of sequences. The topology of the tree is constructed by the neighbor-joining method. The genetic distance matrix is calculated using the minimal evolution method. The external group is the sequence of the virus BVDV-3. Near each node of the dendrogram, bootstrap support is indicated. Investigated isolates are underlined. For reference strains, the name and number in the database GenBank (NCBI) are indicated.

ty of species and subtypes of the virus is quite large. In previous work, among local and imported cattle during acute outbreaks of viral diarrhea of cattle, we detected the circulation of 6 subtypes of BVDV-1 (a, b, c, g, p, and k) and BVDV-2 without differentiation into subtypes [32]. This study confirms the circulation and the prevalence of BVDV-1b in PI and TI animals. In addition, three new subtypes of the first type of virus (BVDV-1d, BVDV-1f and BVDV-1i) were detected, as well as the circulation of new subtypes BVDV-2b and BVDV-2c not previously identified in Russia. The fact of their detection in the territory of Russia should be treated carefully, taking into account the potential pathogenicity of these agents and their lack in commercial vaccines.

Since the livestock farming strategy is changing in Russia towards dairy mega-farms to which animals with different infectious status come from many sources, the study of the genetic polymorphism of the virus is becoming more and more relevant. The comparison of the data on the origin of animals with the results of phylogenetic studies can help in determining the sources, pathways of pathogens in a particular region, as well as in identifying and tracking new and highly virulent strains of viruses. This is especially important in vaccinating

animals when the genetic profiles of vaccine strains and viruses circulating in a specific area do not match [29]. At present, mainly inactivated and live vaccines based on strains of subtypes 1a, 1b and 2a are used in Russia [1, 12]. In some cases, the genetic profile of vaccine strains may not fully correspond to the genetic spectrum of pestiviruses circulating in a particular country or region. The genetic diversity of viral diarrhea viruses that we detected can reduce the effectiveness of specific prophylaxis of the disease. In addition, for the formation of persistent adverse situation on bovine viral diarrhea of cattle and a continuous epizootic process, the cycle mother-calf is important. This leads to the birth of persistently infected offspring, which becomes a permanent endogenous source of the virus in the herd, reducing the effectiveness of specific prophylaxis. Therefore, vaccination will be more effective if the PI animals are completely removed from the herd [1, 4].

Thus, pathogens of two types of bovine viral diarrhea (mucosal disease) circulate at the dairy complexes of Siberia among persistently and transiently infected animals. The phylogenetic analysis revealed seven subtypes of BVDV-1, i.e. 1a (5 %), 1b (35 %), 1c (5 %), 1d (10 %), 1f (20 %), 1i (5 %), 1p (5 %), and two subtypes of BVDV-2, i.e. 2b (10 %) and 2c (5 %). The predominant subtype is BVDV-1b. For the first time in Russia, the circulation of BVDV-2b and BVDV-2c is established among animals of foreign and domestic origin, and the relationship between the belonging of viruses to the subtype and the clinical syndromes caused by them are revealed. Particularly, BVDV-1a causes reproductive pathology, 1b persists in infected animals, 1c, 1d, 1i, 1p lead to respiratory pathology, 1f is found in PI animals and in case of respiratory pathology, BVDV-2b and 2c are causative agents of reproductive pathologies and systemic infection of animals. Our findings may be useful in studying molecular epizootiology of viruses, in elaborating more accurate diagnostic tests that cover a variety of genetic variants, in creating vaccines, and also in more effective programs for infection control.

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GENERATION OF BIOACTIVE PEPTIDES IN MEAT RAW MATERIALS EXPOSED TO PROTEASES OF DIFFERENT ORIGIN

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Abstract

There are plenty of methods nowadays used for the technological correction of meat raw materials and increasing the functionality of meat products. Among them, the enzymatic hydrolyses using proteases of microbial, plant or animal origin causes considerable interest. Sarcoplasmic and myofibrillar proteins of meat products are subjected to proteolysis during enzymatic treatment, resulting in the peptides generation, including those with high physiological activity and a pronounced therapeutic and preventive effect. Usually they are low molecular weight compounds consisting of several amino acid residues. It should be noted that, unlike drugs, such peptides are able to rapidly penetrate the gastrointestinal tract membranes and further into the bloodstream and the rest of the organism. The aim of this work was to study the bioactive peptides generation in various meat raw materials due to enzymatic hydrolyses by enzymes of animal (pepsin, trypsin) and plant (papain, bromelain) origin. *Bos taurus* and *Sus scrofa* skeletal muscle samples were injected with 5 ml of proteases in 50 g of raw meat. The samples were kept for 40 min at 30 °C for exposure to trypsin and pepsin solutions, 30 min at 30 °C for papain and bromelain. The optimum pH value was not established for the enzymes in the samples, the fermentation process was carried out at the native pH of the raw meat. Humidity in the room was 50-55 %. As a control, the corresponding muscle samples without enzymatic treatment were used. According to the results of a one-dimensional gel electrophoresis under denaturing conditions using sodium dodecyl sulfate (SDS-PAGE), obvious quantitative differences were found in the protein profiles of the studied raw materials, and different profiles were obtained for proteases of different origin. A subsequent two-dimensional isoelectric focusing electrophoresis in an ampholine pH gradient (IEF-PAGE) confirmed the obtained results and revealed the used proteases features. Thus, animal proteases, possessing high specificity, affect only a certain part of muscle proteins, but almost entirely destruct them to small peptides (including low molecular weight) and free amino acids. In addition, a greater proteolytic activity of trypsin compared to pepsin was noted. Plant origin proteases affect the majority of muscle proteins with a sufficiently low specificity and destruct them to many fragments, as evidenced by the presence of numerous new protein fractions on 2D-electrophoregram. Using mass spectrometry (MALDI-TOF MS and MS/MS) some short peptides were detected and identified in samples treated with animal proteases. It was practically not possible to detect short peptides after plant proteases treatment due to the insufficient suitability of mass spectrometry to determine the very low molecular weight of generated peptides. Thus, plant origin proteases generate intermediate fractions of some muscle proteins and bioactive peptides more actively and efficiently. The raw materials processing by proteolytic enzymes, in our opinion, can be regarded as the most effective way to obtain biologically active peptides.

Keywords: proteolytic enzymes, enzymatic hydrolyses, biologically active peptides, one-dimensional gel electrophoresis, SDS-PAGE, two-dimensional electrophoresis, IEF-PAGE, MALDI-TOF, mass spectrometry identification

The increase in meat produced in large modern commercial livestock complexes leads to more meat raw with non-traditional technological characteristics. This actualizes the problem of effective meat processing. E.g., intensive pig exploitation and selection for meatiness, keeping without walking and in large groups, early weaning of piglets, significant fluctuations of the microclimate, etc., exceed the adaptive capabilities of animals, reduce their resistance to technological stress and adversely affect meat quality [1]. Hypodynamia, intensive fattening during commercial growing and breeding for meatiness which leads to disturbances of glycogen metabolism are generally accepted causes of exudativeness and dark sticky meat [2]. Targeted enzymatic modification improves nutritional and biological value of such meat providing its more effective use [3-5].

In the last decade, special attention has been paid to the substances of a protein nature, the biologically active peptides [6]. Their presence in raw materials and finished meat products contributes to better digestion of proteins of animal origin [7-9]. Myofibrillar proteins disintegrate into polypeptides under the action of endogenous muscle enzymes, primarily cathepsin D (at low pH), and further into peptides and free amino acids. Peptides are decomposed by endogenous and microbial enzymes into free amino acids, and the degradation is primarily determined by pH values [10].

The proteolytic activity of lactic acid bacteria strains was studied on meat raw materials, including sarcoplasmic and myofibrillar proteins [11-13]. Thus, under the influence of cells and cell extracts of the strain *Lactobacillus plantarum* CRL 681, originally isolated from meat products, the proteolysis of both sarcoplasmic and myofibrillar proteins occurred with the formation of various peptides of hydrophobic nature. During the proteolysis of myofibrillar proteins, the amount of lysine, arginine, and leucine increased to the maximal level, whereas sarcoplasmic proteins mainly released alanine [11]. Similar study was carried out for *L. curvatus* CECT 904 and *L. sakei* CECT 4808. Both strains expressed proteolytic activity towards sarcoplasmic proteins. Adding cells resulted in peptide degradation, whereas adding cell extracts provided formation of hydrophilic and hydrophobic peptides. In addition, the level of produced free amino acids was higher for *L. sakei* strain [12]. The same authors evaluated activity of *L. casei* CRL 705-derived proteinase and aminopeptidase towards muscle proteins. Proteinases of whole cells cleaved sarcoplasmic proteins to a wide range of peptides; partial hydrolysis was also connected with cell extracts. In mixing cells and cell extracts with sarcoplasmic protein extracts, the peptide profiles changed significantly and production generation of free amino acids was higher [13].

Native amino acid sequences, as well as those formed during the autolysis, enzymolysis of peptides, during thermal and other technological treatments, can be functionally active. Bioactive peptides with hypotensive, opioid, antioxidant, antithrombotic, antimicrobial, immunomodulatory and other biological activities, which have a therapeutic or prophylactic effect on the pathogenesis of a number of diseases, are found and studied [14, 15]. Since most of the currently known bioactive peptides do not penetrate from the digestive tract into the blood, their action is probably mediated through the receptors of the intestinal epithelium or is carried out directly in the intestinal lumen [15, 16]. Such peptides are released and activated during digestion in the gastrointestinal tract or during meat processing [17]. In particular, proteolysis (drying, fermentation) in the course of industrial processing releases ACE-I inhibitors and peptides with

antioxidant activity [18]. The activity of peptides depends on the amino acid composition, molecular weight, chain length, type and charge of the amino acid at the N- and C-terminus, hydrophobic and hydrophilic properties, spatial structure, etc. For example, peptides with higher ability to inhibit the angiotensin-converting enzyme (ACE) usually have aromatic or alkaline amino acids at the N-terminus, a greater amount of hydrophobic and positively charged amino acids at the C-terminus [19]. The relationship between activity and peptide structure is currently under study. Many bioactive peptides of natural origin are structurally different from the peptides resulting from the post-translational modification of proteins. They contain non-protein amino acids (β-alanine, γ-aminobutyric acids), D-amino acids, and alkylated amino acids. H-peptide bonds and ring structures are characteristic of peptides with low molecular weight. Together with pyroglutamic acid residues, they provide protection against the proteases with substrate specificity to peptides containing α-amino acids with normal bonds, which makes it possible to preserve the functionality of the peptide until it is digested [3, 14]. Many peptides containing 2-9, less often up to 25 amino acid residues in a strictly determinate sequence [20], are extracted from meat raw materials, and many of them are obtained by proteolysis with enzymes of various origins.

Pepsin, a member of the peptidase family A1, is the predominant digestive protease in the gastric juice of the vertebrates. Unlike some other endopeptidases, it hydrolyzes only peptide bonds, but not non-peptide amide or ester bonds. When an unpurified myosin light chain was treated with pepsin, an ACE inhibitory octapeptide was detected, which was evaluated as a temporarily effective hypotensive substance [21]. Another peptide, obtained by hydrolysis with pepsin of porcine myosin, retained ACE inhibitory activity after heating myosin B at 98 °C for 10 min [22]. It was proven that bioactive peptides can be obtained not only from myofibrillar proteins but also from regulatory proteins, such as troponin and tropomyosin. In 2003, the ACE-inhibitory peptide was isolated from porcine troponin C, hydrolyzed with pepsin. This peptide showed relatively high resistance to digestive proteases, and it can be expected that it will function in vivo as an antihypertensive agent [23]. In another experiment, after the treatment with pepsin, two new ACE-I inhibitory peptides from porcine skeletal troponin were identified. One of them showed the strongest ACE inhibitory activity among the previously detected peptides obtained from troponin [24]. Trypsin belongs to serine proteinases and is synthesized by the pancreas in the form of an inactive precursor (proenzyme) of trypsinogen. Trypsin hydrolyzes peptide bonds formed by the carboxyl groups of L-arginine and L-lysine. Papain is a non-specific thiol protease and the main component of the milk sap protein of the tropical plant *Carica papaya*. Due to its proteolytic properties, it is used in the food industry to soften meat [25, 26]. Papain (300 U/kg) was used to increase the amount of free amino acids in dry fermented sausages [27]. The antithrombotic activity of papain hydrolysate from defatted pork meat (crude fragments and peptides purified by cation exchange chromatography) was estimated in vivo [28]. After the administration to mice per or, the initial peptide fraction with an average molecular weight of 2500 Da showed antithrombotic activity at a dose of 210 mg/kg of body weight, and the fraction 2517 Da purified by cation-exchange chromatography was active at a dose of 70 mg/kg (its activity was equivalent to the activity of 50 mg/kg aspirin). The plant enzyme bromelain is present in large quantities in fruits, leaves, and stems of plants of the *Bromeliaceae* family, of which pineapple is most known (*Ananas comosus*). Like other proteases, it degrades myofibrillar proteins and collagen, which often leads to the softening of meat [29]. The effect of bromelain, papain and collagenolytic enzyme MCP-01 on beef meat at low temperatures (4 °C) was studied. Using

scanning electron microscopy, the differences were found in the destruction of muscle fibers [30].

In this paper, we established for the first time that in processing beef and pork with enzymes of animal (pepsin, trypsin) and plant (bromelain, papain) origin, the formation of bioactive peptides is most likely when using plant enzymes.

The aim of the work was to study peptide profiles of meat raw, presumably having biological activity, under the action of enzymes of animal and plant origin.

Techniques. Bovine cattle (*Bos taurus*) meat (coxal part) and pig (*Sus scrofa*) meat (carbonate) stored after slaughter for 48 hours at 2±2 °C were used. The effects of the following enzymes were studied: pepsin from the gastric mucosa of pigs (10000 U/g) and trypsin from pancreas of a bull (2000 U/g) (HIMEDIA, India); papain from the milk sap of papaya (1100 U/g) and bromelain from the pineapple stem (1310 U/g) (Sigma, USA). In one-dimensional electrophoresis, the enzymes were used at concentrations of 0.5; 1.5; 2.5 %. The samples in which concentrations were effective were further fractionated by two-dimensional electrophoresis.

The solutions of proteases (5 ml per 50 g of meat raw) were injected into the sample of whole muscle (500 g), then allowed for 40 min at 30 °C for trypsin and pepsin preparations, and for 30 min at 30 °C for papain and bromelain. The fermentation was conducted at the native meat pH under 50-55 % room humidity.

The fractional composition of proteins was analyzed by one-dimensional electrophoresis in polyacrylamide gel (12.5 % SDS-PAGE) in a VE-10 chamber (Helicon, Russia) at a constant voltage of 160 V. When the front reached the dividing gel, the voltage was increased to 180 V and the separation was continued for 4-5 hours. The amount of protein introduced is 20 μg per sample. The samples of animal tissue (1 g) were homogenized; proteins were extracted with 3 ml of distilled water. The resulting mass was transferred into a test Eppendorf tube. The slurry was centrifuged for 8 min at 14000 g. One milliliter of the supernatant was placed into a clean Eppendorf tube and 1 ml of the buffer of preparing samples (with dye) was added. The samples were put in a boiling water bath and heated for 2-3 min at 95-100 °C. Then 10-20 μl mixture was added to the gel slots. The marker was a mixture of 11 recombinant proteins (250, 150, 100, 70, 50, 40, 30, 20, 15, 10, and 5 kDa) (Thermo, USA). Coomassie R-250 (Fisher Bioreagents, England) was used for staining. The protein composition was analyzed using the UniProt Protein Database (http://www.uniprot.org/) [31].

The two-dimensional (2D) electrophoresis was performed according to O'Farrell with isoelectric focusing in an ampholine pH gradient (IEF-PAGE); the subsequent detection of proteins was performed by staining with silver nitrate [32]. When making the preparations for 2D electrophoresis, 100 mg of a crushed sample was homogenized in 2 ml of lysing solution (9 M urea, 5 % mercaptoethanol, 2 % Triton X-100, 2 % ampholines, pH 3.5-10, teflon-glass system). The homogenate was clarified by centrifugation (5 min, 800 g); the supernatant fraction containing the protein extract was used for fractionation in equal volumes (50-75 μl).

After the trypsinolysis, the protein fractions were identified by MALDI-TOF (matrix-assisted time-of-flight laser desorption/ionization) and MS/MS (tandem) mass spectrometry (Ultraflex mass spectrometer, Bruker, Germany) with a UV laser (λ = 336 nm) in the mode of detection of positive ions in the mass range of 500-8000 Da with their calibration by the known peaks of autolysis. To study the spectrum of short peptides (with m/z 1500-5000), 100 mg of the preparation was homogenized in 2 ml buffer (5.8 mg KH_2PO_4; 232 mg $Na_2HPO_4 \cdot 2H_2O$; 2.2 g NaCl; 0.5 ml 10 % Triton X-100; 1.87 g KCl) and fur-

ther diluted with water to 50 ml. The homogenate was clarified by centrifugation (5 min, 800 g). In the supernatant fraction, the spectra of the peptides were determined. The analysis of the mass spectra of tryptic peptides was performed using Mascot software, the Peptide Fingerprint option (MatrixScience, USA) at the 0.01 % accuracy of MH^+ mass determining with a search in the NCBI databases [33, 34].

Results. In bovine meat (Fig. 1, A), enzymes of animal origin slightly increased the amount of proteins of a molecular weight of more than 70 kDa (presumably myosin and α-actinin), indicating proteolytic changes in the actomyosin complex. The proteins of 25-70 kDa did not change significantly. In the zone below 15 kDa, new protein bands occurred, which indicates the accumulation of fragments with low molecular weight via cleaving off from major proteins. The effect of pepsin and trypsin on bovine proteins practically did not differ.

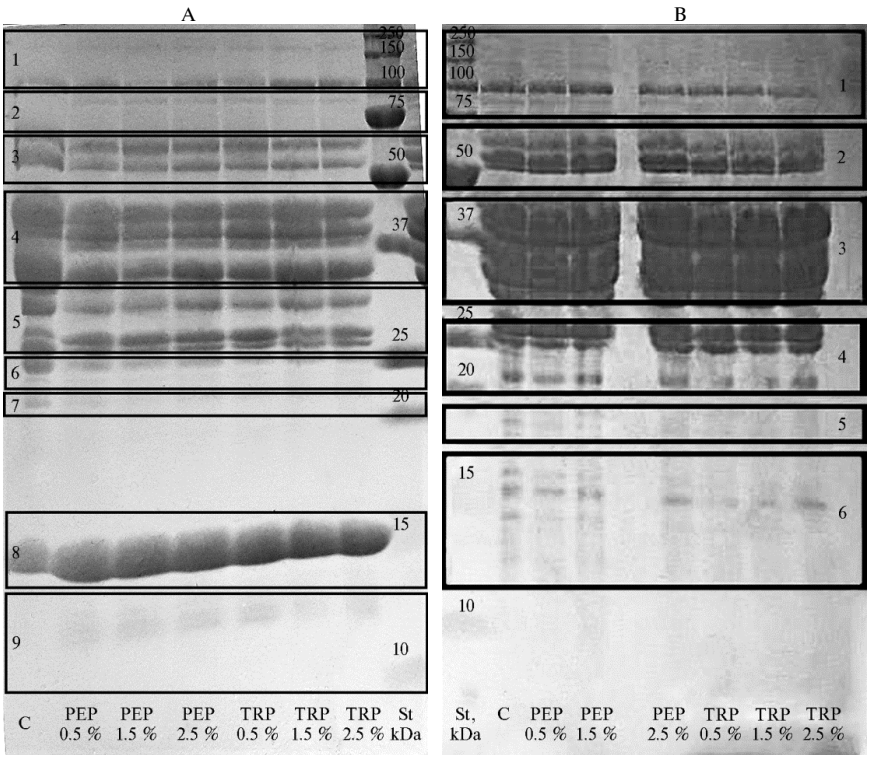

Fig. 1. One-dimensional electrophoresis of proteins derived upon processing extracts of bovine cattle *Bos taurus* (coxal part) (A) **and pig *Sus scrofa* (carbonate)** (B) **skeletal muscles with enzymes of animal origin:** St — protein marker, C — control (without enzymatic processing), PEP — pepsin, TRY — trypsin; 0.5 %, 1.5 %, 2.5 % — the concentration of enzymes.

A: 1 — myosin-10 (222.9-229 kDa); 2 — α-actinin (102-105 kDa); 3 — elastin (64-72 kDa), desmin (53-55 kDa); 4 — α- and β-tubulin (47-52 and 35-52 kDa), α-actin (41.5-42 kDa); 5 — musculoskeletal troponin-T of fast/slow type (30-32 kDa), α/β-tropo-myosin (32.5-32.7 kDa); 6 — musculoskeletal troponin-T of fast type (25-33 kDa), troponin-1 (23-25 kDa); 7 — cofilin 2 (21-22.5 kDa); 8 — hemoglobin (15 kDa); 9 — fragments of high-molecular proteins.

B: 1 — heavy chains of myosin (205-210 kDa), α-actinin (100 kDa), muscle creatine kinase (80 kDa); 2 — elastin (64-66 kDa), α- and β-tubulin (53 and 55 kDa); 3 — G-actin (42 kDa), tropomyosin-1 (39 kDa), troponin-T (35-38 kDa), tropomyosin-2 (32 kDa); 4 — light chains of myosin (16-27 kDa), troponin-1 (23-25 kDa), light chains of myosin-A1 (20.7 kDa); 5 — troponin C (20 kDa), light chains of myosin-2 (18 kDa); 6 — myoglobin (17 kDa), skelemin (15 kDa), fatty-acid-binding protein (14-15 kDa), fragments of myoglobin (8-12 kDa).

In the porcine meat (Fig. 1, B) the content of heavy chains of myosin decreased. A protein of about 18 kDa was significantly degraded (presumably, this corresponds to the light chains of myosin 2). In the samples with pepsin, the intensity of the bands of 14 kDa (presumably ribonuclease) and 8-12 kDa (pre-

sumably α- and β-chemokines), significantly decreased, with trypsin, these bands were absent. It is noted that trypsin has a more pronounced effect on pig meat than pepsin.

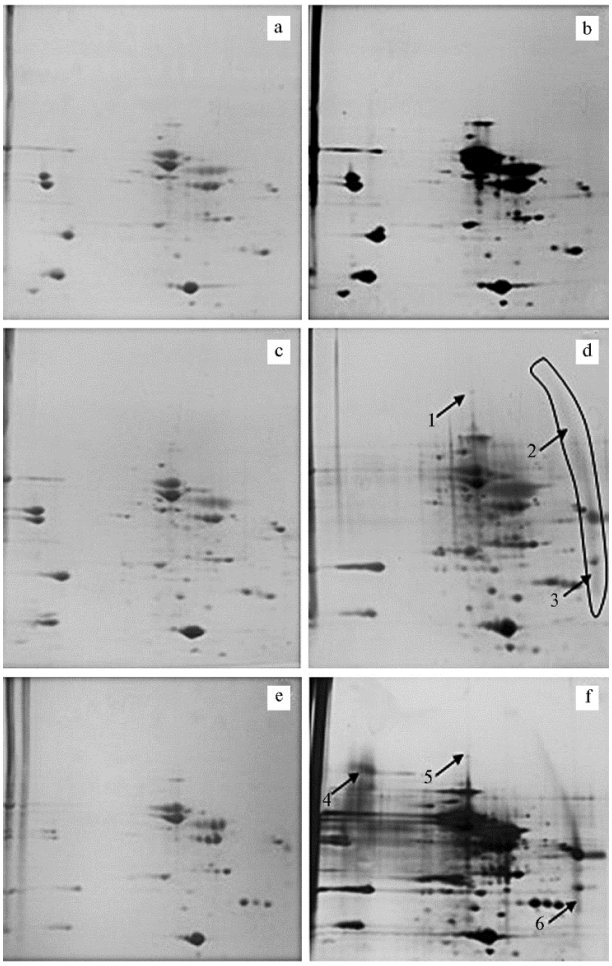

Fig. 2. Two-dimensional electrophoresis of proteins derived upon processing of bovine cattle *Bos taurus* skeletal muscle (coxal part) extract with enzymes of animal origin: a, b — control; c, d — treatment with 1.5 % pepsin; d, e — treatment with 1.5 % trypsin; staining with Coomassie R-250 (left) and silver nitrate (right). The arrows indicate zones of atypical fragments and aggregates of muscle proteins: 1, 5 — track of mitochondrial aconitase 2 fragments (*ACO2* gene product), 2, 3, 6 — aggregate of fragments (60-278 amino acid residues) of isoform 2 of protein 1, containing 4.5 LIM domains (*FHL1*), 4 — fragment (530-1912 amino acid residues) of myosin-1 (*MYH1*).

The computer densitometry upon 2D electrophoresis with Coomassie R-250 staining showed a 15-37 % decrease in the total proteins. A more sensitive staining with silver nitrate made it possible to identify a number of intermediate fragments as bands of degraded tracks of major proteins, e.g. muscle creatine phosphokinase, aldolase A, mitochondrial aconitase, and β-enolase. Moreover, by 2D electrophoresis (Fig. 2), the fragments of the C-terminus of the heavy chain of myosin were detected, normally aggregating at the start of the gel at IEF, which indicates proteolytic changes in the actomyosin complex.

The presence of protein track in the alkaline zone (in the oval, see Fig. 2), which covers the weight range from 400 to 5 kDa, attracts the particular attention. To identify its nature, the fragments of this track were examined in the upper and lower zones. In all cases, it was identified as protein 1 isoform 2, containing 4.5 LIM domains, with a weight of not more than 32 kDa. However, the observed distribution of fragments was significantly wider. That is, during proteolytic processing, this protein apparently retained the core part (positions 60-278) with the formation of aggregates that form a non-ordinary electrophoretic track.

Since the 2D electrophoresis revealed a decrease in the amount of protein material after the exposure to proteases, we studied the changes in the spectra of short peptides (of 8 to 40 amino acid residues) in such samples (Fig. 3). After treatment with proteases, the spectra changed significantly (see Fig. 3). In the control and upon pepsinization, the resultant peptides consisted of 12-40 amino acid residues, and exposition to trypsin led to a pool of peaks, corresponding to lower weights, mainly of 1500-3000 m/z (12-24 amino acid residues) (Table 1).

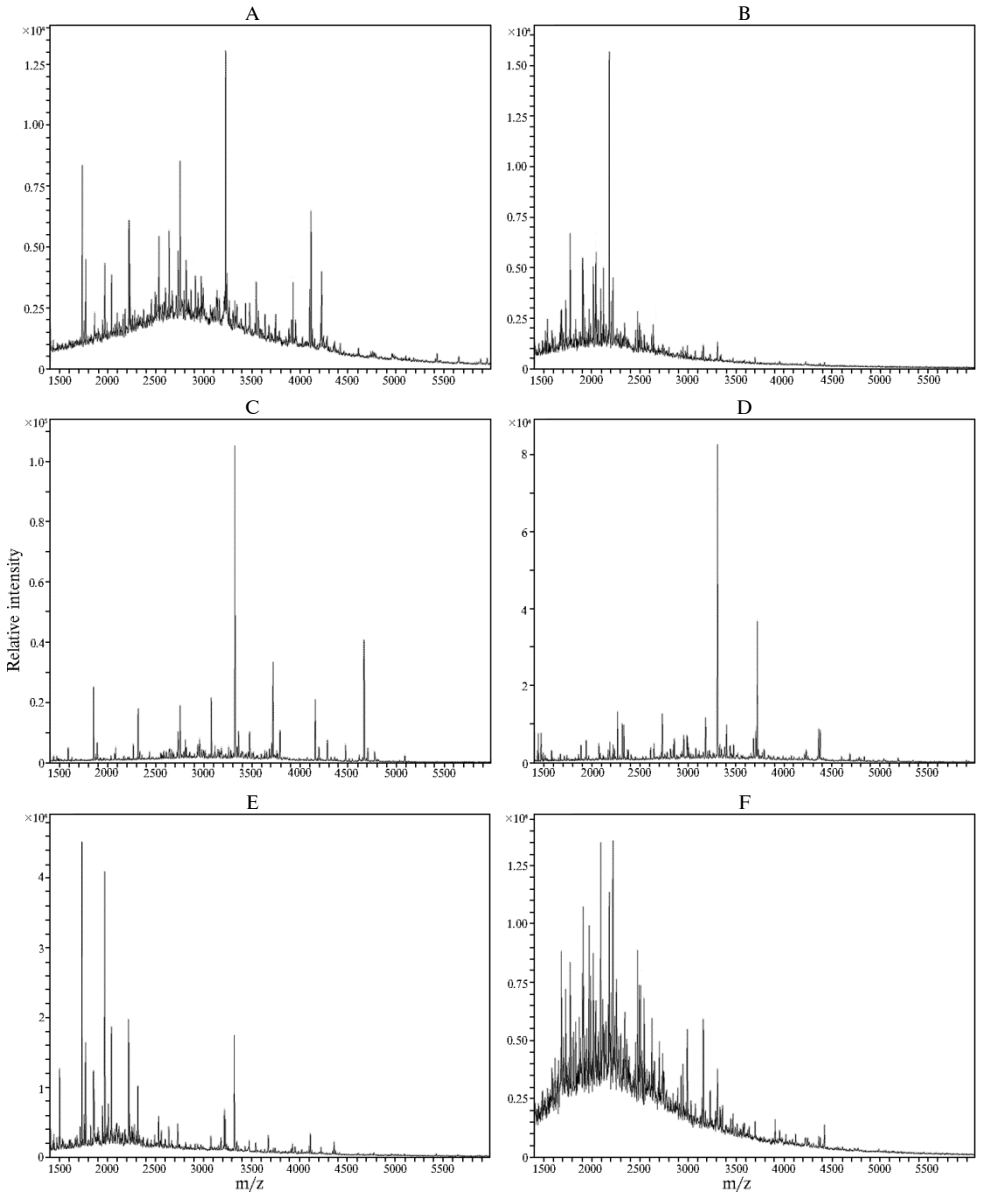

Fig. 3. Tryptic peptide profiles of bovine cattle *Bos taurus* (coxal part) (left) **and pig *Sus scrofa* (carbonate)** (right) **skeletal muscle extracts in the m/z 1500-5000 range:** A, B — control, C, D — 1.5 % pepsin, E, F — 1.5 % trypsin. For description of the identified peptides with m/z 1853.8, 3077.4, 4157.8, 4665.5, 3322.8, 1940.9 and 1952.9 see Table 1.

1. Mass-spectrometric identification (MALDI-TOF MS and MS/MS) of short peptides (m/z 1500-5000) **derived upon processing meat raw extracts with proteases of animal origin**

No. in the Protein Database NCBI	S/M/C	m/z (position in the AA sequence)	Amino acid sequence of identified peptides/proteins
		Beef (pepsin)	
		Myoglobin peptides (*gene MB*)	
NP 776306.1	144/7/100	1853.8 (139-154)	FRNDMAAQYKVLGFHG
		3077.4 (2-30)	GLSDGEWQLVLNAWGKVEADVAGHGQEVL
		4157.8 (71-107)	TALGGILKKKGHHEAEVKHLAESHANKHKIPVKYLEF
		Fructose bisphosphate aldolase peptides A (*gen ALDOA*)	
NP 001095385.1	92/4/26	4665.5 (31-70)	IRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGNTVL
		3322.8 (2-2)1	PHQYPALTPEQKKELCDIAH

Continued Table 1

Beef (trypsin)			
Fructose bisphosphate aldolase peptides A (gene ALDOA)			
NP 001095385.1	86/6/30	3322.8 (2-21)	PHQYPALTPEQKKELCDIAH
Pork (pepsin)			
Peptides of chain A L-lactate dehydrogenase (LDHA)			
NP 001165834.1	124/7/25	1940.9 (92-109)	VVITAGARQQEGESRLNL
Pork trypsin			
Calsequestrin peptide-1 (CASQ1)			
Q05JF3	58/2/51	1952.9 (35-47)	EEGLDFPEYDGVD

Note. AA — amino acids. S/M/C: **S**core (measure of compliance), **M**atch peptides (number of coincident peptides), **C**overage, % (covering the complete amino acid sequence of the protein with the identified peptides).

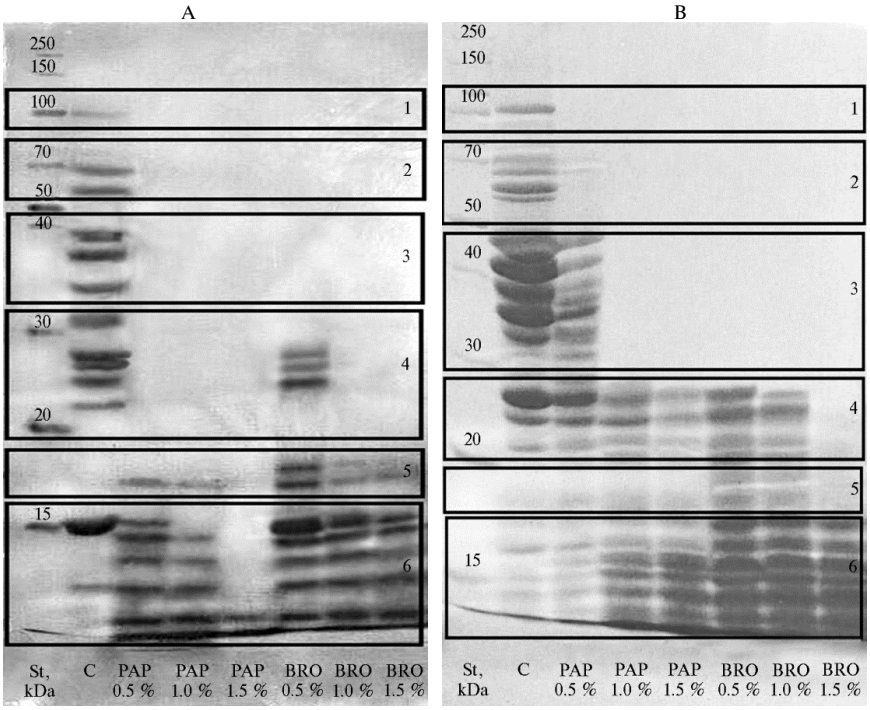

Fig. 4. One-dimensional electrophoresis of proteins derived upon processing extracts of bovine cattle *Bos taurus* (coxal part) (A) **and pig *Sus scrofa* (carbonate)** (B) **skeletal muscles with enzymes of plant origin:** St — protein marker, C — control, PAP — papain, BRO — bromelain; 0.5 %, 1.5 %, 2.5 % — the concentration of enzymes.

A: 1 — α-actinin (103-104 kDa); 2 — elastin (64-66 kDa), desmin (53-55 kDa); 3 — aldolase A (39.5 kDa), musculoskeletal troponin-T of fast type (35-38 kDa), β-tropomyosin (32.5-32.7 kDa); 4 — musculoskeletal troponin-T of slow type (28.6-32 kDa), troponin-1 (23-25 kDa), cofilin (21-22.5 kDa), light chains of myosin-A1 (20.7 kDa); 5 — troponin C (20 kDa), light chains of myosin-2 (18 kDa), myoglobin (17 kDa); 6 — skelemin (15 kDa), protein binding fatty acids (14-15 kDa), myoglobin fragments (8-12 kDa).

B: 1 — α-actinin (100 kDa), muscular creatine kinase (80 kDa); 2 — myoalbumin (70 kDa), elastin (64-66 kDa), catalase (58 kDa), β-tubulin (55 kDa); 3 — G-actin (42 kDa), muscular aldolase (40 kDa), tropomyosin-1 (39 kDa), troponin-T (35-38 kDa), tropomyosin-2 (32 kDa); 4 — light chains of myosin-1 (16-27 kDa), troponin-1 (23-25 kDa), light chains of myosin-A1 (20.7 kDa); 5 — troponin C (20 kDa), light chains of myosin-2 (18 kDa), myoglobin (17 kDa), skelemin (15 kDa); 6 — protein binding fatty acids (14-15 kDa), myoglobin fragments (8-12 kDa).

In the pork, under the influence of plant enzymes (Fig. 4, B), there was significant destruction of proteins with molecular weights above 50 kDa (with papain) and above 30 kDa (with bromelain). The protein fragments of less than 20 kDa accumulated intensively, especially in the samples with bromelain. According to the effect, the plant proteases differed noticeably from the animal ones, probably because of their much lower specificity to potentially attacked proteins.

The protein profile of bovine cattle (Fig. 5) underwent significant destruction: almost all proteins with a weight of more than 20 kDa were destroyed.

The protein fragments significantly accumulated in the range below 20 kDa. At the same time, papain worked more efficiently.

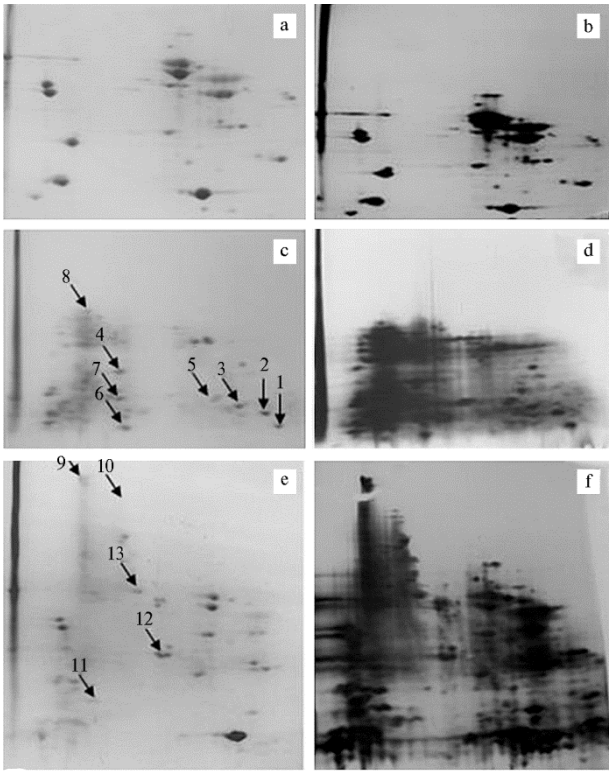

Fig. 5. Two-dimensional electrophoresis of proteins derived upon processing extract of bovine cattle *Bos taurus* skeletal muscle (coxal part) with enzymes of plant origin: a, b — control; c, d — treatment with 0.5% papain; d, e — treatment with 0.5 % bromelain; staining with Coomassie R-250 (left) and silver nitrate (right). The arrows indicate proteins: 1, 2, 3 — fragments of myoglobin (product of the *MB* gene), 4 — a mixture of fragments of actin (*ACTG2*) and myosin 1 (*MYH1*), 5 — canonical myoglobin (*MB*), 6 — fragment of actin (*ACTA1*), 7 — fragment of myosin 7 (*MYH7*), 8, 9, 10 — fragment of myosin 2 (*MYH2*), 11 — musculoskeletal light chain of myosin 1/3 (*MYL1*), 12 — fragment of myosin 1 (*MYH1*), 13 — mixture of fragments of myosin 2 (*MYH2*) and myosin 1 (*MYH1*).

When treating beef with bromelain, the fractions of the α- and β-tropomyosins were detected in a residual amount and myoglobin remained practically intact. Under the influence of papain, the total gel staining remained high due to the appearance of a heterogeneous mixture of fragments of high molecular weight (15-60 kDa). When treated with papain, the fragments of different types of heavy chains (MYH1, MYH2, and MYH7) of myosin were detected, localized in the muscle fibers of the fast and slow types. The action of bromelain turned out to be more specific for fast-type fibers (fractions of MYH7 fragments were not found). The number of bands in one-dimensional gel electrophoresis changed significantly during the processing of pork and beef with plant enzymes (Table 2).

In the extracts treated with plant proteins, spectrum of short peptides (Fig. 6) showed the appearance of additional peaks. When treated with papain, the peptides detected were of m/z up to 4500, with bromelain these were no more than of 3000 (mostly up to 2500). We have constructed the amino acid sequence of the peptide of *Bos taurus* actin family (without additional detail and reference to a gene) resulted from the treatment of beef with bromelain.

2. The number of protein bands on the one-dimensional electrophoregrams derived upon processing cattle *Bos taurus* (coxal part) and pig *Sus scrofa* (carbonate) skeletal muscle extracts with different concentrations of plant enzymes

Molecular weight, kDa	Control 1	Pepsin, %			Trypsin, %			Control 2	Bromelain, %			Papain, %		
		0.5	1.5	2.5	0.5	1.5	2.5		0.5	1	1.5	0.5	1	1.5
						Beef								
> 250	–	–	–	–	–	–	–	–	–	–	–	–	–	–
150-250	1	1	1	1	1	1	1	1	–	–	–	–	–	–
100-149	1	1	1	1	1	1	1	1	–	–	–	–	–	–
70-99	–	1	1	1	1	1	1	4	–	–	–	–	–	–
50-69	2	2	2	2	2	2	2	3	–	–	–	2	–	–
37-49	3	3	3	3	3	3	3	3	–	–	–	5	–	–

Continued Table 2

25-37	6	6	6	6	6	6	6	4	1	1	–	4	2	1
20-24	2	2	2	2	2	2	2	2	2	2	2	3	2	2
15-20	–	–	–	–	–	–	–	4	6	6	4	5	5	5
10-15	2	4	4	4	4	4	4	2	2	2	2	2	2	2
					Pork									
> 250	1	–	–	–	–	–	–	1	–	–	–	–	–	–
150-250	1	1	1	1	1	1	1	–	–	–	–	–	–	–
100-149	1	1	1	1	1	1	1	–	–	–	–	–	–	–
70-99	2	2	2	2	2	2	2	2	–	–	–	–	–	–
50-69	3	3	3	3	3	3	3	1	–	–	–	–	–	–
37-49	3	3	3	3	3	3	3	2	–	–	–	–	–	–
25-37	6	6	6	6	6	6	6	5	3	–	–	–	–	–
20-24	2	2	2	2	2	2	2	2	–	–	–	–	–	–
15-20	3	2	2	1	1	–	–	1	3	3	3	2	1	–
10-15	4	4	4	4	3	3	3	1	4	4	4	4	4	1

Note. Controls 1 and 2 are non-fermented raw materials (for the variant with enzymes of animal and plant origin, respectively). Dashes indicate that there are no protein fractions in the specified molecular weight range, in which obvious changes have occurred.

The peptide was conservative for actins and corresponded to the positions 244-257 of amino acid residues in the canonical musculoskeletal actin of *Bos taurus* (*ACTA1* gene), the m/z position 2066.0 (I/LPDGQVI/LTI/LNERF). Obviously, short peptides in this case should be identified using multidimensional chromatography, which is more adapted to work with short peptides.

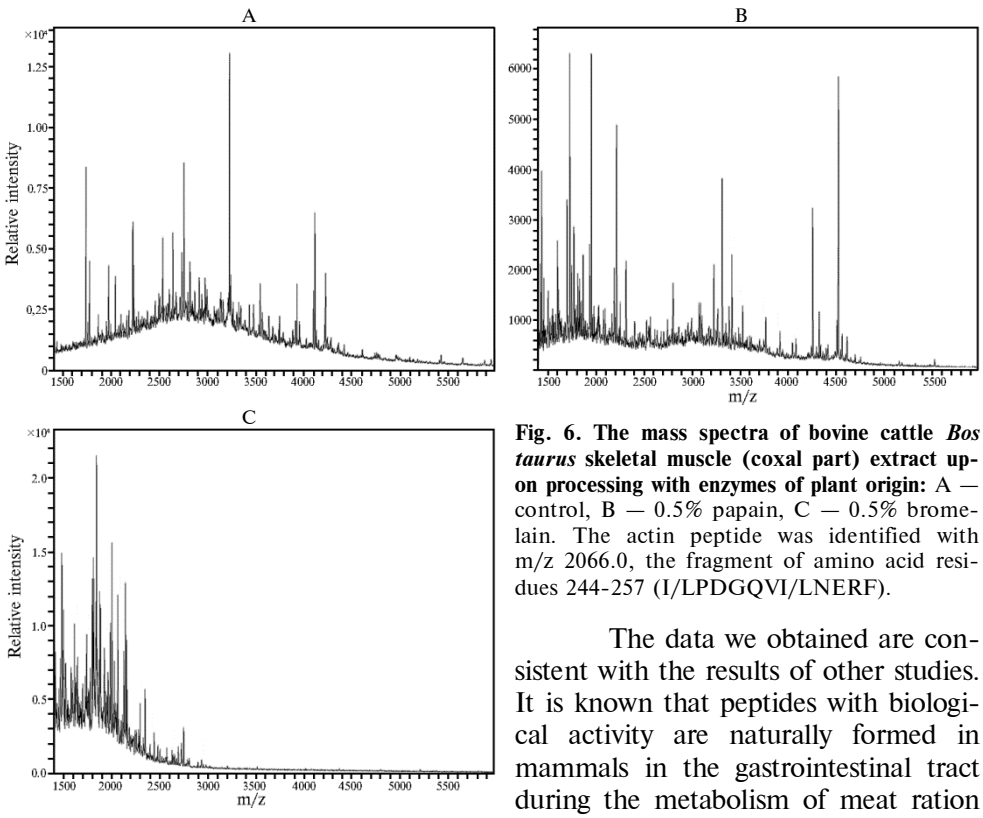

Fig. 6. The mass spectra of bovine cattle *Bos taurus* skeletal muscle (coxal part) extract upon processing with enzymes of plant origin: A — control, B — 0.5% papain, C — 0.5% bromelain. The actin peptide was identified with m/z 2066.0, the fragment of amino acid residues 244-257 (I/LPDGQVI/LNERF).

The data we obtained are consistent with the results of other studies. It is known that peptides with biological activity are naturally formed in mammals in the gastrointestinal tract during the metabolism of meat ration proteins under the influence of digestive enzymes secreted in the small intestine [35-38]. To generate such potentially functional peptides, commercial exogenous proteinases derived from animal tissues (pepsin and trypsin), plants (papain, ficin and bromelain) and microbial sources (Alcalase®, Flavourzyme®, Neutrase®, collagenase or proteinase K) are commonly used to mimic the gastrointestinal digestion [37, 39, 40]. For the production of some biologically active peptides, enzymatic hydrolysis of collagen from meat or by-products of slaughter (cuttings, organs, hemoglobin), in addi-

tion to meat raw materials, is carried out [41]. T. Lafarga et al. [42] studied the release of potential biologically active peptides angiotensin-I-converting enzyme (ACE-I, EC 3.4.15.1), renin (EC 3.4.23.15) and dipeptidyl-peptidase IV (DPP-IV, EC 3.4.14.5) from bovine and porcine proteins, including hemoglobin, collagen and serum albumin. These proteins commonly found in meat by-products (bones, blood, and meat cuttings) play the key roles in controlling hypertension and the development of type 2 diabetes and other diseases associated with metabolic syndrome. The new peptides included the ACE-I inhibitory tripeptide Ile-Ile-Tyr and the inhibitory DPP-IV tripeptide Pro-Pro-Leu, corresponding to the sequences of positions 182-184 and positions 326-328 of porcine and bovine serum albumin, which can be released after hydrolysis, respectively, by papain and pepsin. In other studies [43], the inhibitory and antioxidant activity of angiotensin-I-converting enzyme (ACE-I) of sarcoplasmic proteins isolated from the pectoral muscle (Pectoralis profundus) of bovine cattle (*Bos taurus*) and hydrolyzed by papain was determined. Sarcoplasmic protein hydrolysates were subjected to membrane ultrafiltration and 10 kDa and 3 kDa filtrates were obtained. As a result, 11 peptides were characterized from the total hydrolysates fraction, 15 from the fractions of filtrate 10 kDa, 9 peptides from the fractions of filtrate 3 kDa. The authors identified the similarities between the amino acid sequences of the peptides identified by them and the known antioxidant and inhibitory ACE-I peptides described in the BIOPEP database. Pork myofibrillar proteins were studied as promising sources of biologically active peptides [44]. After simulating gastrointestinal digestion of certain porcine myofibrillar proteins with the use of pepsin, trypsin and chymotrypsin, peptide sequences inhibiting dipeptidyl peptidase IV were most frequently found among intact proteins. All in all, a total of 399 peptides were detected with various activities (enzyme inhibition, antioxidant, hypotensive, stimulating, antiamnesic activity, regulation of body functions).

Thus, we identified a quantitative decrease in the components of beef and pork protein profiles under the action of proteases of various origins. The proteases of animal origin act on the raw materials more evenly and more specifically than the plant ones. For the plant proteases, the formation of intermediate fragments is characteristic, which was not observed in the samples with proteases of animal origin. In all cases, new protein fragments are found, including the ones with low molecular weight, as confirmed by mass spectrometry. By tandem mass spectrometry, we identified some candidate fragments which may have biologically active properties. The greatest changes in the protein profile of meat raw materials are in treating with proteases of plant origin, therefore, in this case, the formation of biologically active peptides can be anticipated.

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LIPID COMPOSITION OF MUSCLE AND FAT TISSUES OF DUROC PIGS (*Sus scrofa domesticus* Erxleben, 1777) — FEATURES AND CORRELATIONS

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Abstract

A key direction in animal genetics and animal breeding is currently the study of metabolic characteristics of animals in combination with their genotyping, which leads to the development of the new specific markers for prediction the individual phenotypic characteristics of animals based on correlation studies. This would further make possible to create new methods for animal phenotyping using lipid analysis techniques to assess the complex effect of environmental and genetic factors (D.P. Lo Fiego et al., 2002; D.P. Lo Fiego et al., 2005; R. Rossi et al., 2002). Data obtained as a result of the analysis of the genetic features of the animal and its metabolic characteristics make it possible to create predictive models for accurate phenotyping of animals and their offspring. In this work we have for the first time performed the comparative non-targeted mass-spectrometry study of the lipid composition of muscle and adipose tissue in Russian Duroc pigs using positive ion registration mode. The aim of the work was to carry out lipidomic analysis of the pig muscle and adipose tissue as an input for predictive models for animal phenotyping. The study was carried out on the samples of adipose and muscle tissue collected post-mortem from 150-180 day-old Duroc boars (n = 9). Samples were taken from three regions of the longest back muscle, three regions of the biceps femoris, and two regions of subcutaneous dorsal fat (72 samples in total). Analysis of the lipid compounds was performed by liquid chromatography coupled with high-precision time-of-flight mass spectrometry, preceded by the methyl tert-butyl ether and methanol extraction of lipids. Type of ionization used was electrospray. A total of 844 mass spectrometry peaks satisfied the quality criteria and were used for the statistical analysis. Peaks were annotated using the LIPID MAPS database search (http://www.lipidmaps.org), with an accuracy of 10 ppm. Statistical analysis shows significant differences in the Pearson correlation for adipose and muscular tissue samples compared or the same tissue samples compared. Correlation coefficients between lipid patterns of adipose and muscular tissue samples are lower (from 0.48 to 0.86, r = 0.69 on average with 95 % confidence interval from 0.61 to 0.79). Correlation coefficients between lipid patterns in two samples of muscle or adipose tissue are higher (from 0.73 to 0.99, r = 0.93 on average with 95 % confidence interval from 0.86 to 0.97). Unpaired t-test shows differences at p-value < 0.01 Data clustering confirms the difference between muscle samples and subcutaneous fat samples. The main classes of lipids detected in the samples were triglycerides (TAG), diglycerides (DAG), phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidic acids (PA), phosphatidylinositols (PI), and lysophosphatidylcholines (LPC). We have found that adipose tissue samples are enriched in triacylglycerols, while muscle tissue samples are enriched in phospholipids. To summarize, we have identified the main lipid types present in different regions of muscle and adipose tissue of pigs, and revealed the similarities and differences in the lipid composition between the two analyzed tissue types, as well as between two different types of

muscles (biceps femoris and longisimus dorsi), and also between muscle and fat tissues. Considering the results obtained in this work we may conclude that liquid chromatography coupled with high-precision time-of-flight mass spectrometry efficiently produces accurate and reproducible lipidomes data. These data may be used in animal breeding, in the search for new genetic markers associated with economically important traits and in breeding programs to evaluate the traits determined by lipid composition.

Keywords: lipidome, animal phenotyping, *Sus scrofa domesticus*, pigs, Duroc boars, high-performance liquid chromatography, mass spectrometry, muscle tissue, adipose tissue

The development of molecular-genetic methods opens up new possibilities for accelerating animal breeding. Since the 2000s, genomic selection has been replacing traditional selection on the basis of BLUP-AM (Best Linear Unbiased Prediction — Animal Model) [1]. The introduction of a genomic assessment system in dairy cattle breeding increased the accuracy of predicting the breeding value of young animals by 15-25 % [2]. In modern animal breeding, the technologies of genotyping of tens and hundreds of thousands of single nucleotide polymorphisms (SNPs) are developed and applied; however, the question of their relationship with certain phenotypic traits often remains open [3]. The synthesis of the most important compounds in the body of an animal directly depends on the presence of mutations in the genes of the corresponding enzymes. In turn, enzyme activity may be associated with certain SNPs and manifest itself in the relative amount of metabolites of a certain type. Often, the function of a defective enzyme is performed by another related enzyme based on the compensating biochemical reactions. A study on the diversity and representation of metabolites in biological samples will help to clarify the issue of the participation of certain SNPs in regulating the activity of biochemical processes in an organism [4], and in the future, to identify associations between SNPs and certain economically valuable traits. This is the reason for the importance of modern high-performance technologies of metabolome research for the development of genomic breeding methods in animal farming.

In pigs, the metabolites that are involved in the formation of the main physicochemical and organoleptic properties of muscle and adipose tissue, which form the indicators of the quality of meat raw materials, are of the greatest interest [5]. The quality and nutritional value of meat depend on its chemical composition influenced by both genetic and paratypical factors. The nutritional value of pork is determined by the fatty acid composition, in particular, the quantitative ratio of free and bound long-chain fatty acids in muscle and adipose tissue.

The composition of intramuscular fat is influenced by several main factors: genetic characteristics, sex, the ratio of body weight and age of an animal, and the diet composition [6]. In pigs, the lipid composition of the tissues, reflecting the phenotypic characteristics of a specimen, directly depends on the method of animal feeding and the individual characteristics of the digestion of the fodder [7]. The composition of the diet also has a great influence [8]. For example, an increased content of polyunsaturated fatty acids in the fodder may increase their content in muscle tissue [9]. It is known that linoleic and linolenic acids coming with the fodder are metabolized in the liver with the formation of polyunsaturated fatty acids that are sensitive to the oxidative process. These changes in the fatty acid composition of intramuscular fat, due to the composition of the diet, can increase the sensitivity of meat to the effects of oxidizing agents [9]. The lipid composition of muscle and adipose tissue, which determines the qualitative characteristics of meat, can be considered as a unique individual characteristic of an animal, reflecting not only its genetic predisposition to assimilate and synthesize lipids but also phenotypical signs [10, 11].

The pool of lipids (lipidome) presented in the muscle and adipose tissue of pigs, in addition to the known fatty acids, includes cholesterol esters, triacyl-,

diacyl- and monoacylglycerols, free cholesterol and its intermediates, as well as various classes of phospholipids, including phosphatidylethanolamines, etc. [12]. It should be noted that the effect of the characteristics of absorbing fatty acids on the composition of muscle and fatty lipidomes is most reflected in the phospholipid fraction, as well as in the length and degree of saturation of fatty acid chains in triacylglycerides [12-14].

To assess the phenotypic lipid and other metabolic characteristics in mammals, the methods of nuclear magnetic resonance spectroscopy and gas or liquid chromatography combined with mass spectrometry detection are most often used [3, 15-18]. Attention is paid to mass spectrometry as the main method of lipid detection, and in particular liquid chromatography coupled with mass spectrometry which makes it possible to simultaneously identify up to several tens of thousands of compounds in a single biological sample [19].

The current trends in selection research in modern pig breeding are assessing the metabolic characteristics of animals in combination with whole-genome genotyping, detecting specific markers to predict an individual phenotype based on the identified correlations, and developing the methods of phenotyping using lipid analysis technologies to assess the complex influence of factors causing phenotypic features of an animal [7, 20, 21]. A quantitative analysis of lipids in the muscle and adipose tissue of pigs in combination with genotyping can be the basis for determining the genes associated with the lipid composition of tissues, which will optimize selection (in particular, for meat quality traits).

In this work, we for the first time applied liquid chromatography with mass-spectrometric detection in the registration mode of positive ions for a detailed study of the lipid profiles of muscle and adipose tissue (molecular phenotyping) in Duroc pigs of domestic reproduction. The similarities and differences in the composition and content of lipids between the studied tissue types were determined; in particular, it was shown that the samples of muscle tissue are enriched in phospholipids, whereas an increased content of triacylglycerols is typical of adipose tissue.

The goal of the study is the lipid analysis of muscle and adipose tissue of pigs using ultra-high performance liquid chromatography—high-precision time-of-flight mass spectrometry (UPLC-MS) to further create predictive models of animal phenotyping.

Techniques. In Duroc boars of domestic reproduction grown on automated feeding stations GENSTAR (Cooperl, France) (CGTs OOO, Verkhnaya Khava, Voronezh Region) and aged 150-180 days (n = 9, carcass weight 89.4±5.67 kg), tissue samples were taken post mortem from the longest back muscle at three points (the 5th-6th rib, LM1; 8th-9th rib, LM2; belt, LM3), biceps femoris at three points (upper part, BF1; middle part, BF2; lower part, BF3), as well as subcutaneous dorsal fat at two points (the 5th-6th ribs, inner and outer layers, respectively scat1 and scat2). A total of 72 samples were analyzed. The tissues were dissected no later than 10 min after the slaughter of an animal. To reduce the activity of enzymes, the samples were taken at a temperature not higher than 4 °C and immediately frozen in liquid nitrogen vapor (–196 °C). The biomaterial was stored and transported at a temperature not higher than –80 °C. For the analysis, 130-145 mg of muscle tissue and 50-65 mg of adipose tissue from each point were used.

The solutions used during extraction were preliminary cooled to 0 °C. The extraction method is based on the use of mixtures of methyl tert-butyl ether (MTBE) with methanol (MeOH) (3:1, v/v) and methanol with water (1:3, v/v) (Scharlau, Spain). Oleic acid $^{13}C_{18}$, palmitic acid $^{13}C_{16}$, stearic acid $^{13}C_{18}$ (Sigma-Aldrich, Germany); 15:0-18:1-d7-diacylglycerol, cholesterol (D7) (Avanti

Polar Lipids, Inc., USA) were isotope-labeled internal standards added at a concentration of 3 μg/ml to the MTBE:MeOH solution. MTBE:MeOH extraction mixture (1000 μl) was added to the sample aliquots, and the tubes were placed in a Precellys® Evolution homogenizer cooled to 7 °C (Bertin Technologies, France). Three cycles of 30 s at 10000 rpm with a 10 s break were used to homogenize muscle tissues. The samples were vortexed for 5 s, transferred to an ultrasonic bath filled with ice (Sonorex Super RK 103 H, BANDELIN electronic GmbH & Co. KG, Germany) and kept for 30 min at 3000 rpm and 4 °C; then the ultrasound treatment was repeated. From each tube (Precellys, Bertin Technologies, France), the liquid phase was carefully recovered and transferred to new tubes. The MeOH:H_2O extraction mixture (700 μl) was added to each tube and vortexed for 5 s. The samples were centrifuged for 10 min at 15000 rpm and 4 °C. After the centrifugation, 200 μl were recovered from the upper phase into the Safe-lock tubes (Eppendorf AG, Germany). The samples were dried with an open lid for 1.5 h in a vacuum centrifuge concentrator (Concentrator plus, Eppendorf AG, Germany) at 30 °C and 1400 rpm (vacuum 20 hPa, V-HV mode). The dry extracts were stored at a temperature not higher than –80 °C.

To prepare the extracts for mass spectrometry analysis, 200 μl of acetonitrile:isopropanol mixture (70:30, v/v) (LC-MS purity) were added to each sample and vortexed for 5 s. The samples were placed in an ultrasonic bath for 10 min. Then they were shaken at 4 °C and 3000 rpm for 10 min. The solutions were centrifuged for 10 min at 15000 rpm and 4 °C. The obtained lipid extracts were diluted by adding acetonitrile:isopropanol mixture (70:30, v/v) (1:50 for muscle tissue and 1:100 for adipose tissue). Prior to the analysis, the 100 μl aliquots of the resultant solutions were transferred to mass spectrometry vials and loaded into the autosampler of a chromatograph.

The lipid extracts were analyzed using UHPLC/Q-TOF-MS, an ultra-high performance liquid chromatography (UPLC) (Acquity I-class, Waters, USA) coupled to a high-resolution time-of-flight mass spectrometry Q-TOF (Maxis Impact II, Bruker Daltonik GmbH, Germany). To separate the extracts, the reversed-phase chromatography was used (an analytical column Acquity UPLC BEH C8 2.1×100 mm, the size of particles 1.7 μm, Waters, USA; a guard column Acquity UPLC BEH C8 VanGuard 2.1×5 mm, 1.7 μm, Waters, USA). The eluent A was 0.1 % formic acid, 10 mM ammonium acetate and 100 % water (LC-MS grade), the eluent B was 0.1 % formic acid, 10 mM ammonium acetate in the acetonitrile:isopropanol (70:30, v/v) (LC-MS grade). The eluent gradient was as follows: 0 min — 45 % A, 1st min — 45 % A, 4th min — 20 % A, 12th min — 15 % A, 15th min — 0 % A, 19.50 min — 0 % A, 19.51 min — 45 % A, 24th min — 45 % A (A + B = 100 %), at a flow rate of 0.4 ml/min. The analytical column was thermostated (60 °C), the autosampler temperature was 4 °C; the volume of injected samples was 3 μl.

Electrospray was used to ionize the substance and deliver it to the mass spectrometer. The drying and spraying gas was N_2, supplied by the generator (flow rates of 2 and 6 l/min, respectively); the voltage of the ionizing spray was 4000 V, with nitrogen was used as the collision gas in the collision cell at a pressure of 0.04 bar. The desolvation line temperature was 180 °C, the radio-frequency of the ion focusing was 300 Vpp in the focusing funnels and 1000 Vpp in the collision cell, the ion accumulation time was 10 ms, and the ion transmission time was 45 ms. The ion spectra were recorded in positive and negative full-scan mode. Mass spectrometry data was processed using the functions from the specialized software packages IPO [22], XCMS [23] and Camera in the R programming language (http://www.r-project.org).

To analyze the mass spectrometry data, the Pearson correlation, Stu-

dent's *t*-test (identifying differences for two samples of the same size with the assumption of equal variances), and the Benjamini-Hochberg method [24] were used to reduce the number of false-positive results for multiple testing. According to the Benjamini-Hochberg method, for p_1, p_2, ..., p_M with the number of conducted tests M, without loss of generality, we can take $p_1 \le p_2 \le ... \le p_M$. Taking a chosen significance level Q, the critical value $c_i = (iQ/M) \cdot N$ is calculated for each p_i and the largest p-value p_k ($p_k < c_k$) is found. Then all p_i for $i \le k$ are considered statistically significant even at $p_i > Q$.

Using the principal component analysis, the clusters of similar samples were identified. The principal component analysis [25] allows constructing a linear transformation that transmits the data into the space with a smaller dimension, where the new coordinate axes are called the main components. The first main component was chosen so that the data dispersion was maximum along it, and the second one was orthogonal to the first one with the maximum possible dispersion along the axis. The subsequent components are chosen in such a way as to be orthogonal to all previous components with maximum data dispersion along them. To calculate the main components, the "scikit-learn" package was used of the version 0.19.1 for the Python 3.5 programming language.

Results. A schematic representation of the sampling points and the results of the studies of their lipid composition are presented in Figure 1, A. A total of at least 10000 chromatographic and mass-spectrometric peaks were detected, each of which can correspond to specific lipid compound.

The peaks were annotated with an accuracy of 10 ppm using the LIPID MAPS database (http://www.lipidmaps.org) [26]. For this, the M+H, $M+NH_4$, M+Na adducts were chosen. A total of 1397 peaks were annotated by at least one lipid, many peaks received multiple annotation, the maximum number of lipids assigned to one peak was 134. In the subsequent work, we used only annotated peaks. After the annotation and filtration of peaks by retention time in the chromatographic column (from 0.6 to 19 min), as well as the exclusion of probable contaminants, 844 peaks remained.

The comparison of lipid profiles showed significant differences between the adipose and muscle tissue samples, whereas the differences between the two different muscle or adipose tissue samples were not significant. The values of the Pearson correlation coefficients for lipid composition for all pairs of samples are presented in Figure 1, B. The dark left upper square and lower right squares show that muscle tissue was characterized by a strong correlation in the pairs of the samples. The same is true for adipose tissue. The correlations found when comparing different types of tissues are significantly weaker. Thus, the correlation coefficients between the pairs of samples from the same tissue (muscle tissue samples were combined into one group) were, on average, 0.93 (the range is 0.73-0.99; 95 % confidence interval is from 0.86 to 0.97). When comparing the pairs of samples of different tissues, the correlation coefficients on average amounted to 0.69 (the range is 0.48-0.86; 95 % confidence interval is from 0.61 to 0.79). The confidence intervals do not overlap; therefore, the correlations between the samples of the same tissue along the lipid profiles are significantly stronger than between the samples of different tissues. The unpaired Student's test also showed that the correlations of lipid composition in the same and in different tissues differ at $p < 0.01$.

Figure 2, A shows the first two main components. It is seen that the samples are grouped in two clusters, i.e. LM + BF and scat1 + scat2, which are linearly separable. This confirms the differences in muscle and adipose tissue samples in the lipid composition.

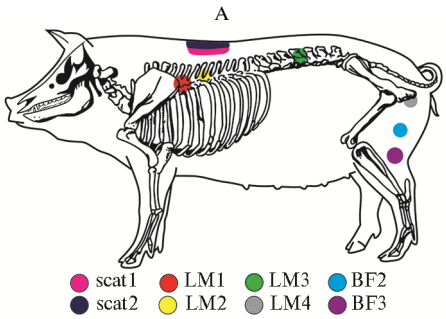
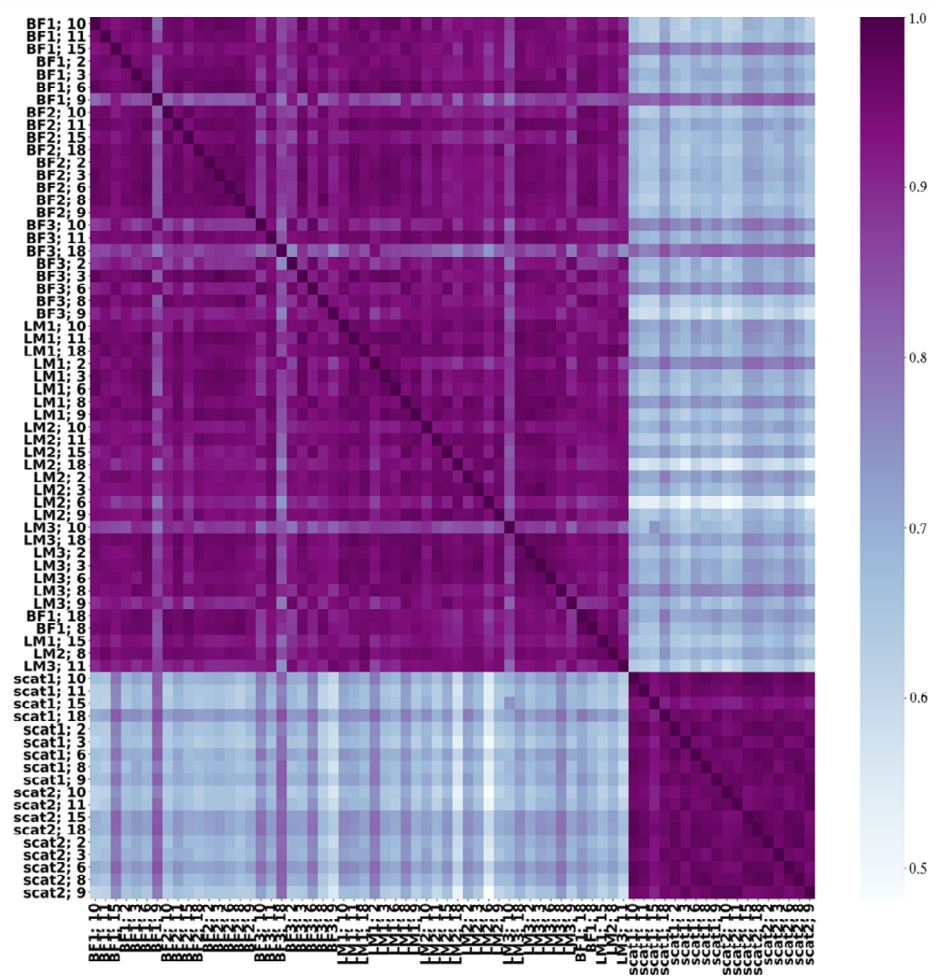


Fig. 1. Lipid composition of tissues in Duroc boars (n = 9, weight 89.4±5.67 kg, age 150-180 days). A: The pattern of sampling for analysis (scat1 and scat2 — subcutaneous dorsal fat, the upper and lower layer, respectively; BF1, BF2 and BF3 — biceps femoris above the back, in the middle part and by a knee; LM1, LM2 and LM3 — the longest muscle between the 5th-6th, 8th-9th vertebrae and at the base of the tale). B: Calculation of the Pearson correlations (r) for the pairs of samples of adipose and muscle tissue (the intensity of violet color reflects the strength of the positive correlation, blue is a weak correlation, white is zero). The r values corresponding to the scale of the intensity of color are indicated in the right. The lipid composition was analyzed by liquid chromatography—time-of-flight mass spectrometry (Acquity I-class, Waters, USA, Maxis Impact II, Bruker Daltonik GmbH, Germany).



We annotated the main groups of lipids: tri- and diacylglycerols (TAG and DAG), phosphatidylcholines (PC, including LPC lysophosphatidylcholines), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidic acids (PA), and phosphatidylinositols (PA) and phosphatidylinositols (PI) for all samples of adipose and muscle tissue (see Fig. 2, B). As it turned out, the adipose tissue samples are enriched in triacylglycerols, and muscle tissue in phospholipids.

The graph of m/z vs. the exit time (Fig. 3, A) shows the differences in the intensity of the peaks of different lipids. The size of the dots on the graph corre-

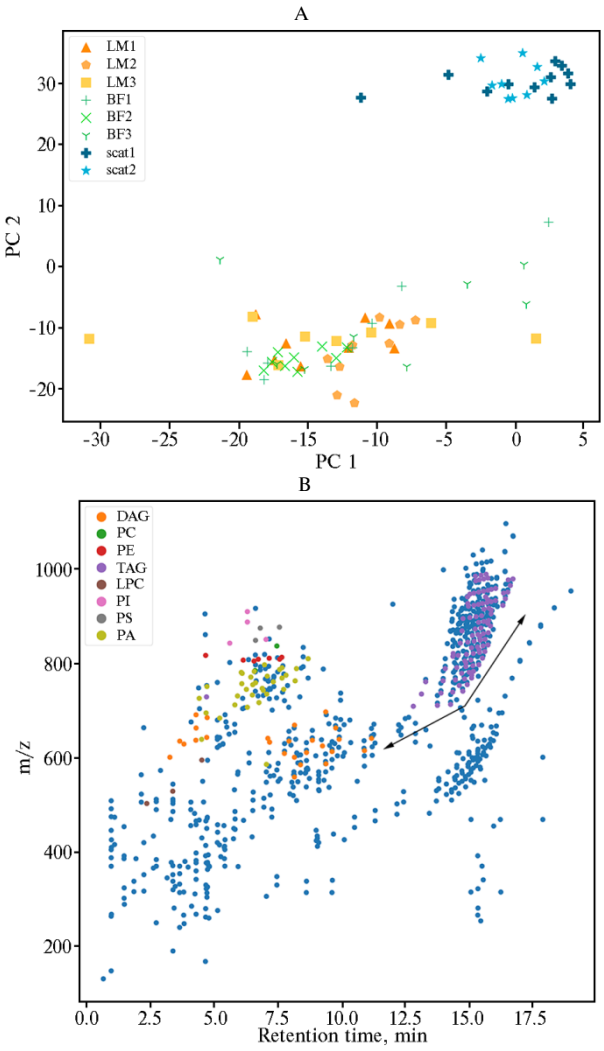


Fig. 2. Clusterization of adipose and muscle tissues sampled from Duroc boars (n = 9, weight 89.4±5.67 kg, age 150-180 days) **according to the lipid composition:** A — principal component analysis (the first two components are shown, see the *Techniques* section), B — distribution of m/z over the time of exit for the main groups of lipids; scat1 and scat2 — subcutaneous dorsal fat, the upper and lower layer, respectively, BF1, BF2 and BF3 — biceps femoris above the back, in the middle part and by a knee, LM1, LM2 and LM3 — the longest muscle between the 5th-6th, 8th-9th vertebrae and at the base of the tail; TAG — triacylglycerols, DAG — diacylglycerols, PC — phosphatidylcholines, PE — phosphatidylethanolamines, PS — phosphatidylserines, PA — phosphatidic acids, PI — phosphatidylinositols, LPC — lysophosphatidylcholines. The arrows indicate the direction of the lengthening of the chain (right, up) and increasing the number of double bonds (left, down). The point corresponds to one sample (B). The measurements were performed by liquid chromatography—time-of-flight mass spectrometry (Acquity I-class, Waters, USA, and Maxis Impact II, Bruker Daltonik GmbH, Germany).

sponds to the average peak intensity for all samples. To find the differences between the tissues, a multiple t-test was used. For each pair of tissues and for each peak, the p-value for the hypothesis of equal averages was calculated. The p-values were adjusted for multiple testing in accordance with Benjamini-Hochberg, the peaks with corrected p-values less than 0.05 were considered statistically significant (see Fig. 3, B). It can be seen that the samples of adipose tissue did not differ significantly from each other, but were significantly different from the samples of muscle tissue. Although in general the differences between muscle and adipose tissue prevailed, some lipids were also statistically significantly different in the two muscles, BF2 and LM2.

The differences between muscle and adipose tissue for all filtered peaks were estimated by the natural logarithm of the ratio between the peak intensity for the averaged muscle and the averaged adipose samples (Fig. 4, A). The differences between the biceps tissues (BF) and the longest muscle (LM) were assessed in a similar way (see Fig. 4, B).

The results we obtained show the effectiveness of liquid chromatography combined with high-resolution time-of-flight mass spectrometry in molecular phenotyping of farm animals, in particular, pigs.

In general, the fatty acid composition only indirectly reflects the fat composition of meat and is insufficient to describe its lipid components [6, 27]. Mass spectrometric analysis in the mode of positive ions gives a more detailed

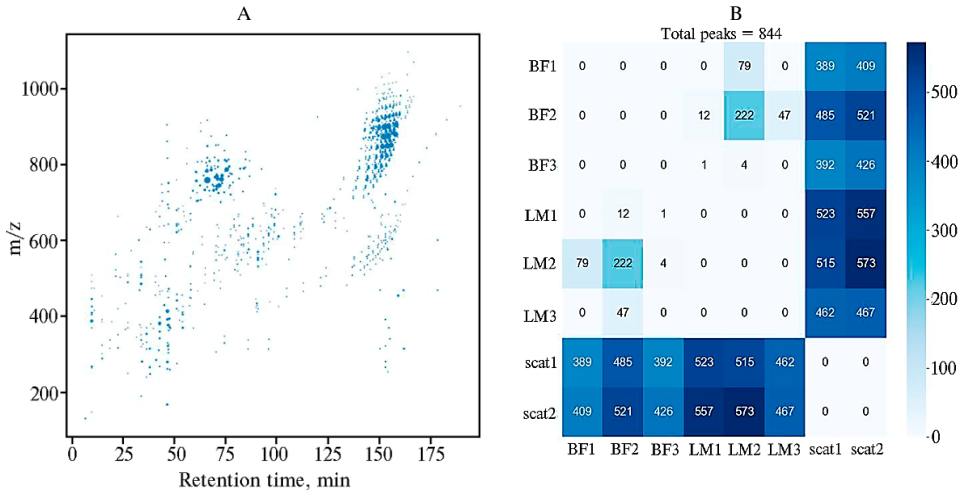


Fig. 3. The diversity of the lipid composition of muscle and adipose tissues in Duroc boars (n = 9, weight 89.4±5.67 kg, age 150-180 days): A — the distribution of m/z vs. the exit time (size of dots corresponds to the average peak intensity for all samples); B — the table reflecting the number of statistically significantly different peaks for the pairs of tissues (at p = 0.05), estimated by Student's *t*-criterion with the Benjamini-Hochberg amendment. The total number of estimated peaks is 844. The intensity of the blue color corresponds to the number of differing peaks, white is the absence or the minimum number of such peaks; scat1 and scat2 — subcutaneous dorsal fat, the upper and lower layer, respectively, BF1, BF2 and BF3 — biceps femoris above the back, in the middle part and by a knee, LM1, LM2 and LM3 — the longest muscle between the 5th-6th, 8th-9th vertebrae and at the base of the tail. The measurements were performed by liquid chromatography—time-of-flight mass spectrometry (Acquity I-class, Waters, USA, Maxis Impact II, Bruker Daltonik GmbH, Germany).

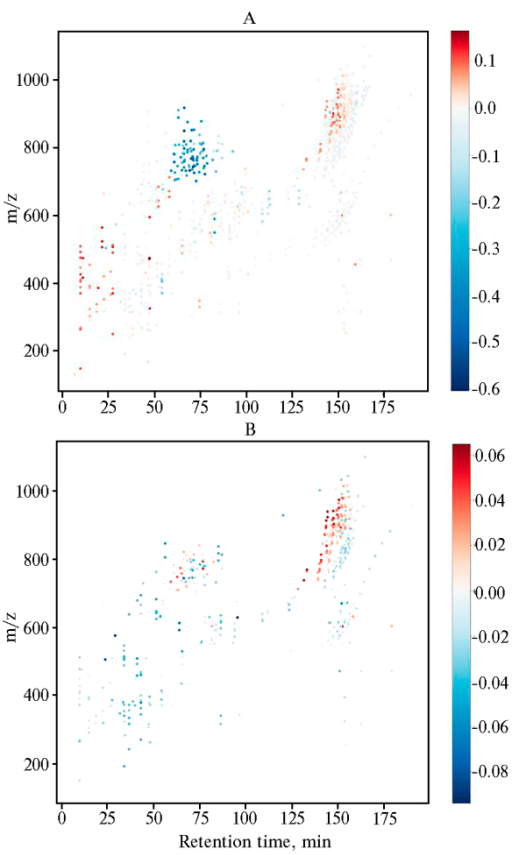


Fig. 4. The graphs of m/z vs. the exit time of lipid peaks for muscle and adipose tissues (A) **and for two fragments of muscle tissue, the averaged sample of biceps tissue BF and averaged sample of the longest muscle LM** (B). The graphs are based on the natural logarithms (ln) of the peak intensity relationships. The color indicates the differences in peak intensity: red — the peak intensity is higher in the fat sample (A) and in the biceps (B), blue — in the muscle sample (A) and the longest muscle (B); white color means that the peak intensities in the compared samples are equal. The measurements were performed by liquid chromatography—time-of-flight mass spectrometry (Acquity I-class, Waters, USA, Maxis Impact II, Bruker Daltonik GmbH, Germany).

characteristic of fat composition of tissues and covers most classes of lipids, i.e. TAG, DAG, PC, PE, PS, PA, PI, LPC, etc. [28]. The results of the analysis show the absence of significant differences in the total fat composition between the muscle areas, but manifest the expected significant differences in the lipid profiles of muscle and adipose tissues. Based on this analysis, it is possible to optimize assessing fat composition of the muscle tissue of animals.

It should be noted, that in pigs, the lipid analysis of tissue samples by UHPLC/Q-TOF-MS was never performed in the mode of positive ion registration. We have found the difference between the correlation coefficients of the lipid composition for the samples from the same tissue ($r = 0.93$) and from different tissues ($r = 0.69$). The reliability of these differences was confirmed by non-overlapping confidence intervals at a significance level of $p < 0.05$ and an unpaired Student's test with a p-value < 0.01. The high correlation of the lipid profiles between the samples from one organ or from two different muscles indicates a good reproducibility of the method. The results show that the muscle tissue samples are enriched in phospholipids, and adipose tissue in triacylglycerols. Triacylglycerols differ in the total length of the chains of their fatty acid residues and in the total number of double bonds. An increase in the total length leads to an increase in the retention time in the chromatographic column, while the addition of a double bond, on the contrary, shortens it. Due to this, chromatographic and mass-spectrometric peaks related to triacylglycerols form the patterns similar to a network on the m/z vs. retention time graph [29]. Unsaturated triacylglycerols are grouped into a single cluster of peaks in the upper left part of the cloud of all peaks belonging to triacylglycerols (see Fig. 2, B). It is interesting that the differences between muscle tissue and adipose tissue, as well as the differences between the two muscles, mainly affect polyunsaturated triacylglycerols, while 0-2-unsaturated fats do not actually change. The use of automatic annotation of chromatographic and mass-spectrometric peaks makes it possible to substantially purify the data from contaminants. The annotation can be further improved by using internal standards or by taking into account the net-like patterns described above. These experiments are beyond the scope of this paper, but we plan to continue the studies in the future.

Thus, the results show that in pigs, muscle tissue is enriched in phospholipids, and adipose tissue in triacylglycerols. In general, the lipid profiles of different tissues differ, while in the same tissue they show similarity. The samples taken from different points of the same muscle do not differ significantly from each other, whereas the difference for different muscles is statistically significant at $p < 0.05$ (in unpaired Student's test, p-value < 0.01). UHPLC/Q-TOF-MS makes it possible to quickly, reliably, efficiently and objectively determine the molecular phenotypes of farm animals, in particular, their lipid profiles, to qualitative assess individuals during selection.

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GENE CONSTRUCT-BASED SERINE PROTEASE OF *Bacillus pumilus* AS A FEED ADDITIVE FOR POULTRY FARMING

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Abstract

Bacterial enzymes, in particular proteinases, as dietary additives in poultry farming, improve digestibility of feed nutrients and, as a consequence, make animal diets cheaper. This explains why bioadditives are being actively developed worldwide. Proteinases break down proteins and reduce the negative effect of digestive inhibitors thus allow the costs for purchasing synthetic amino acids to be lower. Bacteria and microscopic fungi, including those with gene constructs developed to increase yield and improve properties of the expressed enzymes, may be producers. Bacterial serine proteinases have a high thermostability and are resistant to inhibitors of animal origin. In this paper, we report for the first time about the production of highly purified secreted subtilisin-like serine proteinase from *Bacillus pumilus* upon expression of the recombinant vector in *B. subtilis* strains and evaluate the main physicochemical and biological characteristics of the synthesized product. The goal of our study is to obtain, by using the expression system, the highly purified subtilisin-like serine proteinase from *B. pumilus* as a promising feed additive for the poultry industry. The substrate specificity of the produced serine proteinase, i.e. the depth of hydrolysis, corresponds to the specificity of subtilisins, the enzyme cleaves the bonds formed by the carboxyl groups of the hydrophobic amino acids leucine, phenylalanine and tyrosine, as well as a number of hydrophilic amino acids. An investigation of the effect of temperature and pH on serine proteinase activity showed that in the presence of calcium ions at a final concentration of 5 mM, the temperature optimum of the enzyme reached 50 °C. The enzyme remaines stable in the pH range from 7 to 10. The proteinase activity was studied at various pH values to simulate the conditions of the gastrointestinal tract of chickens. In a weakly acidic medium (pH 5.5, goiter) proteinase completely retains its activity (100 %), at pH 2.9 (stomach) the enzyme activity decreases by 40 %, and upon transition again to alkaline conditions (pH 6.5-8.0, small intestine and large intestine), the enzyme restores activity up to the values exceeding control by 13 %. Thus, the enzyme can remain active throughout the whole digestive tract of broiler chicks. The proteinase activity was not inhibited by natural inhibitors, such as a trypsin inhibitor, which would also allow the enzyme to function in the gastrointestinal tract of chickens. In experiments on the effect of chicken bile from 0.01 % to 0.05 % for 1 hour at 40 °C on the microbial proteinase, the enzyme completely preserved its activity. With an increase in the concentration of chicken bile to 1 %, the enzyme activity decreased by 10 %. To study the toxicity of proteinase, 1-day-old Cobb 500 broiler chickens were observed for 10 days. The dietary proteinase at 100 EU/kg concentration showed no toxicity, and all the indices of the poultry remained normal. We found that in the early period, during 0-10 days of growth when the chickens are fed with Start ration, a dosage of 5 EU/kg of proteinase is effective. In the late stages of poultry growth (21-42 days), the use the Finisher mixed feed supplemented with bacterial proteinase at a dose of 15 EU/kg is optimal. In both cases, the dietary proteinase increases poultry weight gain by 13.9 % and 7.9 %, and also improves feed conversion by 14 % and

8.5 %, respectively. Thus, the amount of the introduced enzyme must be adjusted depending on the age of birds and the feed composition. The main indicators of Cobb 500 broiler chickens' growth when using recombinant proteinase allow us to conclude that this proteinase is promising as a feed additive.

Keywords: *Bacillus pumilus*, recombinant subtilisin-like serine proteinase, substrate specificity, stability, activity, effects of pH and temperature, fodder additive, broiler chickens, Cobb 500.

Economic benefits from more complete assimilation of cereal feeds by increasing the digestibility of nutrients remain a pressing issue in commercial poultry farming [1-5]. Enzymes, including bacillary proteinases, can help to solve the problem. Proteinases increase the digestibility of protein components, necessary for growing broilers, and also destroy the bonds between proteins, starch or fiber, which positively affects starch digestibility by increasing its bioavailability [6-10]. The use of microbial proteases also improves the digestibility of feeds with a high content of non-starch polysaccharides [11-13]. Dietary multienzyme complexes (protease/β-glucanase/pectinase) resulted in an increase in live weight of laying hens, in egg weight, in a darker yolk, and also had a positive effect on the digestive organs [14]. In addition, exogenous proteinases, due to their effect on anti-nutritional components, for example, by destruction of the inhibitors of trypsin and lectins in soybean meal, increase the digestibility of feed nutrients [15, 16]. Exogenous proteases act as a prophylactic agent, reducing the amount of undigested protein, which is a factor in colonization of the intestine by pathogenic microorganisms, leading to the development of coccidiosis and necrotic enteritis in chickens [17, 18]. It is known that undigested proteins are factors leading to dysbacteriosis which causes necrotic enteritis [19, 20]. The protease additives improved the productivity of broilers infected by *Eimeria* spp., the causative agent of necrotic enteritis [21]. The complex preparations of living bacteria or spores in combination with exogenous proteases have a growth-promoting and protective effect on chickens [22, 23].

A search for new producers and design of effective recombinant microbial enzymes used as feed additives is an important biotechnological task [24-26]. Effective expression systems are developed to obtain feed additives based on microbial proteinases in the required quantity [27]. The inexpensive components of the media for bacilli culture, as well as the safe status of these microorganisms, determine the prospects for their use in poultry farming.

This paper is our first report on synthesis of highly purified secreted subtilisin-like serine proteinase of *Bacillus pumilus* upon expression of recombinant vectors in *B. subtilis* strains, with characterization of the main physicochemical and biological parameters of the recombinant product, which determine its promising use as a feed additive.

The goal of the study is to produce, using the expression system, highly purified subtilisin-like serine proteinase of *Bacillus pumilus* as a feed additive for poultry.

Techniques. Natural isolate *B. pumilus* 7P, its streptomycin-resistant mutant *B. pumilus* 7P/3-19, and plasmids pCS9 with gene for *B. pumilus* subtilisin-like protease were provided by S.V. Kostrov (Institute of Molecular Genetics RAS, Moscow), pGP382 was brought by courtesy of Dr. Prof. T. Mascher (Ludwig-Maximilians-Universität München, Germany). A protease-deficient strain *B. subtilis* BG 2036 (by the courtesy of Prof. E. Ferrarri, Genencor Int., Inc., USA) was a recipient. Recombinant vectors pTN 3036 (pLIKE-rep + *aprBp*), pTN 3050 (pLIKE-rep + SP_{Pac} + *aprBp*), pTN 3093 (pLIKE-rep + SP_{Yngk} + *aprBp*) and pTN 3801 (pGP382 + *aprBp*) were used to transform the protease-deficient strain *B. subtilis* BG 2036 to obtaine strains *B. subtilis* MRB044, *B. subtilis* MRB045, *B. subtilis* MRB046 and *B. subtilis* MRB072, respectively. The strains and plasmids used are stored in the museum of the Laboratory of Microbial Bio-

technology of the Kazan Federal University.

The growth medium composition was as follows (g/l): bacteriological peptone (Sigma, USA) — 20, $CaCl_2 \cdot 2H_2O$ — 0.6, $MgSO_4 \cdot 7H_2O$ — 0.5, NaCl — 3, $MnSO_4$ — 0.1, Na_2HPO_4 — 0.2, NH_4Cl — 0.2. The strains *B. subtilis* containing recombinant constructs were cultured with erythromycin and lincomycin (10 and 25 μg/μl, respectively), the recombinant strain *B. subtilis* pCS9 with 20 μg/μl erythromycin. The subtilisin-like proteinase production was evaluated by the ratio of proteolytic activity to the biomass and expressed in conditional units.

The proteinase activity was determined by the hydrolysis of azocasein (Sigma, USA) as described [28, 29]. The enzyme activity unit (EU) was the amount of enzyme hydrolyzing 1 μg of substrate per 1 min under the experimental conditions. The specificity of the proteinase was estimated by the effect on the β-chain of oxidized sheep insulin [30]. Measurements were conducted on an xMark spectrophotometer (Bio-Rad, USA).

A bioreactor Biotron LiFlus SP30L (Biotron, Inc., Korea) was used for proteinase production. Fifteen liters of the medium was sterilized in the reactor for 30 min at 121 °C; the pH of the medium was adjusted to pH 8.5 automatically and maintained by the addition of 2 N NaOH through the peristaltic system of the bioreactor. Three hundred milliliters of a 16-hour inoculum (2 % of the medium, v/v, OD_{600} 3.0), the antibiotic erythromycin (to a final concentration of 10 μg/μl) and a defoaming agent Sofeksil 1250 (Sofeks, Moscow) were introduced in the fermenter. Bacteria was cultured for 24 hours at 37 °C with constant aeration (the flow rate of 10 l/min, O_2 content is not lower than 20 %) and stirring (150-900 rpm). After 24 h growth, when the enzyme activity reached the maximum (4.4 EU/ml, OD_{600} 6.0) the cells were removed by centrifugation (5000 rpm, 15 min, Beckman Avanti JXN-26, Beckman Coulter, Inc., USA). Proteinase was purified on a column with carboxymethylcellulose (CMC) (Sigma, USA). The supernatant after the centrifugation was diluted 10 times with distilled water (pH was adjusted to 6.3), then mixed with CMC and equilibrated with 0.02 M Na-acetate buffer (pH 6.3). The mixture was kept for 90 min with constant stirring for the sorption of the enzyme. Then CMC aggregate was precipitated, the supernatant fluid was removed and the sorbent was used to fill the column. The column was rinsed with the same buffer, the protein was eluted with 0.2 M Na-acetate buffer (pH 6.3), and the enzyme activity was measured in the resulting fractions.

The molecular weight of the produced proteinase was determined by SDS-electrophoresis [31].

The physicochemical properties of the proteinase were evaluated by the temperature optimum in the presence and in the absence of calcium ions (5 mM $CaCl_2$). To assay thermostability, enzyme solutions in 0.05 M Tris-HCl buffer (pH 7.2) were incubated at the temperatures from 0 to 70 °C for 30 min, then the activity was determined at 37 °C as described above. To assess pH optimum, the enzyme activity in 0.05 M Tris-HCl buffer was determined. When studying pH stability, protein solutions were incubated in 0.05 M Tris-HCl buffer for 1 h at 25 °C, then azocasein was added and the activity was determined at 37 °C as described above.

The effect of inhibitors on a proteinase was studied using phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), o-phenanthroline (a specific inhibitor of metalloproteinases) and ovomucoid (trypsin inhibitor). The protein solution was incubated with an inhibitor for 1 h at 37 °C in Tris-HCl buffer (pH 7.2) and the proteolytic activity was determined as described above.

To imitate the conditions of the gastrointestinal tract (GIT) of chickens,

Britton-Robinson universal buffer was used [32]. A series of four aliquots of universal buffer (0.04 M) was prepared with different pH values (2.9; 5.5; 6.0; 6.3 and 8.0). The enzyme was transferred from one solution to another by the method of successive dilutions and kept in each for an appropriate time; total enzyme dilution 1:200. The sequence of aliquots corresponded to the sequence of the digestive tract sections of chickens: pH 5.5 (50 min), ingluvies simulation; pH 2.9 (90 min), stomach simulation; pH 6.5 (30 min), small intestine simulation, pH 8 (70 min), large intestine simulation. The buffer temperature throughout the experiment was 40 °C.

Bile, obtained from 10-day-old broiler chickens, was diluted with 0.02 M Na-acetate buffer (pH 6.3). The samples containing bile in the enzyme solution from 0.01 % to 5 % were kept at 40 °C for 60 min. The samples were taken every 15 min to measure the activity. An enzyme solution in 0.02 M Na-acetate buffer (pH 6.3) without bile was control. The control solution of the enzyme was kept at 40 °C for 1 h and the proteinase activity was determined.

The properties of proteinase as a feed additive were studied under the conditions of a peasant farm enterprise (Srednee Azyakovo village, Medvedevskii District, Mari-El Republic). For the experiment, 225 1-day-old Cobb 500 chickens with an average live weight of 0.049±0.003 kg were selected, out of which a control group (75 birds) was formed, which received standard mixed feed ration, and two experimental groups (75 birds each), where recombinant proteinase was added to the mixed feed at a dose of 5 EU/kg (group I) or 15 EU/kg (group II). The experiment continued for 42 days. The chickens aged 0-10 days received the Start mixed feed, 11-20 days — the Growth, 21-42 days — the Finisher feeds in accordance with growing technologies (Algoritm investitsii LLC, Yoshkar-Ola, Mari El Republic). The enzyme solution was added to dry feed by spraying with constant stirring. The chickens were kept in ventilated cellular batteries at 35-36 °C. Weight gain in chickens was evaluated daily, from the initial to the final day of the experiment. The amount of the consumed feed was counted per chicken. The feed conversion ratio was calculated as the ratio of the amount of consumed feed to the increase in body weight.

The toxicity of the proteinase preparation was studied on 1-day-old Cobb 500 chickens weighing 0.047±0.001 kg, out of which a control group (15 birds) was formed, which received only mixed feed, and an experimental group (15 birds), where proteinase was added (100 EU/kg feed) for 10 days. During the experiment, the chickens were weighted, and their behavior was controlled and excrements were surveyed. After 10 days, 3 chickens were randomly selected from each group and euthanized by inhalation anesthesia with chloroform to examine the internal organs.

The statistical processing of the results included the calculation of the mean value (M) and the standard error of the mean (±SEM). The significance of differences was assessed by Student's t-test. The differences were considered statistically significant at $p < 0.05$.

Results. We compared the expression of subtilisin-like extracellular serine proteinase of *B. pumilus* in natural and recombinant strains. The wild-type strain *B. pumilus* 7P is a natural isolate with an increased production of extracellular ribonuclease and other enzymes, including the proteinase, *B. pumilus* 7P/3-19 is its streptomycin-resistant mutant. A recombinant strain *B. subtilis* pCS9 was obtained, which carries the multicopy plasmid pCS9 containing the proteinase gene *B. pumilus* (*aprBp*) with its own signal peptide under the control of its own promotor. To clone *aprBp*, an optimized LIKE expression system based on the promotor *liaI B. subtilis* was also used, which is regulated by a two-component antibiotic-induced system LiaRS [33, 34]. As part of the pLIKE-rep vector, the

gene *aprBp* was introduced into *B. subtilis* MRB044 with its own signal peptide (pTN 3036, pLIKE-rep + *aprBp*), into *B. subtilis* MRB045 with the nucleotide sequence of the signal peptide of penicillin-amidase (penicillin amidohydrolase, EC 3.5.1.11) gene of *B. megaterium* (pTN 3050, pLIKE-rep + SP_{Pac} + *aprBp*), and into *B. subtilis* MRB046 with the sequence of the recombinant signal peptide of glycoside hydrolase (EC 3.2.1.-) gene of *B. megaterium* (pTN 3093, pLIKE-rep + SP_{Yngk} + *aprBp*). pGP382 expression vector with a strong constitutive promoter (P_{DegQ}) [35] was also used to clone *aprBp* gene [35]. The *degQ* gene encodes the protein (46 amino acid residues) involved in the phosphorylation of the two-component system DegS/DegU that controls the synthesis of proteinases [36]. *B. subtilis* MRB072 contained plasmid pGP382 with the *aprBp* gene and the Strep tag in the composition of a protein affinity purification vector (pTN 3801, pGP382 + *aprBp*). The comparison of expression showed *B. subtilis* pCS9 (Table 1) to be the most effective proteinase producer, which was used to continue the experiments.

1. Proteolytic activity in the culture medium of natural isolates *Bacillus* and recombinant strains with *B. pumilus* subtilisin-like serine proteinase gene *aprBp* in various constructions ($n = 10$)

Strain	Activity, EU	Productivity, units
B. pumilus 7P	1.50±0.02	0.50±0.01*
B. pumilus 7P/3-19	2.90±0.01	0.93±0.02*
B. subtilis pCS9	3.50±0.01	1.14±0.02*
B. subtilis MRB044	0.25±0.05	0.10±0.01
B. subtilis MRB045	0.30±0.02	0.12±0.02
B. subtilis MRB046	0.42±0.03	0.17±0.02*
B. subtilis MRB072	0.50±0.01	0.20±0.04

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

2. Purification of recombinant subtilisin-like proteinase expressed in *Bacillus subtilis* pCS9 with *B. pumilus* gene *aprBp* in the plasmid pCS9 ($n = 5$)

Stage of purification	Volume, ml	Protein mg/ml	Activity			Purity	Yield, %
			EU/ml	total, EU	specific, EU/mg		
Culture medium	12800	870±20*	4.4±0.07*	56320[a]	0.005	1.0	100
Ion-exchange chromatography on carboxymethylcellulose	470	383±10*	33.8±0.1*	15886[a]	0.088	17.6	28.2

N o t e. [a] — mean values of activity.

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

After culture of *B. subtilis* pCS9 in the bioreactor and purification of the proteinase, a preparation with total activity of 15886 EU was obtained from joined electrophoretic fractions with high activity (Table 2). SDS electrophoresis confirmed the presence of 28 kDa protein. Thus, using CMC chromatography, a highly purified recombinant subtilisin-like proteinase preparation was obtained in 0.2 M Na-acetate buffer (pH 6.3). Since the buffer components are non-toxic to chickens, their presence in the enzyme solution was not an obstacle for the use in experiments with poultry.

The temperature optimum of the recombinant enzyme was 37 °C (Fig. 1, A). For the practical use, it is important that in the presence of calcium ions at a final concentration of 5 mM, the temperature optimum of the enzyme increased to 50 °C. In our experiment, the enzyme activity increased on average by 40 % at 50 °C and by 60 % at 55 °C (see Fig. 1, B). The proteinase remained stable in the temperature range from 0 to 40 °C. The optimum acidity was pH 9.5. The proteinase retained stability in the range of pH 7-10. At pH 3 and pH 11, the drop in activity did not exceed 40 % (see Fig. 1, B). The data on thermo- and pH stability of the protein testify to the possibility of its use as a feed additive. When distilling an aliquot of the enzyme with a solution of a specific inhibitor of serine proteinas-

es PMSF (1:1000), the enzymatic activity was completely suppressed, and it did not change in the presence of metalloproteinase inhibitors EDTA and o-phenanthroline (1:100). These data indicate that the enzyme belongs to the class of serine proteinases. The proteinase activity was not inhibited by the trypsin inhibitor, thence, it was suggested that the recombinant enzyme will be able to function in the digestive tract of chickens.

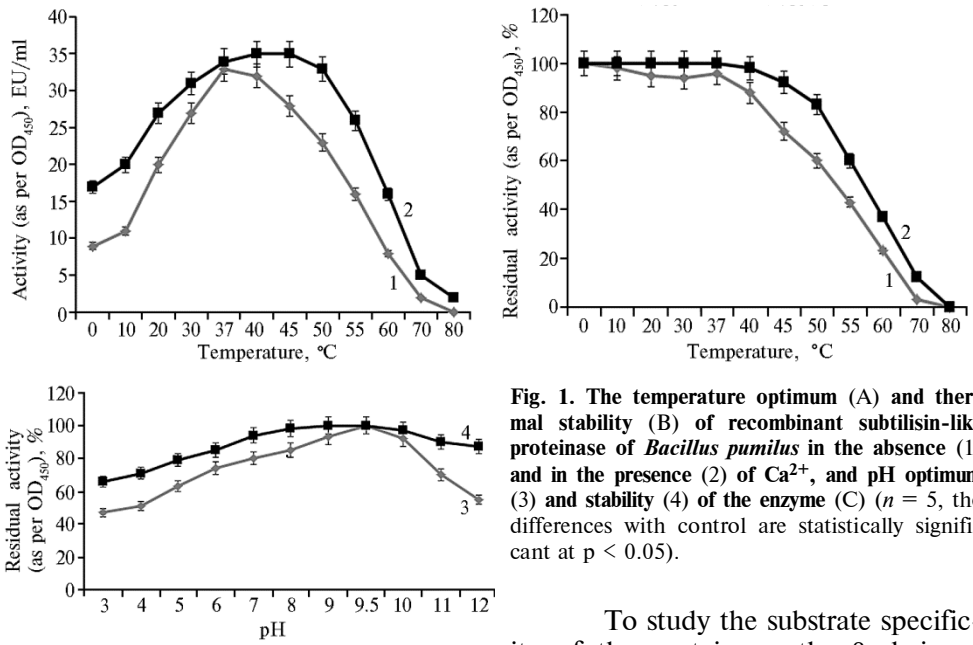

Fig. 1. The temperature optimum (A) **and thermal stability** (B) **of recombinant subtilisin-like proteinase of *Bacillus pumilus* in the absence** (1) **and in the presence** (2) **of Ca^{2+}, and pH optimum** (3) **and stability** (4) **of the enzyme** (C) (n = 5, the differences with control are statistically significant at $p < 0.05$).

To study the substrate specificity of the proteinase, the β-chain of the oxidized insulin was used. The hydrolysis of the of_the β-chain led to numerous peptide fragments detected by thin-layer chromatography (the data not given), indicating a broad substrate specificity well-known for subtilisin-like enzymes, e.g. proteinase K, esperase of *B. lentus* and subtilisin BPN′ of *B. amyloliquefaciens* [37]. The enzyme hydrolyzes the bonds formed by carboxyl groups of hydrophobic amino acids (Phe1-Val2, Leu11-Val12, Leu15-Tyr16, Phe25-Tyr26, etc.) and also hydrophilic amino acids (Asn3-Gln4, Gln4-His5, Cys7-Gly8, Ser9-His10, Tyr16-Leu17, etc.). Consequently, the obtained proteinase of *B. pumilus* has wide substrate specificity and the ability to deeply hydrolyze protein substrates, which also determines the perspective nature of the enzyme as a bioadditive splitting protein components of feeds.

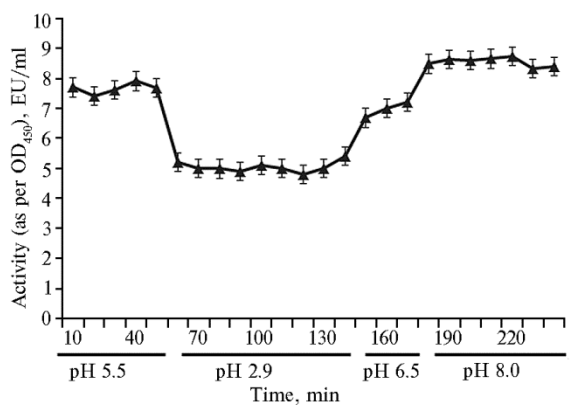

Fig. 2. The activity of the recombinant subtilisin-like proteinase of *Bacillus pumilus* at different pH values, simulating conditions in the gastrointestinal tract of chickens (n = 5, the differences with the control are statistically significant at $p < 0.05$).

To work effectively in the digestive tract of poultry, proteinase should remain active at elevated temperatures (40 °C) and aggressive pH values, ranging from an acidic to an alkaline one. The experiment with simulating the gastrointestinal condi-

tions of chickens (pH, time and temperature) showed that proteinase successfully functions in such conditions (Fig. 2). In a weakly acidic medium (pH 5.5), the enzyme remained activity within the control one. In a strongly acidic medium (pH 2.9, stomach simulation), the enzyme activity decreased by 40 %, and in alkaline conditions (pH 6.5-8.0, small and large intestine simulation) it increased by 10-13 % compared to the control. These data showed that proteinase can remain active throughout the entire digestive tract of poultry.

The study of the activity and stability of the enzyme under the action of bile for 1 h at 40 °C showed that at its concentration from 0.01 to 0.05 % the enzyme activity remained within the control value. When increasing the concentration up to 1 %, the enzyme activity decreased by 10 %, and at a concentration of 5 %, the residual activity of the enzyme was 60 % (Fig. 3). Therefore, the resulting bacterial enzyme is able to maintain catalytic activity when exposed to bile under the conditions of the gastrointestinal tract of chickens.

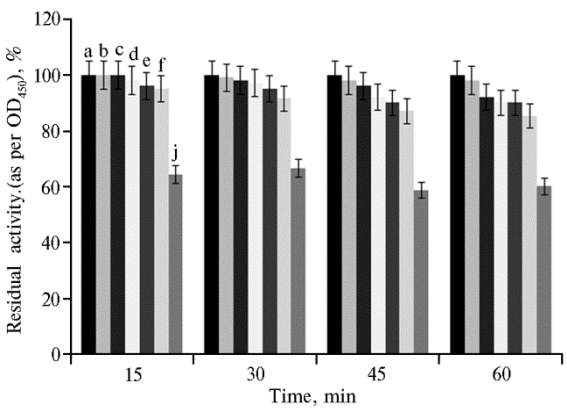

Fig. 3. The activity of recombinant subtilisin-like proteinase of *Bacillus pumilus* at different concentrations of bile: a — 0.01 %, b — 0.05%, c — 0.10%, d — 0.25%, e — 0.50%, f — 1%, g — 5% (n = 5, the differences with the control are statistically significant at $p < 0.05$).

When assessing the toxicity of the preparation, the chickens were kept in cages. The chickens were preliminarily examined by a veterinary specialist to identify the sick and weakened ones (they were excluded from the experiment).

3. The main zootechnical indicators in Cobb 500 broiler chickens upon the addition of recombinant proteinase of *Bacillus pumilus* in the feed (M±SEM, physiological experiment, a peasant farm enterprise, Mari El Republic)

Indicator	Control (n = 25)	Group I, 5 EU/kg feed (n = 25)	Group II, 15 EU/kg feed (n = 25)
Live weight gain, kg:			
0 day	0.049±0.003	0.049±0.003	0.049±0.003
1-10 days	0.201±0.007	0.229±0.008	0.214±0.005
11-20 days	0.364±0.014	0.402±0.014	0.391±0.010
21-42 days	1.551±0.032	1.668±0.038	1.674±0.039
Total	2.165±0.044	2.348±0.044	2.328±0.037
Consumption of feed per chicken, kg:			
Start (0-10 days)	0.343	0.336	0.347
Growth (11-20 days)	0.729	0.705	0.719
Finisher (21-42 days)	3.112	3.127	3.071
Total	4.184	4.168	4.137
Conversion of the feed:			
Start (0-10 days)	1.71	1.47	1.62
Growth (11-20 days)	2.00	1.75	1.84
Finisher (21-42 days)	2.01	1.88	1.84
Total	1.98	1.81	1.82
Poultry survival	100 %	100 %	100 %

N o t e. In group I and group II, the subtilisin-like proteinase of *Bacillus pumilus* was added to the main (control) ration (see *Techniques* section). The differences with the control are statistically significant at $p < 0.05$.

The Start feed was used in the form of grits. The proteinase concentration was 12.5 ml/kg feed per 15 birds. For 10 days (the period of observation), all chickens remained healthy, active, ate the feed well, physiological abnormalities and the changes in behavioral reactions were not observed. The live weight

of the chickens in the experiment remained within the control. The excrements of broiler chickens were normal. After the postmortem examination, damage and pathological changes were not revealed in the internal organs. These results confirmed that the bacterial proteinase preparation was safe and not toxic for the poultry.

When determining the nutritional value of the feeds (Growth, Start, Finisher) in adding bacterial proteinase, the calcium content (about 1 %) was sufficient to stabilize the activity of the introduced enzyme. For 42 days (the period of observation), all chickens remained healthy, active, ate the feed well, their behavioral reactions did not change. The preservation of livestock was 100 % in the control and experimental groups. By the end of fattening, the live weight of the poultry, which was given proteinase as an additive, was higher compared to control, i.e. in group I (5 EU/kg) by 8.7 % ($p < 0.05$), and in group II (15 EU/kg) by 7.7 % ($p < 0.05$) (Table 3). During 0-10 days (Start feed), the increase in the live weight of chickens in group I and group II was higher than in the control by 13.9 % ($p < 0.05$) and 6.5 % ($p < 0.05$), respectively (see Table 3). The feed conversion improved in both groups (by 14.0 and 5.3 %, respectively, $p < 0.05$). From day 11 to day 20 (Growth feed), the weight gain in group I was 10.4 % higher ($p < 0.05$), in group II 7.4 % higher ($p < 0.05$) compared to the control. The feed conversion improved (by 12.5 and 8.0 %, respectively, $p < 0.05$). When using the Finisher feed (21-42 days), there was a 7.5 % gain in group I ($p < 0.05$), and a 7.9 % gain in group II ($p < 0.05$). During this period, the feed conversion in the experimental groups improved by 6.5 and 8.5%, respectively ($p < 0.05$).

Thus, subtilisin-like proteinase of *Bacillus pumilus*, expressed in *Bacillus subtilis* pCS9 with *aprBp* gene in pCS9 plasmid, shows wide substrate specificity, stability (withstands the fluctuations of pH, temperature, high concentrations of bile), high activity (is capable of remaining activity in both the upper and the lower intestines of Cobb 500 chickens) and non-toxicity to poultry. These properties are necessary in the conditions gastrointestinal tract of broilers, since the enzyme should remove the substrate that could disturb the digestion and microflora balance as the chyme moves along the whole intestine. The obtained data allows conclusion that in the early stages of growth (0-10 days) with the use of the Start feed, the effective dose of proteinase is 5 EU/kg feed (there is a tendency to improving the feed conversion). The same is noted later (21-42 days) with the addition of proteinase at a dose of 15 EU/kg to the Finisher feed. The resulting recombinant bacillary proteinase can be considered as a potential feed additive to increase the live weight gain and reduce the feed consumption when growing broiler chickens.

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ISOLATION AND IDENTIFICATION OF ROOT NODULE BACTERIA FROM GUAR *Cyamopsis tetragonoloba* (L.) Taub.

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Abstract

Cyamopsis tetragonoloba (guar) belongs to the family *Fabaceae* and is one of the promising crops for cultivation in Russia. Beans contain a large number of protein and fatty oil content, green beans can serve as a valuable source of food and feeds (as seed flour and not ground granulated feeds), but the plant is more in demand as a source of guar gum, which is a polysaccharide formed by galactose and mannose (galactomannan) and is contained in the endosperm of the seeds of this plant. Guar gum is widely used in various industries: food, textile, cosmetic, oil and other. Guar comes from India, where approximately 80 % of the world's production of guar gum is obtained. However, due to high demand, the plant is cultivated throughout the world in areas with a suitable climate (the USA, Sudan, Kenya, Pakistan, Australia), including in the south of the Russian Federation. It is known that the productivity of leguminous crops depends not only on climatic conditions, but also on the effectiveness of symbiosis of plants with nodule bacteria (rhizobia), which is determined by the nitrogen-fixing activity and competitiveness of strains, as well as their complementarity to a particular variety. The use of rhizobia for inoculation of plants is especially important when they are introduced to new habitats, so knowledge of its microsymbionts is necessary for successful cultivation of guar in Russia. This paper is the first to report on isolation of the nodule bacteria of the species *Bradyrhizobium elkanii* from root nodules of the guar plants grown in a pot experiment with the use of soil samples from India. We determined the taxonomic position and genetic heterogeneity of the isolated strains. The 16S rRNA gene (*rrs*), ITS-region between the 16S and 23S rDNA and three "housekeeping" genes *atpD*, *dnaK* and *recA* of 10 isolates of nodule bacteria were sequenced. According to the results of the *rrs* sequence analysis, all isolates are assigned to the species *Bradyrhizobium elkanii* (family *Bradyrhizobiaceae*), whose representatives are microsymbionts of a wide range of leguminous plants, including the tribe *Indigofereae*, to which the guar belongs. However, the representatives of the species were not previously described as a microsymbiont of *Cyamopsis tetragonoloba*. Sequencing of the ITS-region and the "housekeeping" genes confirmed the species identity of the isolates and demonstrated their genetic heterogeneity. Thus, the study of nodule bacteria from guar has expanded our knowledge of the phylogeny of its microsymbionts and will allow us in the future to select the most effective strains that improve nitrogen nutrition and plant growth. Knowledge of the rhizobial microsymbionts of guar will help maximize the symbiotic potential of this agronomically valuable culture for its stable and highly productive cultivation.

Keywords: *Cyamopsis tetragonoloba*, guar, root nodule bacteria, 16S rRNA gene, ITS region,

Cyamopsis tetragonoloba (L.) Taub. (guar, pea tree, or Indian acacia) is an annual leguminous crop with a high content of protein from the tribe *Indigofereae* of the family *Fabaceae*. Besides *C. tetragonoloba*, the genus *Cyamopsis* includes 4 more species (*C. dentata, C. psoraloides*, *C. senegalensis* and *C. serrate*) with a smaller industrial value [1, 2]. It is grown as a vegetable crop and can be used as forage for animals. Guar is a nitrogen-fixing plant; it also serves as a good predecessor in crop rotation. Guar natural gum has a certain importance, which is used as a natural stiffener and emulsifying agent in food, medical, textile and pulp-and-paper industries, in the production of cosmetics, explosives, and as a high-viscosity surfactant in the coal and oil-and-gas industries [3]. The demand for it shows a stable growth: according to the data provided for 2014-2016, the annual requirement for natural gum is about 1.5 million tons, and in 2016, the import of guar natural gum to Russia exceeded 15 thousand tons [4]. The native land of *Cyamopsis* and, accordingly, the basic supplier of guar natural gum is India, though the plant is also cultivated in Pakistan, Sudan, Africa, Australia, Ceylon, Afghanistan and the USA [1]. In Russia, guar was delivered in the mid-twenties [5], but it did not find wide applicability because of insufficient knowledge in the technology of its cultivation [6]. Recent years have been noted for the interest in guar industrial cultivation in the North Caucasian Region of Russia, in the Krasnodar Territory, the Rostov Region and in Crimea [3].

It is known that the productivity of leguminous crops depends on the efficacy of their symbiosis with nodule bacteria, which is defined by nitrogen-fixing activity, virulence, competitiveness, and complementarity (specificity) of strains of microsymbionts to a particular variety of the plant. The application of active strains as inoculates provides intensive nitrogen-fixing, promotes photosynthesis intensifying and, as a consequence, leads to the augmentation of plants productivity [7]. The use of rhizobia for inoculation is especially important in the cultivation of leguminous crops when they are introduced to new habitats, where there are no necessary microsymbionts in the soil. For example, the attempt of cultivation of cultural soya (*Glycine max*) in geographical regions of Russia, atypical for the variety, showed that nodules on roots were practically not formed, and the increase in crop yield and the protein content in vegetative mass and grain demanded introducing specific microsymbionts with seeds [8]. According to past years' research, inoculation of guar growing in Sudan with the strains of *Bradyrhizobium* spp. made an appreciable positive impact on the development of plants, greatly increased the number of nodules, dry mass of plants, nitrogen general content, and seed yield [9].

We believe that successful guar introduction in Russia requires (along with studying suitable research of soil-environmental conditions and development of technologies of cultivation) research on microsymbionts of this crop and the subsequent selection of effective strains.

This paper is the first to report on the isolation of the rhizobia species *Bradyrhizobium elkanii* from the guar nodules. Also, we have defined the taxonomic position and genetic heterogeneity of the isolated strains by sequencing 16S ribosomal DNA, ITS-region and "housekeeping" genes *atpD, dnaK* and *recA*.

The work purpose is obtaining and phylogenetic analysis of microsymbionts of plants *Cyamopsis tetragonoloba*.

Techniques. Seeds of guar (obtained from Vavilov All-Russian Institute of Genetic Resources of Plants, St.-Petersburg) were scarified and superficially sterilized in 98 % H_2SO_4 during 10 min, then carefully washed with sterile tap water and couched on filter paper in Petri dishes at 25 °C in a dark room for 3 days. Seedlings were transferred into plastic pots (12 pots, 4 seedlings per each) with

100 g of sterile vermiculite. Aqueous extracts were prepared from 12 soil samples collected in the state Rajasthan (India), and a 5 ml extract was added per pot.

Plants were grown during 45 days in a phyto-room with a 60 % relative humidity at a 4-level light exposure and temperature mode: night (darkness, 18 °C, 8 h), morning (200 μmol · m^{-2} · s^{-1}, 20 °C, 2 h), day (400 μmol · m^{-2} · s^{-1}, 23 °C, 12 h), and evening (200 μmol · m^{-2} · s^{-1}, 20 °C, 2 h), lamps L 36W/77 FLUORA (Osram, Germany). After the end of the experiment, the roots of the plants were taken out from vermiculite and washed thoroughly with tap water. The formed nodules were separated from the roots, superficially sterilized for 1 min in 70 % ethanol and homogenized in sterile tap water.

Strains of rhizobia were traditionally isolated [10] from the nodule homogenates with the use of modified mannitol-yeast agar YMSA [11] with 0.5 % succinic acid. Strains were cultured at 28 °C. All isolates are deposited to the Russian Collection of Agricultural Microorganisms (RCAM, the Station of the Low-Temperature Computerized Storage of Biological Samples, Liconic Instruments, Liechtenstein) [12]. The information on strains is accessible in the RCAM Internet database [13].

Rhizobia strains were phylogenetically assessed based on sequencing 16S rRNA gene (*rrs*) and ITS-region between 16S and 23S rRNA genes. The primer pairs used were fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′)—rD1 (5′-AAGGAGGTGATCCAGCC-3′) for 16S rDNA gene amplification (a fragment of about 1500 bp), and FGPL-132 (5′-CCGGGTTTCCCCATTCGG-3′)—FGPS1490-72 (5′-TGCGGCTGGATCCCCTCCTT-3′) for ITS-region (800 bp). For more correct taxonomic identification and study of the genetic heterogeneity of strains, "housekeeping" genes *atpD*, *recA* and *dnaK* were sequenced. Amplification of "housekeeping" genes was carried with primers atpD352F and atpD871R, recA63FD and recA504RD [14], dnaK1466Fd and dnaK1777Rd [15]. The PCR products were extracted from gel and refined [16] for sequencing (genetic analyzer ABI PRISM 3500xl, Applied Biosystems, USA). Homologous sequences were searched in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) with BLAST software [17]. A phylogenetic tree was constructed with MEGA5 software using the Neighbor-Joining method [18]. Pairs of sequences were compared according to the number of differing nucleotides. To estimate levels of clusters support, the bootstrap-analysis was carried out (1000 replicates). The obtained sequences are deposited in the GenBank database under the numbers MH938226-MH938235 for *rrs* gene, MH938704-MH938713 for ITS-region, MH982271-MH982280 for gene *atpD*, MH982261-MH982270 for gene *dnaK* and MH982251-MH982260 for gene *recA*.

Results. Ten bacterial strains were isolated from the root nodules of guar, a strain per soil sample. In two samples, nodules were not found on the roots of plants. According to 16S rRNA gene sequencing, all strains are assigned to the species *Bradyrhizobium* and form a monophyletic statistically authentic cluster with a 99 % support level (Fig. 1). In addition to isolates, the cluster included typical strains *B. elkanii* USDA 76T, *B. jicamae* PAC68T, *B. lablabi* CCBAU 23086T, *B. pachyrhizi* PAC48T and *B. tropiciagri* SEMIA 6148T. However, the *rrs* homology of new isolates was maximum (100 %) only with two typical strains, the *B. elkanii* USDA 76T and *B. pachyrhizi* PAC48T (Table). As for the strain *B. tropiciagri* SEMIA 6148T, the *rrs* gene similarity was lower and varied from 98.4 to 99.3 %; *rrs* homology for *B. jicamae* PAC68T and *B. lablabi* CCBAU 23086T was below 99 % (see Table).

To more correctly identify taxonomic position of the isolates and study their genetic heterogeneity, the analysis of three "housekeeping" genes sequences was carried out: *atpD* and *recA*, coding β-subunit of membrane ATP synthase

and DNA recombinase, respectively [14], and *dnaK* which codes chaperone preventing protein aggregation and providing their refolding upon thermal damage [15]. Figure 2 provides a dendrogram constructed on the basis of *atpD*, *dnaK* and *recA* nucleotide sequences.

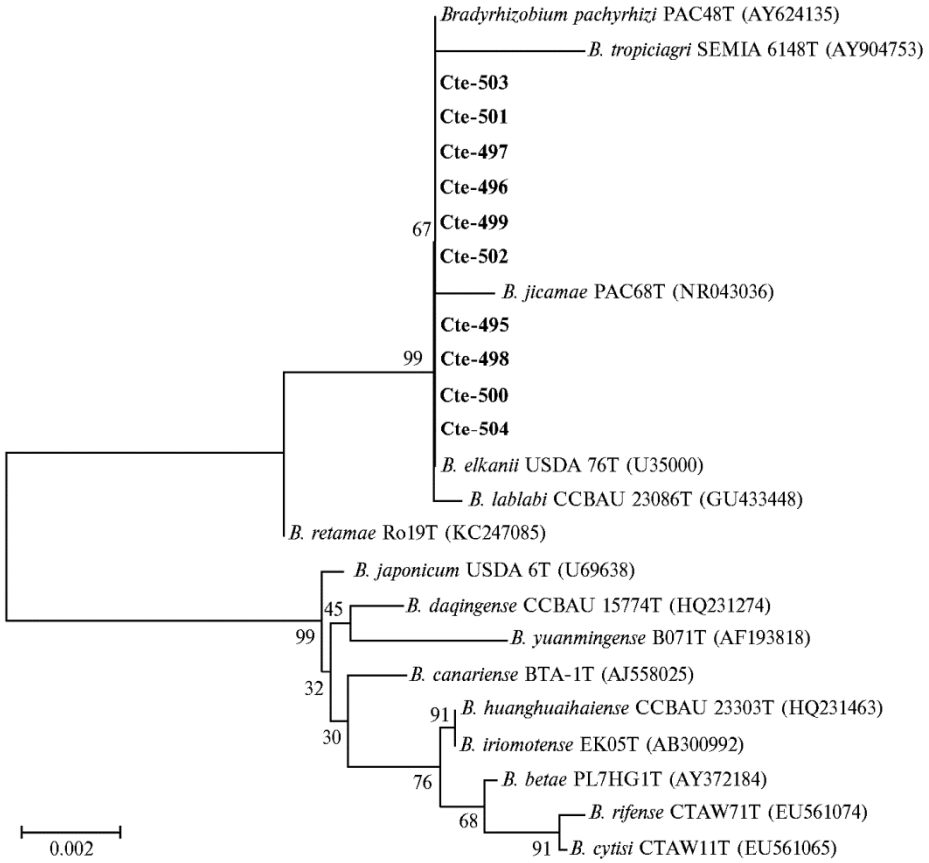

Fig. 1. *rrs*-Phylogram of isolates from guar *Cyamopsis tetragonoloba* nodules and the members of related *Bradyrhizobium* species. Typical strains are marked with the letter T. Guar strains are marked with bold (the Neighbor-Joining method).

Gene sequence homology (%) of isolates from guar *Cyamopsis tetragonoloba* nodules and the closest typical strains of genus *Bradyrhizobium*

Typical strains	Locus	Iso;ate C									
		495	496	497	498	499	500	501	502	503	504
B. elkanii USDA76T	*rrs*	100	100	100	100	100	100	100	100	100	100
	ITS	92.2	93.0	92.3	92.6	92.9	92.1	94.2	91.3	94.3	94.3
	atpD	96.1	96.5	97.4	97.8	97.8	96.8	96.0	96.2	96.3	97.0
	dnaK	98.8	98.8	98.4	97.9	98.4	98.7	98.4	98.7	98.7	99.2
	recA	94.4	95.0	94.5	94.5	94.7	94.5	95.7	94.4	96.1	94.6
B. pachyrhizi PAC48T	*rrs*	100	100	100	100	100	100	100	100	100	100
	ITS	90.6	89.8	90.6	90.7	90.8	90.2	87.9	90.6	87.9	88.3
	atpD	96.1	95.3	95.1	95.7	95.1	94.9	95.7	95.8	95.8	95.1
	dnaK	99.6	99.6	99.2	98.6	99.1	99.0	98.7	99.3	99.0	99.2
	recA	93.8	93.9	93.9	93.9	93.6	93.8	94.8	93.9	94.7	93.6
B. tropiciagri SEMIA6148T	*rrs*	99.3	99.3	98.8	99.0	99.1	99.2	98.4	99.3	99.3	99.2
	ITS	92.1	91.9	91.8	91.5	91.9	91.5	91.4	91.1	90.8	90.8
	atpD	96.9	95.7	95.7	96.2	96.3	95.3	96.6	96.8	96.8	95.3
	dnaK	96.6	96.7	96.3	96.7	96.8	96.7	96.1	97.0	96.4	96.5
	recA	94.7	95.2	95.2	94.8	94.4	94.8	95.5	94.6	95.9	94.4

All isolates were clustered at 100 % support level together with typical strains *B. elkanii* USDA 76T, *B. pachyrhizi* PAC48T and *B. tropiciagri* SEMIA

6148T. Within this group, there are two statistically authentic subclusters formed by isolates Cte-501 and Cte-503, and Cte-495 and Cte-502 (support levels of subclusters are 100 and 98 %, respectively). The maximum homology on the gene *atpD* between new isolates and typical strains was 97.8 % for *B. elkanii*, 96.9 % for *B. tropiciagri* and 96.1 % for *B. pachyrhizi* (see Table). The homology with *dnaK* varied from 98.4 to 99.2 % for *B. elkanii*, from 98.7 to 99.6 % for *B. pachyrhizi* and from 96.1 to 97.0 % for *B. tropiciagri*. According to *recA*, the maximum homology between isolates and typical strains was 96.1 % for *B. elkanii*, 95.9 % for *B. tropiciagri* and 94.8% for *B. pachyrhizi* (see Table 1).

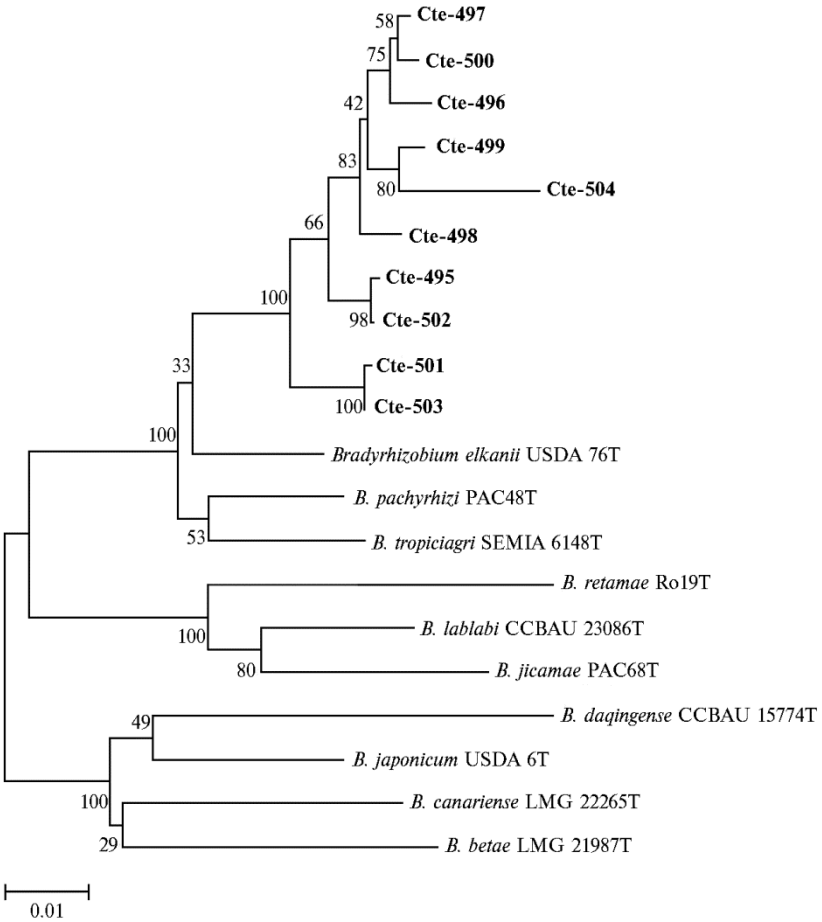

Fig. 2. Phylogram constructed for *atpD, dnaK* and *recA* genes of the isolates from the guar *Cyamopsis tetragonoloba* nodules and the members of related *Bradyrhizobium* species. Typical strains are marked with the letter T. Guar strains are marked with bold (the Neighbor-Joining method).

The analysis of the ITS-region sequence is often used to additionally identify microorganisms [11, 19]. Comparison of ITS-region showed that all isolates clustered together with a typical strain *B. elkanii* USDA 76T at 100 % statistical support level (Fig. 3). The maximum homology of ITS-region was between thee new isolates and typical strains *B. elkanii, B. tropiciagri* and *B. pachyrhizi*, 94.3, 92.1 and 90.8 %, respectively (see Table).

The name of the species *Bradyrhizobium elkanii* was offered in 1992 [20] for a homologous group of strains within the existing species *B. japonicum*, described for nitrogen-fixing soya microsymbionts *Glycine max* [21]. Both species belong to a predominant genus capable to nodule the majority of varieties of the tribe *Genisteae* [22]; the most known representatives are lupine (*Lupinus*), broom

(*Cytisus*) and greenweed (*Genista*). However, the strains *B. elkanii* were also discovered in nodules of leguminous plants from the tribe *Indigofereae*, which guar belongs to, i.e. in cow pea (*Vigna unguiculata*, *V. radiate*), rosewood (*Dalbergia odorifera*) and beggarweed (*Desmodium incanum*) [23-25]. Earlier on, two isolates from *Cyamopsis*, XBD2 SARCC-388 and ENNRI 16A) capable of effective nitrogen fixation and identified as *B. japonicum* and *Bradyrhizobium* sp., respcetiely, were described [26, 27]. However, the guar was primarily studied for enriching the natural gum properties [28], resistance to illnesses and selection of highly productive varieties [2, 29]. Probably, it is due to no necessity of inoculation because of the sufficient indigenous microsymbionts in places of traditional cultivation of the crop. However, introduction of guar to new habitats makes the plant-microbe symbiosis, which provides plants with nitrogen nutrition, the major factor to effectively cultivate this crop.

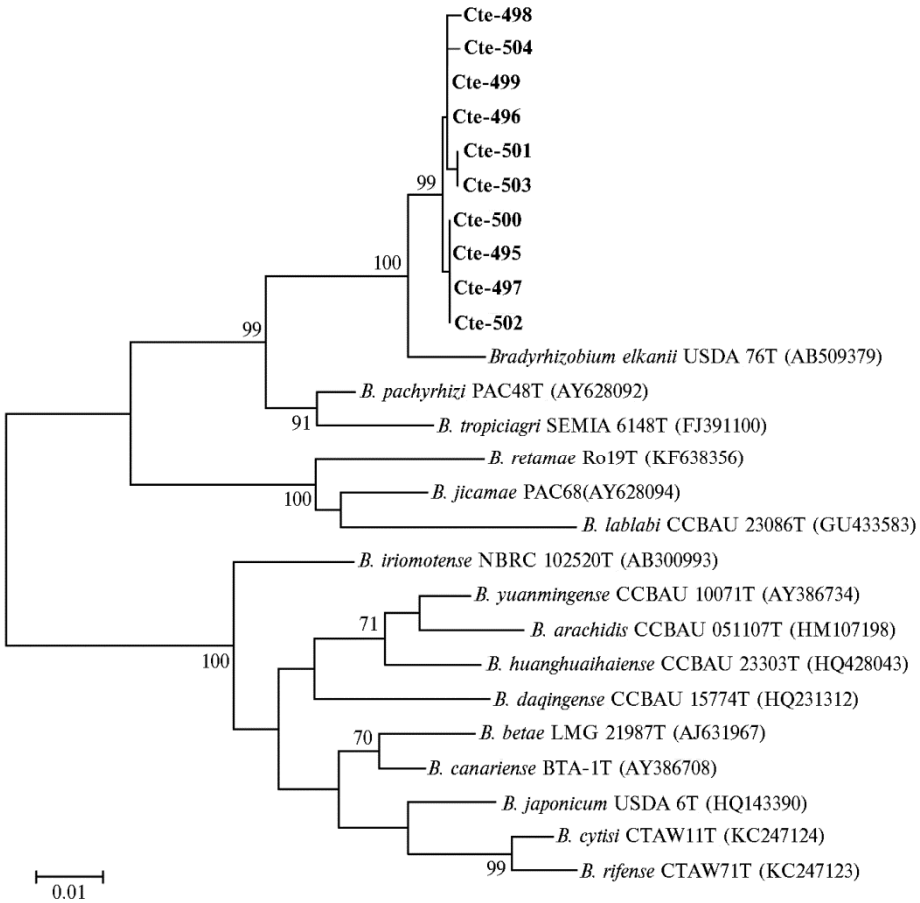

Fig. 3. ITS-phylogram of isolates from guar *Cyamopsis tetragonoloba* nodules and the members of related *Bradyrhizobium* species. Typical strains are marked with the letter T. Guar strains are marked with bold (the Neighbor-Joining method).

Thus, by sequencing *rrs* gene, ten strains isolated from guar root nodules in pot tests, are referred to the species *Bradyrhizobium*. Additional identification (sequencing the ITS-region and three "housekeeping" genes, *dnaK, recA* and atpD), allowed us to specify the taxonomic position of isolates and to show their relation to species *Bradyrhizobium elkanii*. Earlier members of this species were not known as microsymbionts of *Cyamopsis tetragonoloba*. The sequencing of ITS-region and "housekeeping" genes show genetic heterogeneity of natural population of guar microsymbionts that can testify to distinctions in symbiotic

mutual relations between plants and the isolated strains. Further study of genetic diversity, morphological, physiological, biochemical features and economically useful traits of the guar nodule bacteria will allow us to widen knowledge about phylogeny of microsymbionts of this crop which is rather new to Russia and also to select strains effectively improving plant growth and quality of production in new regions of cultivation.

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SCREENING OF SWEET CLOVER (*Melilotus* Adans.) SPECIES DIVERSITY FOR RESISTANCE TO CHLORIDE SALINIZATION

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Abstract

Saline soils are widespread in the world, including in CIS countries. Species of sweet clover (genus *Melilotus* Adans.) which are valuable fodder plants, e.g. at insufficient moisture on chestnut saline soils, are also among the best phytomeliorants in the legume family. However, during early stages of growing the sweet clover has poor salinity tolerance. Here, we modified earlier developed test and screened accessions of the VIR World Collection (Vavilov All-Russian Institute of Plant Genetic Resources) for resistance to chloride salinity, and for the first time identified among them the forms with high salinity resistance. In the research we used a laboratory method of assessing salt tolerance. Samples of white and yellow sweet clover were grown under controlled conditions, in water culture, according to the following scheme: control (without salinity); salinity of 3 atm. NaCl; the salinity of 5 atm. NaCl. The length of root and sprout of seedlings were measured after 5-day exposure to the stressor. For each sample we calculated the indices of the root length and the sprout length as the ratio of the relevant medium parameters of plants in the test to that in the control. The higher the index value, the more salt-tolerant the sample is. In our tests, chloride salinity caused growth inhibition of roots and shoots samples of both species. The degree of negative growth response intensifies with an increase in NaCl concentration. It was shown that salt stressor intensifies intra- and interpopulation variability of sweet clover growth indicators. It is established that the root length index is more informative diagnostic criterion for laboratory screening for salt tolerance among sweet clover plants. The studied fragment of the collection includes 36.2 % of resistant samples of white sweet clover and 29.6 % of yellow sweet clover, i.e. the adaptive capacity towards excess of sodium chloride in the root zone of the studied species is approximately the same. Salt-resistant varieties of white clover come from Far East (Ryadovoi and Diomid), Kazakhstan (Shedevr 75), Novosibirsk (Lucernovidnyi D-20) and Ivanovo (wild sample k-15650) regions; of yellow sweet clover — from Western Siberia (Omskii 8, Omskii 916, Novosibirskii 1, Severotatarskii, Khorog 2155). Selected salt tolerant forms can serve as initial parental forms for creation of yellow and white sweet clover varieties resistant to chloride salinity during early stages of plant development. This will allow better use of the potential of these species for forage, and for bioremediation goals. According to the obtained data, salt-tolerant sweet clover samples often originate from a region with widespread saline soils that should be considered to determine the strategy of search for forms with edaphic resistance valuable for breeding. Many of the selected salt-tolerant varieties are also characterized by other important breeding traits: high productivity, intensive regrowth after cutting, precocity, biotic and abiotic resistance to viruses, drought, low temperatures, unfavorable overwintering, valuable biochemical properties such as high protein content and low coumarin level.

Keywords: chloride salinity, *Melilotus*, sweet clover, salt tolerance, growth indicators, variability, root length index

Saline soils of different types and salinization degrees occupy about 550000 km^2 of the former USSR territory. In the Russian Federation, they are most widespread in the North Caucasus, in the Lower Volga Region, in the Southern Urals, and in Western Siberia. As an effective method for improving the state of saline soils, one can consider salinity control by means of cultivation of resistant plant species [1, 2]. Leguminous plants are widely used in soil remedia-

tion though, according to some data, display a moderate salt tolerance [3]. Meadows formed on saline soils by perennial leguminous grasses promote soil their enrichment with nitrogen, restoration of fertility and recovery to agriculture.

Species of the genus *Melilotus* Adans. (sweet clover) are among the best phytomeliorants in the family *Fabaceae*. Sweet clover species grow in saline soils where the traditional leguminous pants do not survive [2, 4]. The origin of the genus is Eurasia and North Africa; the representatives of the genus are resistant to heat and cold [5, 6]. Twenty-six species of sweet clover are described; the genus is presented by 13 species in the former USSR area [7]. The following species are integrated into agriculture: sweet clover *M. albus* Medik., yellow melilot *M. officinalis* (L.) Pall., Volga sweet-clover *M. wolgicus* Poir., plaster clover *M. suaveolens* Ledeb., dentated melilot *M. dentatus* (Waldst. et Kit.) Pers., hairy melilot *M. hirsutus* Lipsky, and Caspian melilot *M. caspicus* Grun. [8]. Some samples of dentated melilot and Indian melilot are noted for high salt tolerance [9-11]. In the world, melilot sowings occupy 1.2-1.4 million hectares and are mostly concentrated in Canada and the CIS (Western and Eastern Siberia, the Volga Region, Kazakhstan, Yakutia, the Southern Ural Mountains, the North Caucasus, Bashkiria, the Far East). In the CIS countries, these species are widespread almost in all floral zones [12]. Melilot is a perspective fodder, phytomeliorative, medicinal and melliferous crop; the increasing attention is given to it as green manure [13, 14]. The wide application of melilot is due to its high ecological plasticity. Due to the deeply penetrating root system, melilots can grow in a wide range of edaphic conditions; melilot is drought-resistant, winter-hardy, not exacting to soils fertility [15]. The greatest green mass productivity among perennial leguminous crops is most typical for sweet clover and yellow melilot; sweet clover is more valuable as a fodder [15].

Disorder of the activity of nodule bacteria responsible for the fixation of atmospheric nitrogen [16] becomes one of the main causes of growth oppression of leguminous plants in saline soils. Melilot in the conditions of soil salinization is capable to form rather big green mass yield and to enrich the soil with nitrogen, which makes it applicable for the subsequent cultivation of other crops [15]. Now, research on the use of this culture in the saline soils is conducted in the Central [16], Volga [17, 18], Southern [19-22], North Caucasian [23] and Siberian [24] regions.

Melilot, as well as many other crops [25], displays high sensitivity to the raised salts content in the soil. In this connection, search for melilot forms with high salt tolerance in the initial growth of plants (seed germination and sprout growth) is considered relevant for research.

The collection of melilot of VIR (Vavilov All-Russian Institute of Plant Genetic Resources, St.-Petersburg) contains about 1000 samples (species, varieties, selection and wild-growing samples). The collection is studied for a series of valuable traits, i.e. winter hardiness, biochemical composition, morphological variability [26], resistance to sour soils [27, 28]. Now in Russia, it is admitted to use 15 species of white melilot and 8 species of yellow melilot. In laboratory screening for salt tolerance, one often uses sprouts growth indexes as a test parameter [29, 30], depending both on the stress factor intensity (salts content in the roots zone) and on the genotype properties [31, 32]. In the conditions of chloride salinity, ions of Na and Cl coming into cells compete with the major nutritious elements, first of all, with ions of potassium, calcium and magnesium, which leads to toxic and metabolic stresses in plants, their oppression, and sometimes to destruction at early stages of ontogenesis [33-35]. Now mechanisms of acclimatization of plants in the conditions of the raised salt content in soil [37] have been revealed for some leguminous plants (*Medicago truncatula*

Gaertn.); they are studied in details [38].

In this work, using a modified screening methodology, we for the first time found among VIR collection accessions the forms high-resistant to salinization which are perspective for use as a source of salt tolerance in breeding.

The purpose of the studies is revealing a specific diversity of the collection samples of melilot for resistance to chloride salinity at the early stages of ontogenesis.

Techniques. Fifty-eight samples of white melilot and 27 samples of yellow melilot of various ecogeographical origins, including cultivated and wild-growing forms, were studied. Samples were grown under artificial lightening (10 klx, 16 h photoperiod, day temperature +21 ... +22 °C, night temperature +17 ... +18 °C). The seeds stratified with sandpaper (50 pieces per sample) were put in germinator cells with a mesh bottom. Germinators were placed in containers with distilled water for 2 days so that the grid touched the water surface; above they were covered with a polyethylene film. Not germinating seeds were rejected. Then germinators with seeds starting to germinate were transferred into solutions according to the trial schema: control (distilled water); stress background 1 (NaCl solution with osmotic pressure 3 atm.); stress background 2 (NaCl solution with osmotic pressure 5 atm.). After 5 days, the root and sprout length was measured.

For statistical data processing, Statistica 6.0 software (StatSoft, Inc., the USA) and Systat 10.2 software (Systat Software, Inc., the USA) were used. For each sample, the mean value (M), the standard error of the mean (±SEM) and the variation coefficient (Cv) were calculated. The informative value of indicators was estimated by comparison of the intrapopulation variability of the initial traits (the mean variation coefficient within the populations Cv_1) and their interpopulation variability (the variation coefficient among the means of the populations Cv_2). According to the mean values of the root and sprout length, the root and sprout length indexes (IR and IS) were calculated as a proportion of the index in a trial variant to the control (the higher the index value, the more the sample is resistant to salinization). For splitting plants into groups according to salinity resistance for the totality of the calculated indexes, confidence intervals of the means were calculated. Samples having the index value below the low level of the confidence limits were considered low-tolerant, those from the low and the upper levels of confidence limits were moderately tolerant, and those above the upper level of confidence limits were deemed tolerant.

Results. There are various laboratory methods of plant testing for salt tolerance at the early stages of ontogenesis [39]. To choose an optimum method, we have considered available data on the use of morphometric indexes in diagnostics, in particular, the lengths of sprouts and the roots of seedlings [40-42]. The method to estimate melilot salt tolerance developed earlier [43] assumes the registration of seed germination after salt exposure, that is, it covers earlier stages of plants development, from seed swelling to root emergence. In our opinion, screening indexes, in this case, can depend on seeds quality. According to the developed modification, the diagnostics are carried out for photosynthesizing sprouts, with prior rejection of poor seeds. Besides, such modification provides high performance and reproducibility of the trial.

In the aqueous culture, there was a significant genotypic variability of growth indexes in all variants of the trial. In control (absence of salinization), the most intensive growth of roots was typical for the varieties of sweet clover Kinelskii (Samara Region), Neosypayushchiisya (Krasnodar Territory), Bashkirskii (Republic of Bashkortostan), Yantar (Irkutsk Region), Mestnyi (Austria). Yellow melilot demonstrated the intensive growth of roots in the absence of salt

stressor in the varieties from Canada (Goldtop and Yukon). Wild-growing samples of sweet clover from Kazakhstan (k-48543), the Republic of Yakutia (k-46992), the varieties of yellow melilot Yukon and Norgold (Canada), No. 28, No. 29 (the USA) showed high rate of sprout length growth under the control conditions.

The chloride salinity in the roots zone, as a rule, led to the intensifying intrapopulation and interpopulation variability of growth indexes (with the exception of the interpopulation variability of sprout length in yellow melilot) (Table 1). Both in control and salinization groups, the intrapopulation variability of root length and sprout length mostly exceeded the interpopulation one. Note, in both species in all trial variants, intrapopulation variability of root length was higher compare to sprout length.

Under salinization, the intrapopulation variability of root length increased more significantly than sprout length; therefore, for salt tolerance diagnostics, the length of roots was used. A similar result was obtained in seeking for a diagnostic criterion for the melilot estimation for resistance to aluminum toxicity in sour soils [27, 28].

1. Intrapopulation and interpopulation variability of root and sprout length in species of sweet clover *Melilotus albus* Medik. and yellow melilot *M. officinalis* (L.) Pall. depending on cultivation conditions

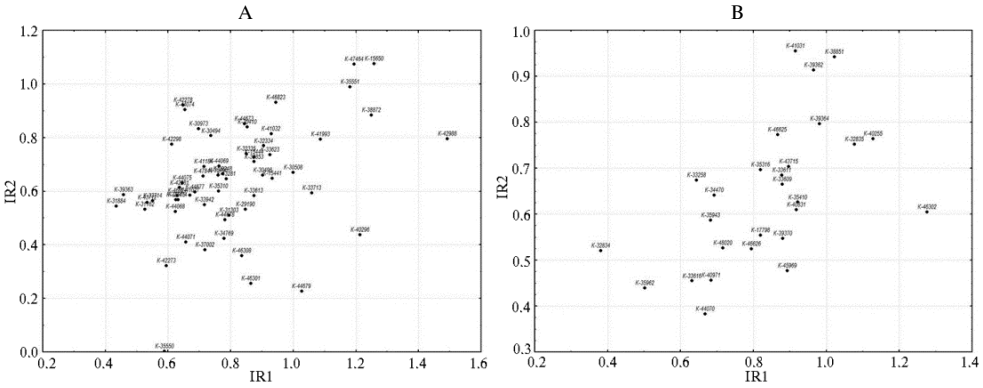
Indicator	*Melilotus*											
	albus			*officinalis*			*albus*			*officinalis*		
	root length						sprout length					
Osmotic pressure of background, atm.	0	3	5	0	3	5	0	3	5	0	3	5
Intrapopulation variability (Cv_1, %)	45	49	46	34	47	41	24	26	28	23	25	26
Interpopulation variability (Cv_2, %)	21	26	34	22	29	30	19	22	25	26	26	21

In salinization, the repression of sprout growth increased with NaCl concentration increasing. The root length index in sweet clover was 0.44-1.49 (Cv = 3.5 %) at osmotic pressure of 3 atm. and 0.003-1.07 (Cv = 4.2 %) at 5 atm.; for yellow melilot, these values were 0.38-1.28 (Cv = 4.4 %) and 0.38-0.95 (Cv = 4.6 %), respectively.

For allocation of the samples in groups according to their salt tolerance, we used the confidence limits of the mean of the roots length index. The samples were distributed in three conditional groups: tolerant, moderately tolerant and low-tolerant. According to the screening, 36.2 % samples were tolerant to chloride salinity among sweet clover samples and 29.6 % samples among yellow melilot. The greatest part of white melilot (39.7 %) and yellow melilot (37.1 %) samples were low-tolerant. Sweet clover samples that showed a higher salt tolerance originated from the Primorye territory (Ryadovoi and Diomid), Kazakhstan (Shedevr 75) and the Novosibirsk Region (Lutsernovidnyi D-20), that is, from regions with saline soils. High salt tolerance was characteristic of the wild-growing sample k-15650 from the Ivanovo region (Table 2, Fig., available http://www.agrobiology.ru). The root length index of this sample at the maximum NaCl concentration (as well as of the variety Diomid) was the highest. Among the samples of yellow melilot, the greatest salt tolerance was in varieties from Western Siberia Omskii 916, Novosibirskii 1, Severotatarskii, Khorogskii 2155, Omskii 8 (the originators are the Siberian Research Institute of Forages and the Siberian Research Institute of Agriculture) (see Table 2, Fig.). They can be involved in breeding for adaptability to saline soils.

Some wild-growing samples and varieties with high salt tolerance at early ontogenesis also have other valuable traits. So, the variety Shedevr 75 (Aktyubinsk experimental station) is characterized by precocity, fast regrowth after mowing, high drought and winter hardiness, resistance to virus diseases, a high

content of protein and a low content of coumarin. The variety Ryadovoi from the Primorye Territory is characterized by the intensity of regrowth after mowing, high winter hardiness, less hard seeds, belongs to low coumarin varieties. The highly productive variety Diomid (Primorskiy Research Institute of Agriculture and VIR Far East Experimental Station) obtained from wild-growing samples by mass selection shows fast regrowth in spring, high fodder properties; it is resistant to drought and unfavorable factors of overwintering, rather resistant to fungal diseases. The variety Omsk 8 shows intensive regrowth after mowing, high productivity, drought resistance, winter hardiness and a high content of protein. Important traits for selection, such as high productivity, intensive regrowth after mowing, precocity, bio- and abiotic resistance (to viruses, drought, low temperatures, unfavorable factors of overwintering), and valuable biochemical properties (high content of protein, low coumarin) are typical for many of the salinity-resistant varieties studied (the data are not shown).



Allocation of samples of sweet clover *Melilotus albus* Medik. (A) **and yellow melilot *Melilotus officinalis* (L.) Pall.** (B) **according to salt tolerance at two salinization environments:** IR1 — the root length index at osmotic pressure of 3 atm., IR2 — the root length index osmotic pressure of 5 atm.

2. Samples of sweet clover *Melilotus albus* Medik. and yellow melilot *Melilotus officinalis* (L.) Pall. with high salt tolerance at early ontogenesis

No. in the VIR catalog	Variety (origin)	Root length, mm (M±SEM)			Root length index IR	
		control	3 atm. NaCl	5 atm. NaCl	3 atm. NaCl	5 atm. NaCl
	Sweet clover					
k-15650	Wild (Ivanovo Region)	2.7±0.20	3.4±0.24	2.9±0.21	1.26	1.07
k-35551	Ryadovoi (Primorye Territory)	2.8±0.37	3.3±0.44	2.8±0.72	1.18	0.99
k-38872	Shedevr 75 (Kazakhstan)	1.6±0.22	2.0±0.34	1.4±0.16	1.25	0.88
k-42988	Lutsernovidnyi D-20 (Novosibirsk Region)	3.2±0.59	2.0±0.45	2.5±0.33	1.49	0.79
k-47464	Diomid (Primorye Territory)	2.0±0.38	2.4±0.35	2.2±0.31	1.19	1.07
	Yellow melilot					
k-38851	Omskii 916 (Omsk Region)	3.5±0.11	3.6±0.17	3.3±0.13	1.02	0.94
k-39362	Novosibirskii 1 (Novosibirsk Region)	3.3±0.20	3.2±0.32	3.0±0.26	0.97	0.91
k-39364	Severotatarskii (Novosibirsk Region)	3.6±0.27	3.5±0.32	2.8±0.25	0.98	0.80
k-40255	Khorogskii 2155 (Novosibirsk Region)	3.0±0.16	3.4±0.19	2.3±0.12	1.13	0.76
k-41031	Omskii 8 (Omsk Region)	2.1±0.19	1.9±0.35	2.0±0.24	0.91	0.95

The chloride salinity, as a rule, led to intensifying the intra- and interspecific variability of melilot sprout growth indexes. It is necessary to notice similar regularity for other plant species under the influence of unfavorable environmental factors. For example, under the drought conditions, variability of some traits among spring wheats was higher [44].

The intraspecific variability of root length of melilot seedlings under salinization increased to a greater degree compared to that of sprouts, which testifies to a higher informative value of the root length index to estimate salt tolerance

of melilot and other crops [44]. Linear characteristics of roots are used in studying plant resistance to other abiotic stressors, in particular, to the aluminum toxicity of sour soils [45], drought [46] and low temperatures [47].

The samples tolerant to salinization among the studied sweet clovers and yellow melilots makes 36.2 and 29.6 %, respectively, that is, the adaptation potential of these two species to the excess of sodium chloride in the root zone is approximately identical. Similar results are obtained in Australia while studying the influence of soil salinization on the growth and development of annual and perennial species of melilot introduced there [48].

According to the data obtained in this trial, salinity-resistant species and samples of melilot often originate from regions with widely spread saline soils. This fact must be considered in a strategy of seeking forms valuable for selection for salt tolerance. Perspective varieties may derive from crossing parental forms with high green mass productive and tolerance to salt stress [49]. Despite the available varietal diversity of melilot, the search for new genotypes always remains relevant to create more productive cultivars better adapted to unfavorable environmental factors [50].

Thus, the samples of white and yellow melilot with high initial intensity of plant growth under controlled conditions (in absence of salinization) are for the first time revealed by us in the VIR collections. The presence of sodium chloride in the root zone negatively affects growth indexes of plants. The more severe the stress, the more apparent inhibition of root and sprout growth occurs. Several tested samples and varieties can serve as a new initial material to create yellow and white melilot varieties with tolerance to chloride salinity in early ontogenesis, which will provide better use of their potential for fodder production and bioremediation. Among salinity tolerant varieties that we revealed there are those with high productivity, intensive regrowth after mowing, precocity, bio- and abiotic resistance, and valuable biochemical properties.

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