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MECHANISMS OF IMMUNE RESPONSE AND PROSPECTS FOR DNA VACCINES AGAINST AFRICAN SWINE FEVER

(review)

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Abstract

The agent of African swine fever (ASF) is a large envelope virus (ASFV) belonging to family *Asfarviridae* and containing a double-stranded linear DNA of 170 to 190 kb in size coding for more than 150 proteins, most of which are involved in host-virus interactions (L.K. Dixon et al., 2004). Its virulent isolates cause a contagious hemorrhagic disease with 100 % mortality both among domestic pigs (*Sus scrofa domesticus*) and wild boars (*Sus scrofa*). The disease control is complicated by the lack of any specific preventive methods (R.J. Rowlands et al., 2008; D.A. Chapman et al., 2011; P. Rahimi et al., 2010). The attempts to protect pigs against ASF with experimental live and inactivated subunit vaccines developed by standard methods failed (S. Blome et al., 2014). This paper discusses immunological mechanisms to provide the specific defense base on potentially protective virus-specific proteins, and immunogenic and some protective properties of ASFV gene-based DNA constructs. Immune protection at ASF is due to cytotoxic T-lymphocytes (CTL) and antibody-dependent cell-mediated cytotoxicity (ADCC) effectors against viral proteins located on infected monocyte/macrophage. There is a synergism of these effectors (A.D. Sereda, 2013). Based on i) the location, structure and functional properties of viral proteins, ii) the polypeptide specificity of blood antibodies after injecting pigs with ASFV attenuated or virulent strains, iii) the effects of pig immunization using purified proteins from infected cells or the recombinant proteins, and DNN constructs, p30, p54 and CD2v proteins are considered as potentially protective (S.D. Kollnberger et al., 2002; M.G. Barderas et al., 2001; J.G. Neilan et al., 2004). A significant disadvantage of the candidate DNA vaccine is a relatively low immune response, especially in large mammals. There were attempts of overcoming the problem using various strategies (J. Rajcani et al., 2005, M.A. Liu et al., 2006; L.H. van Drunen et al., 2004; J.A. Leifert et al., 2004). To target the lymphocytes expressing receptors CD48 and CD58 to the protein CD2 of the antigen presenting cells (APC), the secretory part (s) of ASFV protein HA (or CD2v) has been used (A. Brossay et al., 2003; K. Crosby et al., 2004). The addition of sHA gene to the DNA construct enhanced both humoral and cellular responses in pigs against fused recombinant proteins p30 and p54 (F. Ruiz-Gonzalvo et al., 1996). An increase in the humoral response due to targeting p30 and p54 fused to one chain of the antibody recognizing the invariant epitope of pig class II main histocompatibility complex (MHC) was demonstrated. However, the enhancement of the humoral immune response to p30 and p54 rather often resulted in earlier death of pigs infected with virulent strains. To stimulate the specific $CD8^+$-T-cell responses, a pCMV-UbsHAPQ construct coding for antigenic determinants p30, p54 and sHA fused with cellular ubiquitin (Ub) was developed. The immunization using pCMV-UbsHAPQ did not induce an instrumentally determined antibody response though provided partially pig protection against ASFV challenge (J.M. Argilaguet et al., 2011). The potential of the DNA constructs was confirmed by pig immunization using ASFV DNA libraries ($ASFV^{Ublib}$) coding for viral genome short fragments combined with the cellular ubiquitin gene (A. Lacasta et al., 2014). In the 4029 clones, about 76 % of the viral genome (130 kb) were covered. As many as 60 % of $ASFV^{Ublib}$-immunized pigs survived after infection with an ASFV virulent strain. According to ELISA, none of the $ASFV^{Ublib}$-immunized pig had detectable specific antibodies to ASFV proteins prior to the challenge. The $CD8^+$-T-cells comprised the only cell sub-

population among the studied ones that showed a statistically significant growth in the survived pigs starting from day 5 post immunization. The opportunities for a vaccination strategy based on the use of BacMam viruses that are baculovirus vectors encoding viral antigens under the control of cell-active promoters of vertebrates have been analyzed (J.M. Argilaguet et al., 2013). Immunization with recombinant baculovirus (BacMam-sHAPQ) encoding two ASFV full-length immunodominant proteins p30 and p54 fused to a carboxyl terminus of the extracellular domain of a viral hemagglutinin sHA resulted in no viraemia or clinical signs of the disease in 66 % of the pigs. Moreover, BacMam-sHAPQ-immunized animal had no ELISA-detectable virus-specific antibody prior to challenge. Thus, the prospect for development of DNA vaccine against ASFV seems to be encouraging.

Keywords: DNA vaccines, African swine fever, protective proteins, antibody, cytotoxic T-lymphocytes

The agent of African swine fever (ASF) is a large envelope virus (ASFV) belonging to family *Asfarviridae* and containing a double-stranded linear DNA of 170 to 190 kb in size coding for more than 150 proteins, most of which are involved in host-virus interactions [1]. Its virulent isolates cause a contagious hemorrhagic disease with 100 % mortality both among domestic pigs (*Sus scrofa domesticus*) and wild boars (*Sus scrofa*). First ASF outbreak was registered in 2007 in Caucasus region of Georgia [2]. From there disease had spread to Armenia, Azerbaijan, Nagorny Karabakh, Iran, Abkhazia, and Russia, and further to Ukraine, Belarus, Baltic States, and Poland [3]. The disease control is complicated by the lack of any specific preventive methods and preparations despite permanent attempts of their developing. All traditional preparations, i.e. viral strains inactivated by UV radiation, freon, ionic and nonionic detergents, β-propiolac-tone, etc., extracts from cell cultures of lung, marrow, and spleen macrophages of the infected pigs, purified virions fixed on bull erythrocytes, mycobacteria, γ-globulin, infected macrophages fixed by glutardialdehyde, etc. [4-6], were ineffective. At ASF epizooty during years 1962-1963 at Iberian Peninsula, large-scale vaccination with attenuated ASFV strains in Portugal and Spain has resulted in 3-6-fold increased number of sites at risk of ASF outbreaks, 10-50 % mortality of the vaccinated livestock, and clinical manifestation of the disease during post-vaccination and farther periods [7]. Such complications of the disease could be due to insufficient information on the attenuated strains or their antigenic non-conformity to the circulated virulent virus since there had been a report of several immunologic types of ASFV at Iberian Peninsula [8]. In USSR, laboratory attenuated strains and their various formulations (so called live vaccines) for temporary pig protection from ASFV of seroimmunotypes I-V have been obtained based on the concept accepted in the 1970s [9]. The disadvantages of live vaccines include emergence of carriers of the attenuated strain manifested to some degree, likelihood of partial restoration of vaccine strain virulence, development of subclinical infection sometimes turning into a chronic form, and insufficient protectiveness in animals with low immunity, for instance bred sows. Further infection of animals, vaccinated by attenuated strains, with virulent ASFV strains of homologous seroimmunotypes usually resulted in virus carriage in survived pigs [9-11].

These findings demonstrate that eradication of the disease requires taking strict veterinary and sanitary measures involving total slaughter of pigs in the infection foci and in the first threatened zone. Nevertheless, something is expected from DNA vaccinations. Here, we would consider immunologic special protection mechanisms, potentially protective virus-specific proteins, approaches to construction of DNA vaccinations bearing ASFV genes, as well as research findings of their immunogenic and protective properties

Immunologic mechanisms of specific protection. For development of next generation vaccines, it is important to know mechanisms of specific protection, and the role and interrelation of humoral and cell immunity at ASF. ASFV-specific antibodies are considered as important protection compo-

nent. Transfer of serum and colostrums from the survivors to intact pigs prior to their infection with corresponding virulent isolate might delay clinical manifestations, decrease viraemia, and increase the percentage of survived animals. Out of animals, which had received anti-ASFV immunoglobulin (Ig), 85 % had survived after the infection with virulent ASFV strain E75 compared to 100 % mortality in the control where pigs had received either Ig against pseudorabies or normal pig Ig, or phosphate buffer solution. In addition to the significantly delayed and poorly manifested fever, animals receiving anti-ASFV Ig remained clinically healthy after infection. There was a 10000-fold reduction of average and maximum virus titers as compared to the control group, in which clinical signs of ASF have been manifested as early as 4 days following infection [12].

It should be noted that humoral immunity response mechanisms remain debatable over a long time. Although in 1980s the opinion prevailed that ASFV does not induce neutralizing antibodies [13], based on findings published in 1990s it was hypothesized that virus neutralizing antibodies are critical in protection against ASF [14]. It has been reported on specific virus neutralizing antibodies to ASFV structural proteins p30, p54 and p72 [15]. The pigs of three groups were immunized three times with ASFV proteins p30 and p54, which were expressed in cells of insects infected by recombinant baculoviruses with inserted genes for p30 or p54 proteins. Group I was challenged with recombinant protein p30, group II was challenged with p54, and group III was challenged with combination of p30 and p54. The immunoblotting had confirmed that serum of immunized pigs contained antibodies specific to injected recombinant proteins. Within 15 days following the last immunization, the animals were intramuscularly infected with high-virulent strain ASFV E75 in dosage of 10^2 HAU_{50}. Mortality rate in the non-immunized group reached 100 % on days 5 to 6; animals immunized by either p30 or p54 died on days 5-10, in this the development of viraemia in these animals was similar. Upon combined immunization with p30 and p54, disease development widely varied. Of six infected pigs, two pigs became ill 3 days after control pigs but survived; three pigs, which became ill 10 days after the animals immunized separately by each protein, died on days 21-27 following the challenge, and one animal showed no clinical symptoms of the disease and viraemia. Only in one pigs out of three survived animals, ASFV was found by PCR on day 46 after immunization only in supramaxillary lymph nodes, whereas outcomes were negative in other samples of immune system organs. The authors have associated partial protective effect of combination of p30 and p54c proteins with their ability to induce antibodies which can prevent virion attachment or penetration into target cells [16]. The opposite hypothesis had also been practically and theoretically validated. Subject to such hypothesis, formation of immune complexes promotes penetration of virus into target cells, the monocytes (macrophages), due to activation of phagocytosis. The above-mentioned effects of serum antibodies in vivo have been associated with antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism [17, 18].

It was shown that cytotoxic T-lymphocytes (CTL) are important for maintenance of virus-specific protection in the early period following ASFV infection [19, 20]. Cytolysis percentage of macrophages infected by homologous isolate was higher than that for heterologous isolates, which evidenced on immunotype specificity of CTL [21]. Critical role of virus-specific CTLs in protection against ASF was most visibly proved by the fact that in vivo exhaustion of $CD8^+$ T-cells by monoclonal antibodies abolishes protective immunity formed after inoculation of attenuated strain OUR/T88/3 [22].

Therefore, immune protection against ASFV is likely due to effect of

killer T-lymphocytes and ADCC against viral proteins at surface of infected monocytes (macrophages) [20, 23]. Synergy of CTLs and antibodies in ADCC had been proved by in vitro tests with the use of homological cultures of leukocyte cells obtained before and on day 6 post injection of high dose of the attenuated ASFV strain. Antibody-mediated protection mechanisms had exceeded those of CTLs in ability to restrict ASFV reproduction [24]. It appears that virus-specific protection against ASF is ensured by not just one, but several proteins, which all induce either humoral or cell immunity effectors [25].

Potentially protective proteins. Given i) localization, structure, and functions of virus induced proteins manifested in virion envelope and cytoplasmic membrane of infected cells, ii) polypeptide specificity of pigs' blood antibodies against virus proteins at early stages after injection with attenuated or virulent ASFV strains, iii) effect of immunization with proteins purified from the infected cells, recombinant proteins, and with DNA constructs, the p30, p54, p73 and CD2v proteins have been previously considered as potentially protective [16, 17, 25-27].

Inner membrane of ASFV virion includes proteins p12 (dimer), p22, p54 and p30, outer membrane which derived upon budding of virus particle through cell plasmatic membrane consists of p12, p24 and CD2v [28]. Proteins p12, p30 and p54 are critical at ASFV attachment and penetration into target cells. It is assumed that antibodies to p54 block virion binding to cell surface, whereas antibodies to p30 inhibit virus entering inside the cell [15, 16]. Based on radioimmuneprecipitation data and immunoblotting, p30, p54 and p72 have been characterized as highly-immunogenous (inducing antibodies) [29-31]. Processes determining the results of pig ASFV infection which can be instrumentally recorded take place from day 3 to day 10. ACCD was observed within 3-6 days after high-dose inoculation of the attenuated strains FC-135, and primary CTLs were detected from day 4 to dat 10. As late as on days 3-6 following injection of the attenuated, low-virulent or virulent ASFV strains, radioimmuneprecipitation had revealed 30 and 36-39 kDa polypeptide antibodies in blood which were p30 and non-glycosylated p54, respectively [17]. Less sensitive immunoassay and immunoblotting with the use of recombinant p30 as an antigen allowed detection of the antibodies since days 7-8 after infection with various virulent ASFV strains [32-34].

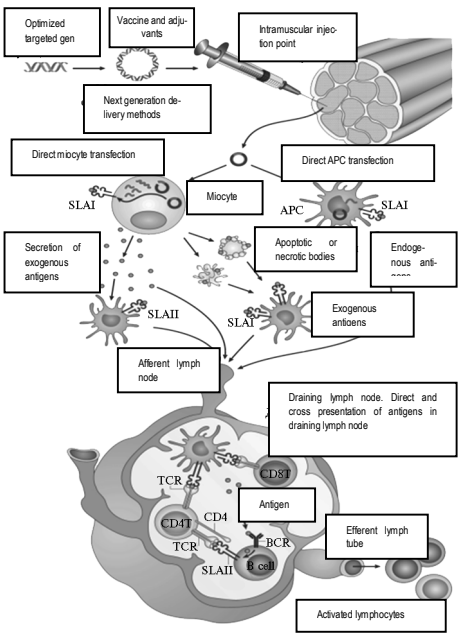
Proteins p30, p73, major glycoprotein GP 110-140 (or CD2v, HA) have been considered as potential antigens for CTL [35]. CTL induction might explain partial protection against ASFV after immunization with recombinant proteins p30 and p54 expressed in baculovirus [16], with hemagglutinin sHA ASFV or serotype specific HP 110-140 in liposomes [36].

Enveloped viruses, as a rule, have protective glycoprotein defining serotypical specificity. For ASFV, major glycoprotein GP 110-140 owns serotypical specificity [35]. Based on similarity of physical and chemical properties, this protein corresponds to CD2v encoded by *EP402R* gene, which, as we know, directly participates in hemadsorption upon ASFV infection of the susceptible cells [37]. In confirmation of this fact, it had been established that data of genotyping for locus encoding CD2v corresponded to distribution of ASFV isolates and strains by seroimmunotypes [38]. Assumedly, this protein could be main CTL inductor. Crucial role of CD2v (GP 110-140) in protective immunity upon ASF infection had been practically validated. Fourfold immunization of pigs with purified GP 110-140 in liposome composition had protected 67 % of animals from death, but not from the future repeated infection by virulent strain F-32 [36]. Immunization with recombinant baculovirus with ASFV gene for CD2v had protected against further control challenge with virulent strain. Intramuscular injection of DNA constructs containing genes for p30, p54 and extracellular domain

CD2v had prevented mortality of up to 67 % pigs infected in furtherance with virulent ASFV strain [39, 40].

Thus, many researchers consider p30 and p54 proteins and CD2v as crucial elements for induction of the immune protection against ASF.

DNA vaccines. DNA immunization first described in 1990s [41] is used upon development of vaccines against cancer, infection and autoimmune diseases. It is conceptually important that DNA vaccines are potentially safe for animals and induce not only humoral, but also cell immunity (Fig.) [42, 43].



Induction of cell and humoral immunity by DNA vaccinations: SLAI and SLAII are pig antigens with histocompatibility of I and II grades; APC — antigen presenting cells; TCR and BCR — specific receptors of T- and B-cells for identification and linkage of certain antigen; B cell — B-lymphocytes; CD4T — T-helpers; CD8T — T-suppressors (cytotoxic T-lymphocytes) (by reference 42).

Sufficient disadvantage of candidate DNA vaccines is relatively low induction of immune response, especially in big mammals. Several approaches have been tested to overcome this problem [44-48].

Targeting. Antigen targeting was successful in many systems [46]. First attempts to induce protective immune response against ASFV with the use of DNA constructs encoding p54 and p30 in form of fusion protein PQ were unsuccessful [49]. Similarity between ASFV hemagglutenin HA (CD2v) and molecule CD2 of leukocytes [37, 50] had allowed assumption that CD2v is capable of targeting lymphocytes which express receptors to CD2 (CD48 and CD58) toward viral antigens in antigen presenting cells [51, 52]. Addition of sHA had strengthened both humoral and cell response to PQ with optimal result after 3 intramuscular injections. Enhanced immune response to sHA injection may also be due to presence of T-helper cell epitopes in such molecule. The other strategy is based on targeting of viral antigens to the points of antigen presentation [53, 54] with the use of single-chain variable fragment (ScFv) antibodies [55], which specifically identify cell antigens on surface of antigen presenting cells [56, 57[. This strategy had appeared to be effective in induction of the immune responses against numerous different antigens both upon immunization with recombinant subunit proteins, as well as upon DNA vaccination [58, 59].

Targeting of recombinant viral antigens fused with a chain of antibody recognizing invariant epitope of MHC molecule II (APCH1) enhances immune response to subunit vaccines in mice and rabbits [59]. Potential of APCH1 had been demonstrated in vitro upon Vero cell transfection with plasmid pCMV-APCH1GFP, which encodes pig ScFv fused to green fluorescent protein (GFP). Following transfection, fused protein secreted by Vero cells was able to bind to pig macrophages in a specific manner. Effectiveness of APCH1 as a genetic adjuvant had been confirmed in vitro at immunization of pigs by plasmid pCMV-APCH1PQ, in which APCH1 gene is fused with chimeric open reading frame (ORF) for PQ [26]. DNA constructs encoding only PQ have been causing production of antibodies in high titers in mice, but not in pigs, whereas injection of

pCMV-APCH1PQ had induced in pigs both synthesis of antibodies specific to PQ, and T-helpers targeted on histocompatibility II antigen, which denoted adjuvant effect of APCH1 molecule. However, such candidate DNA vaccine, as compared to earlier described candidate subunit vaccine based on the same ASFV antigens, did not protect pigs from future ASFV infection [16, 26].

Ubiquitinylation. Regardless of the instrumentally registered response to immunization with pCMV-sHAPQ, pigs were not protected from control infection (clinical signs and viraemia kinetics have been indistinguishable from the same in control animals). Lack of protection had coincided with induction of specific antibodies, which had not neutralized ASFV in vitro (earlier, in vivo protection was associated with them) [16]. This had confirmed inability of the candidate DNA vaccines to induce neutralized antibodies against p30 and p54, whereas non-neutralizing antibodies, as it was found, could even aggravate development of the infection [49].

pCMV-UbsHAPQ construct encoding antigen determinants p30, p54 and sHA fused with cell ubiquitin had been designed to avoid the unwanted antibody induction and to stimulate specific $CD8^+$-T-cell responses. As it was expected, immunization with pCMV-UbsHAPQ did not induce humoral response in pigs, but had ensured partial protection against control infection with ASFV, thus confirming the importance of T-cell response for protection from this virus. The attained protection had not been enhanced by repeated injections of DNA vaccine that, possibly, reflects the lack of booster effect for T-cell response induced after the first immunization. In particular, upon 2-fold immunization by pCMV-UbsHAPQ only 2 of 6 pigs survived, whereas upon 4-fold immunization only one pig survived. Possibly, boostering could have negative effect on protection. According to authors, 4-fold immunization with pCMV-UbsHAPQ could have resulted in low induction of antibodies not neutralizing virus and aggravating the disease [49], which in its turn, may reduce protective effect of the induced $CD8^+$-T-cells.

Expression library immunization (ELI). ELI is considered as perspective approach to development of protection means against particularly menacing infections [60, 61]. To improve immunogenicity, one could develop modified ELI-vaccines either with targeting of encoded antibodies to induction points of the immune mechanisms or with activation of intracellular destruction and presentation of antigens [62, 63]. Protectiveness of $ASFV^{Ublib}$, the DNA library represented by ubiquitin-fused short fragments of ASFV in plasmid pCMV-Ub for enhancing induction of specific CTLs, had been studied [64]. Obtained 4029 clones (total 130 kbp) covered nearly 76 % of viral genome. Suboptimal plasmid dosage at injection in animals is 0.15 μg, optimal is 600 μg. Following challenge with strain ASFV E75, all control pigs (5 animals) died within 10 days, whereas out of 5 pigs immunized with $ASFV^{Ublib}$ 3 animals died immediately and 2 animals died on day 10, that was later, that death of control animals had commenced. Upon re-testing, the same protection level in animals had been denoted with confirmation of the protective potential of $ASFV^{Ublib}$. Generally, during two tests 6 of 10 immunized pigs had survived lethal challenge with ASFV. Surviving animals had lower virus titers in blood and nasal discharge as compared to $ASFV^{Ublib}$-immunized animals died from ASF, and also to control animals. All animals had demonstrated development of the standard symptoms, including fever, however clinical state and temperature in surviving pigs became normal on day 10 to 11 after infection, whereas final restoration had accompanied by lack of viraemia starting from day 21 after infection. Virus had not been detected in such individuals in any of the studied tissues, including pharyngeal lymph nodes, tonsils, and spleen that had been confirmed by the lack of ASF-specific macro- and micro-

scopic affections at postmortem examination. In ELISA, specific antibodies to ASFV proteins had not been found in any of the $ASFV^{Ublib}$-immunized individuals prior to control challenge, which had confirmed the findings for plasmids encoding ubiquitinized potentially protective proteins (p30, p54, CD2v). Among all studied cell sub-populations in surviving pigs, statistically important population growth had been found only in $CD8^+$-T-cells starting from day 5, which, evidently, confirms development of specific $CD8^+$-T-cell responses prior to control infection with ASFV and highlights their role in protection against ASFV. Lack of complete protection had not resulted in appearance of carrier-animals since virus titers in blood, nasal liquids, lymph nodes, tonsils and spleen of the surviving individuals had been lower than test sensitivity that had minimized the threat of ASFV transfer to susceptible recipients.

BacMam Viruses. Vaccination opportunities for optimization of the induced immune responses had been defined based on the use of BacMam viruses, which represent baculovirus vectors encoding virus proteins under the control of vertebrata promoters, thus ensuring high transgene expression in mammalian cells [65-67]. Although arthropod cells are hosts for baculoviruses, BacMam viruses may also promote effective transduction of vertebrata cells, in which initially baculoviruses are non-replicable. Therefore, in terms of safety BacMam viruses are suitable for use as vaccine vectors [68-73]. Effectiveness of BacMam vaccination had been demonstrated in small laboratory animals with established ability to induce humoral and cell reactions [68, 74-77].

Upon use of recombinant BacMam encoding three proteins of ASFV isolate E75 under control of the vertebrata-specific promoter pCMV [39], new recombinant baculovirus (BacMam-sHAPQ) encoded two full-size immunodominant proteins p30 and p54 fused with carboxyl terminus of extracellular domain of viral hemagglutinin (sHA; HA in positions 21-204). Prior to that, in had been demonstrated that chimeric protein (sHAPQ) had induced humoral and cell immune responses in pigs after DNA injection [40].

Expression of sHAPQ in vitro in mammals had been proven by indirect immunofluorescence in cells KOP/R transduced with the use of BacMam-sHAPQ. Immunogenicity of BacMam-sHAPQ had been determined after 3-fold injection of 10^7 BFU with 15-day interval (in control wild type of baculovirus was used). Afterwards, all animals had been infected with homologous isolate E75 at 10^2 HAU_{50}. All control animals had fallen sick, with various clinical signs of ASF and short fever peak (< 41.5 °C). On day 10, virus titers in blood achieved the maximum level and then slowly reduced until death by day 17 after infecting. In 4 of 5 pigs immunized by BacMam-sHAPQ no signs of viraemia and clinical signs of disease have been detected (prior to infecting, no one animal had virus-specific antibodies). Moreover, kinetics of specific humoral reactions after infecting with ASFV was identical in both immunized and control groups. According to authors, lack of specific antibody induction could not be due to protein expression defects or antigen specificity of fused protein, since the induced antigen (sHAPQ) had caused active humoral responce upon immunization of pigs by candidate DNA vaccine [40]. Inability of BacMam-sHAPQ to induce specific antibodies is not related to the initial error of BacMam strategy since such vector is successfully used with many other antigens [73, 74, 78]. One of the causes could be due to any defect in in vivo antigen presentation to B-cells as it was indicated for other antigens [79, 80]. Induced cell responses had been assessed with the use of IFNc-ELISPOT [49], for which periphery blood mononuclear cells (PBMC) produced within 15 days following each immunization or within 17 days following challenge had been stimulated during 16 hours by isolate E75 (10^6 HAU_{50}/cm^3). As it was expected, specific responses lacked in the control prior to infecting, while specific

T-cell responses were manifested in 4 of 6 pigs immunized by BacMam-sHAPQ. It is completely coherent with data obtained with the use of attenuated ASFV strain when direct correlation had been established between the protection and induction of IFNc-secreting T-cells [11].

Thus, upon BacMam-sHAPQ vaccination, protection of pigs against sublethal homologous infection with ASFV is possible at absence of antibody induction. Besides, induced protection and stimulation of T-cells are directly related.

Development strategy of DNA vaccines against ASF. In veterinary, preparations for DNA vaccination of horses against Western Nile virus and salmon fish against infectious hemorrhagic necrosis have already been licensed [81]. Virus-neutralizing antibodies against herpes virus-1 in bulls, bovine diarrhea viruses, distemper, classic swine cholera, aftosa, hepatitis B in ducks, infectious bursal disease, infectious hematopoietic necrosis, Japanese encephalitis, reproductive and respiratory syndrome in pigs, pseudo rabies, rabies, vesicular stomatitis, and hemorrhagic septicemia have been found upon immunization by candidate DNA vaccines [82, 83].

If perspectives of the classical live inactive sub-unit vaccines against ASF are comprehendible, approaches to obtainment of specific protection means based on DNA technologies require further elaboration. Development of DNA vaccine against ASF first of all requires determination of purposes and minimum requirements. Application of DNA vaccine is feasible for temporary preservation of the livestock at large pig-breeding farms in the focus of infection and first threatened area for the product processing period, as well as in African countries, where this disease is enzootic. DNA vaccine is also required due to the fact that it is impossible to fully exclude the probability of occurrence of any dangerous for humans or economically significant animal-based natural or inherent virulent ASFV strains or similar pathogens. We believe that DNA vaccine shall protect against death, contamination, viraemia upon infection by homologous virulent isolate and from its acceptance; shall not cause reactions in place of injection; at 1-2-fold injection may induce protection within 14-21 days for the term of up to six months; shall be stable upon normal conditions of storage and transportation. Effective DNA vaccine presupposes in vitro measured induction of both CTLs and antibodies which participate in ADCC, are specific to the narrow scope of protective protein epitopes and not detectable in immunofluorescent assay or neutralization reactions. Herewith, optimal relationship between the induced immune cellular and antibody-mediated protective mechanisms is critical. Use of ELI-libraries could, probably, allow for determination of protective epitopes for both CTLs and ADCC [60]. It is planned to optimize delivery methods of DNA constructs to immune competent cells, to ensure targeting of translation products and immune competent cells to antigen presenting cells, and to select immunestimulants. For that end, one should use electroporation [84], additional simulating molecules, for instance, synthetic oligodeoxynucleotides containing non-methylated CpG motifs [85] or prime-booster immunization strategy [86, 87]. Operation of the body immune system is corrected by brain and vegetative nervous system [88]. Thus, finding of the means and methods of selective effect on separate sub-populations of immune system cells for realization of the protective potential of candidate DNA vaccines is also relevant. Besides, new molecular mechanisms of virulence and protection of pathogens from the host immunity, which would be discovered during research, should also be accounted for.

Thus, mechanisms of immune response to ASFV and virus-host relationships yet require clarification. Nevertheless, research findings of development of DNA vaccine against ASFV are encouraging, including DNA constructs bearing sHA gene, pCMV-UbsHAPQ encoding antigen determinants for p30, p54 and sHA fused with cell ubuquitin, DNA-library ASFV^{Ublib}, and use of recombinant

baculovirus.

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EVOLUTION OF THE METHODS FOR ESTIMATION BIODIVERSITY IN REINDEER (*Rangifer tarandus*)

(review)

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Abstract

Reindeer *Rangifer tarandus*, the only member of the genus *Rangifer*, is an important component of the food security of the indigenous people of the Russian North, and is an indispensable part of the Arctic ecosystems (A. Savchenko, 2014; V.G. Loginov, 2014). To-date, due to a number of unfavorable natural and anthropogenic factors, population number of both domestic and wild reindeer is sharply decreasing. This leads to a loss of the genetic diversity, which is sufficient for survival in new habitats (Y.A. Stolpovsky, 2010). In this regard, it is significant to monitor the genetic diversity of resource breeds and wild reindeer populations with use of genetic markers. The review summarizes the results of the genetic diversity studies of reindeer using different molecular genetic analysis methods. The first genetic studies of reindeer began with the assessment of serum transferrin polymorphism in the 1960s (B. Gahne et al., 1961; M. Braend, 1964). Types of transferrin were distinguished from each other by the band position and mobility in gel electrophoresis (A.V. Soldal et al., 1979; K.H. Roed, 1985; P.N. Shubin et al., 1988). With the development of genetic technologies, DNA markers gained popularity (M. Çalişkan, 2012). The so-called "anonymous" markers (initially RAPD and later ISSR) became the first DNA markers used to investigate the biodiversity of reindeer populations (V.V. Goncharov et al., 2009; N.V. Kol et al., 2006; T.M. Romanenko et al., 2014; G.Y. Bryzgalov, 2016). Since the publication of the complete nucleotide sequence of the control region of the mitochondrial genome of reindeer subspecies of Eurasia and North America, analysis of the polymorphism of mitochondrial DNA (mtDNA) has become widespread (M.A. Cronin, 1992; E. Randi et al., 2001; A.V. Davydov et al., 2007; M.V. Kholodova et al., 2009; A.N. Korolev et al., 2017). The method is a highly informative for revealing the phylogeny and origin of breeds and populations by the maternal line (Ø. Flagstad et al., 2003; N.A. Akopyan et al., 2016). Microsatellites have found great implementation in applied studies of genetics of reindeer (establishment of genetic structure, characteristic of allele pool, identification and differentiation of individuals) (K.H. Røed et al., 1998; B.I. Jepsen et al., 2002; R. Courtois et al., 2003; M.A. Cronin et al., 2003; K.A. Zittlau, 2004; P.D. McLoughlin et al., 2004; A.D. McDevitt et al., 2009; A.I. Baranova et al., 2016). For Russian reindeer populations, a multiplex panel of nine microsatellites was developed (V.R. Kharzinova et al., 2015). It is successfully using in the routine testing of reindeer, including the detection of hybrids between wild and domestic forms (V.R. Kharzinova et al., 2016). However, with the development of new high-throughput technologies and new-generation analytical equipment (A. Vignal, 2002; E.K. Khlestkina, 2013), DNA chips based on genotyping of multiple SNPs come to the fore in genetic studies of farm animals (F.J. Steemers et al., 2007; S. Mastrangelo et al., 2014; T.E. Deniskova et al., 2015; B. Slim et al., 2015, N.A. Zinovieva et al., 2016; T.E. Deniskova et al., 2016, R. Yonesaka et al., 2016). To-date, despite the fact that there is no the specific DNA chip for reindeer, the use of the Bovine SNP50 BeadChip, designed for cattle, is the most effective and highly informative method for studying the reindeer genome (V.R. Kharzinova et al., 2015; V.R. Kharzinova et al., 2016; V.R. Kharzinova et al., 2017).

Keywords: *Rangifer tarandus*, reindeer, genetic diversity, genetic marker, SNP, DNA chip

Ecosystem stability stems from preservation, increase, and use of biodiversity. Information about genetic variability and processes involved in the origin and preservation of species play a critical role in understanding of the structure and evolution of populations [1]. Maintenance of the optimal genetic variability and heterozygosity in animal populations preserves their ability to adapt to natural environment (climate changes, negative impact of hazardous substances). High genetic diversity ensures evolutionary adaptiveness of the animals [2-4]. Levels of genetic variability within and among the populations correlate with their demographic history, as well as with environmental factors [5]. Information about genetic structure of the animal population not only enables to assess the importance of the fundamental evolutionary factors (selection, mutation, migration, genetic drift) under stress conditions, but is also important for restoration and rational use of the species [6]. Based on the aforesaid, it is important to develop methods and approaches providing a more objective overview of the genetic diversity of species and breeds. This is of particular importance due to the decreased number of wild and domestic animal breeds observed during the last 100 years [7]. According to the second report "The State of the World's Animal Genetic Resources for Food and Agriculture" (Food and Agriculture Organization, FAO), nearly 17 % or 1458 breeds of farm animals in the world are on the verge of extinction, whereas the risk status of many others (58 %) is unknown due to the lack of information about the size and structure of populations [8].

Decrease in abundance has affected all species, including the reindeer (*Rangifer tarandus*), playing a key role in life of peoples of the Far North. Reindeers are not only the priority element of the Arctic societies, but also the crucial component of food security for the peoples of northern Siberian territories [9]. For the peoples of the North, deer breeding forms the basis of way of living; it is a continuous process with regular alternation of used pasture grounds. Domestic reindeer population of four breeds provides indigenous community with food and necessary materials for houses and cloths [10]. Wildlife reindeer species are under serious threat since ecological changes in the environmental conditions (i.e. melting of glaciers due to climate warming) along with destabilize the population size. Decrease in population size means decrease of biological diversity and may result in loss of the unique and valuable cultures of small indigenous groups and in ethnic disaster [11, 12]. Among the objects in agricultural sphere, reindeer is one of the least genetically studied species.

Present overview summarizes genetic diversity findings obtained with the use of various methodologies for the reindeer *Rangifer tarandus* Linnaeus, 1758, the only species of genus *Rangifer* Smith H., 1827 and a member of subspecies *Odocoileinae*. Reindeer studies with the use of DNA-micro-matrices based on BeadArray platform are described for the first time. This approach based on genotyping multiple SNP markers enables to assess biological diversity of such unique member of natural fauna of the Northern Russia across the entire genome and not merely at the level of a certain gene.

First method used to describe biological diversity and genetic differences within and among reindeer populations was gel electrophoresis, and the first marker was serum protein transferrin (Tf) polymorphism of which was identified yet in 1959 [13]. Various Tf types differed from each other by location of bands in the gel due to different electrophorestic mobility. Tf locus remains the most frequently used protein marker for assessment of reindeer biodiversity during the period preceding discovery of DNA polymorphism [14-17]. Six various Tf types in reindeers, in one of which 3 alleles were detected, have been reported in 1961 [14]. In 1964, 8 Tf alleles were detected in three domestic, one semi-domesticated and one wildlife population of reindeer in Norway [13]. In further-

ance, number of Tf alleles identified in the Norwegian population had increased up to 12 [17]. Among 9 proteins studied in reindeer of Spitsbergen Island only transferrin was polymorphic and, accordingly, was suitable for assessment of biodiversity [15]. Two alleles not found in the Norwegian animals were found in reindeer population of Spitsbergen Island, based on which they were separated into the specific phylogenetic group, *R. tarandus plutyrhynchus* [15, 16].

Specific attention was paid to studying of Tf locus in the Russian population of reindeer. P.N. Shubin [18] had described 5 alleles of Tf locus, number of which had increased up to 13 in further studies [19]. Upon comparative study of wildlife and domestic populations in Taymyr, the most number of Tf alleles was identified in wildlife reindeer leading to the conclusion about low identity of two populations [20]. Ten genetic variants of Tf were identified in Nenets species [21]. High polymorphism of Tf locus had defined wide use of Tf electrophoretic screening for identification of the events precedent to the evolution of reindeer populations, including assessment of their passing through the bottleneck [22].

Discovery of DNA structure and development of the methods for determination of its variability had resulted in stepwise replacement of protein polymorphism study by DNA sequence analysis. DNA markers are relatively easy to find, they are located along the entire genome, are fully independent of environmental conditions, and, in fact, can detected at any stage of body development [1].

There is an abundance of genetic locus, polymorphism of which may be used in molecular marking. The most conservative ones may be used for global classifications [23], and the most variable ones allow assessment of diversity within a population [24]. Discovery of various polymorphism types is based on modification of the Polymerase Chain Reaction (PCR) technique which enabled to use various types of DNA markers in many science domains and agricultural sectors [25]. Evolution of molecular markers had resulted in emergence of the new knowledge about biodiversity of reindeer populations, including estimates of genetic variability, genetic structure, extent of differentiation and phylogenetic relationship of breeds and populations of this species.

Thus, with the use of the earliest marking techniques, RAPD (random amplified polymorphic DNA), it was found that Nenets breed of reindeer is characterized by higher levels of polymorphism and heterozygosity than Ewenki breed [26]. DNA inter simple sequence repeats (ISSR) test had enabled to establish genetic structure of reindeer population on the Kolguev Island and to study genetic diversity of artificially created and maintained population of domestic reindeer at natural territorial habitats on the Kaninsko-Timanskaya tundra of Nenets Autonomous Okrug [27, 28]. Average pairwise indices of similarity and average heterozygosity values of the analyzed population were calculated during study of ISSR polymorphism in Tuvin population of reindeer [29]. ISSR technique formed the basis for description of the genetic structure of the Chukot breed [30]. Heterozygosity level of intermicrosatellite DNA comprised 0.851-0.876, which is illustrative of the genetic diversity of relevant locus.

Sequence analysis of mitochondrial DNA (mtDNA) is widely used for assessment of the level of genetic diversity, phylogenetic relationship within and between reindeer populations. High polymorphism, matriliny and lack of mtDNA recombination also enable the effective application of such approach for determination of the historical origin of breeds and populations [31]. The most popular is use of the control region (CR) of mtDNA and its hyper variable part — D-loop. Full nucleotide sequence of CR in Eurasian and North American reindeer subspecies was published in 2003 [32].

Polymorphism analysis of D-loop fragment had enabled to describe di-

versity of reindeer population found in the Tojinsky region of the Republic of Tyva [33]. Prevalence of a single haplotype was established regardless of quite high level of genetic diversity. It is assumed that species with such variant of mtDNA come of one doe. Significant differences between the Chukot breeds and breeds from the Siberian tundra and North American caribou were found based on mtDNA haplotype analysis, including hyper variable part, left domain of the control region of D-loop [34]. Authors explain this by temporal gap in the origin of forms and unequal genetic exchanges between populations. High haplotype diversity as compared to other island forms was found based on mtDNA analysis in animals from Novaya Zemlya Archipelago, Kolguev Island, and from other places. Two haplotypes characteristic of the Kolguev Island were found in animals from Novaya Zemlya. Nowadays, such population involves 7 haplotypes, i.e. almost the same amount as that typical for other groups occupying much larger continental areas. Presence of 1-3 haplotypes is usually typical for island forms [35].

Study of the control region of mtDNA in wildlife reindeer inhabiting the European part of Russia highlighted quite high level of haplotype diversity of such population (0.914). Phylogenetic analysis had demonstrated close relationships between the European reindeer and wildlife Siberian reindeer. Common haplotype of the reindeer from the Murmansk region with wildlife reindeer inhabiting South-Western Norway had been described. The assumption was made that in the near past wildlife reindeer of the European North of Russia formed common population with reindeer inhabiting northern region of the Asian part of Eurasia [36]. Study of D-loop of mtDNA in reindeer from the continental part of the European North-East of Russia (eastern regions of Archangelsk area, the Republic of Komi, and Nenets Autonomous Okrug) revealed relatively high genetic diversity values. Phylogenetic analysis had enabled to found close relationship of such reindeer with animals from the Siberian tundra. Influence of domestic reindeer on formation of the genetic diversity in wildlife reindeer was generally acknowledged to be insignificant. Genetic lines of the extinct group of forest reindeer in Nizhny Novgorod area were found among the recent groups of reindeer from the European North-East of Russia [37]. During study of the polymorphism of mitochondrial DNA in *Cervidae* species, including 51 reindeer populations inhabiting the Europe and Asia, it was found that red deer had originated from the territory between the Kyrgyzstan and North India [38]. P. Gravlund et al. [39] had demonstrated polygenetic origin of three high arctic species: *R. t. pearyi* (Canadian Archipelago) and *R. t. eogroenlandicus* (Eastern Greenland, extinct since 1900 A.D.) are closely interrelated and, possibly, came from the North Arctic; *R.t. platyrhynchus* (Spitsbergen) came of forest Eurasian deer. Analysis of mtDNA variability in *Cervidae* species was reported in a series of studies [40-42].

Studies show that mtDNA polymorphism analysis in reindeer serves high informative tool for description of the genetic diversity, clarification of phylogeny and differentiation of breeds and populations within the species.

Microsatellites are also widely used for molecular and genetic analysis of diversity of the animal breeds and populations. This is the class of short tandem repeats (STR) of DNA [43-45] present both in non-coding and coding genome regions, as well as in chloroplast [46] and mitochondrial genomes [47]. These are widely used in assessment of the genetic structure of reindeer breeds and populations. Applied significance of STR is shown in publications of the scientists from America [48-50], Norway [51, 52], Canada [53-55], Great Britain [56], Denmark [57], Ireland [58], and Russia [59-61]. Use of 13 STRs had enabled to describe genetic diversity of various reindeer subspecies inhabiting Norway, Canada, Western Greenland, Spitsbergen, Alaska, and Finland [62]. Due

to the isolated geographical habitat, population of reindeer from Spitsbergen had the least values by the average number of allele per locus and geterozygosity level, and was less than other populations by number of polymorphic STRs (5 of 13). Based on analysis of 11 microsatellites, A. Mcdevitt et al. [58] had studied structure of two reindeer populations from the North America. Use of 14 STRs had enabled to identify genetic differentiation between two continental reindeer populations in Spitsbergen with habitat at distance nearly 45 km from each other [56]. M. Ball et al. [63] had analyzed polymorphism of 11 microsatellite loci in forest reindeer population (*R. t. caribou*) of the Central Canada. J. Kushny and J. Coffin [64] studied genetic diversity of three reindeer populations in Canada (*R. t. groenlandicus*, *R. t. pearyi*, *R. t. caribou*) with the use of 4 microsatellite loci. Using 16 microsatellite loci, A.I. Baranova et al. [65] reported clear separation of reindeer from the continental part of the Eurasian area and Arctic islands. Reindeers from the Asian and European regions of Russia were genetically more close to each other than to Kamchatka reindeer breeds. Moreover, they found quite poor separation of reindeer from eastern Eurasia in some habitats (Tomsk region, Khanty-Mansy Autonomous Okrug, Taymyr, Yakutia, and Chukotka), that evidences on their close genetic relationship.

We have developed multiple locus panel with 9 STRs for better performance and informativity of STR-analysis to control validity of origin and assessment of biodiversity of the Russian reindeer populations (Even, Ewenki, and Nenets breeds, and Tuvin population) [59]. It enabled to study sample allele pools of two great reindeer populations, domestic Nenets breeds and Taimyr wild reindeer population, and to identify the extent of genetic introgression between them. Although cluster analysis had shown high genetic segregation of both forms, several individuals of mixed genetic origin were found [66]. According to S.A. Kotovaya et al. [67], STR markers remain the most valuable genetic tool for i) studying population variability, ii) identification of possible substructures of animal populations inhabiting either the same territory, or geographically isolated or distant territories, iii) identification of the population structure of wildlife and domestic animals of the same species; iv) gene mapping and assessment of the gene flow between the groups of animals, and v) establishing the paternity and identification of species.

Alongside, obtainment of new information about the animal genome, improvement of the methodological approaches, development of high-performance genome analysis technologies, and creation of advanced analytical equipment had enabled the use of various genetic markers for allele pool studies [68]. Nowadays, single nucleotide polymorphism (SNP) analysis is mostly demanded for these purposes. There are many SNP detection techniques, starting from the analysis of restriction fragment length polymorphism (RFLP) (RFLP analysis) and ending by pyrosequencing [69]. However implementation of projects for determination of entire nucleotide genome sequences in most species of the farm animals had resulted in creation of DNA micromatrices (DNA-chips), which enabled SNP markers to take the leading position in animal genome studies. DNA chips are sets of the large number of oligonucleotides at miniature solid substrates designated for analysis of DNA sequences [70]. BeadArray-based technique of parallel full-genome genotyping of multiple SNPs (up to hundreds thousands) is the most popular [71]. Information on biodiversity [72-74], evolutionary relationships, extent of introgression and variability of animal breeds and populations had been obtained based on full-genome SNP screening with the use of DNA chips of various density [75, 76].

Recently, DNA chips for the most popular species of the farm animals (cattle, sheep, goats, pigs, and horses) and chicken are developed by Illumina Inc.

(USA) (http://www.illumi-na.com) and Affymetrix Inc. (USA) (http://www.affymetrix.com). Nevertheless, study of biodiversity in members of *Cervidae* genus with the use of native DNA chip seems to be an impossible task due to the lack of information on entire genome. However, several authors had demonstrated possibility of using commercial DNA chips developed for related domestic animals to study biodiversity and structure of subspecies population [82-85], as well as to differentiate horned ungulates (*Bovidae*) from deer family (*Cervidae*) [80]. For *R. tarandus* species, whole-genome genotyping started from testing of two commercial average density chips, Bovine and OvineSNP50 BeadChip [81]. It was established that Bovine SNP50 BeadChip developed for cattle is more effective for genome reindeer scanning since its use enables to detect larger number of polymorphic SNPs as compared to the chip created for domestic sheep. One of the last publications regarding reindeer genetics study with the use of multiple SNP markers had provided for population and genetic description of three breeds raised at the territory of the Republic of Sakha—Yakutia (Even, Ewenki, Chukot, and Khargin breeds) [82]. It was shown that individuals of the Chukot breed leave behinds their congeners of two other breeds by extent of the genetic diversity, but, however is characterized by less number of the unique polymorphisms. Besides, regardless of the identified clear segregation of each breed, Even and Ewenki breeds are closer genetically.

Therefore, we have provided detailed description of how approaches to study genetic diversity of the reindeer evolved from the simplest (transferrin) to modern high-performance (DNA chips). Regardless of the successful application of the later, wide implementation of the new techniques (e.g. next generation sequencing, NGS) and cheapening of the existing sequencing techniques (Sanger method) allow studying the entire nucleotide sequence of genome of the unique species, *Rangifer tarandus*, thus opening new perspectives for its studies.

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BIOFORTIFICATION OF HEN EGGS: VITAMINS AND CAROTENOIDS
(review)

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The dependence of the vitamin content in the egg from the content in the chicken feed represents the saturation curves (V.M. Kodentsova et al., 2005; K. Hebert et al., 2005; S. Leeson et al., 2004; A.L. Shtele, 2004; S. Grobas et al., 2002). The effectiveness of the various forms of vitamins for enrichment of chicken feed and the use for this purpose of herbal supplements had been analyzed (P. Mattila et al., 2004; P.H. Mattila et al., 2011; M. Hammershøj et al., 2010, J.A. Moreno et al., 2016). The optimal content of vitamins and carotenoids in the poultry feed results in an increase to the maximum level of vitamins and carotenoids in the egg that makes them a significant source of vitamins D, E, B and carotenoid for humans. One such egg can provide up to 40-50 % of the recommended daily intake of vitamins D, B_{12}, A, pantothenic acid, 30 % of vitamin E, 20 % of folate, 10 % of vitamin A, 12 % of vitamin B_2, and up to 30 % of the adequate level of lutein intake. The advantage of biofortification is biotransformation in the chicken's body of synthetic vitamins added to food into their natural form, which deprives the arguments of opponents of enrichment of food products with synthetic vitamins. Comparison of the addition of different forms of vitamins showed that D_3 in the diet more effectively increased the vitamin content in egg yolk (P. Mattila et al., 2004). If the chicken feed contains vitamin D only as $25OHD_3$, then vitamin D in the form of cholecalciferol may be completely absent in the yolk (P.H. Matvila et al., 2011). Irradiation of chicken with ultraviolet light or free-range farming in the natural sunlight may provide an original, safe and natural alternative to produce vitamin D-enriched eggs (A. Schutkowski et al, 2013; J. Kühn et al., 2014, 2015) and chicken meat without the risk of overdose of this vitamin. By increasing the content of lutein in the yolk of a chicken egg, the bioavailability of this carotenoid can be substantially increased as compared to plant sources (G.J. Handelman et al., 1999). The enrichment of eggs with vitamins meets the criteria for the fortified foods (V.M. Kodentsova et al., 2010). Increasing the level of all vitamins in hen diet resulted in a simultaneous increase in the content of all vitamins in eggs (H. Zang et al., 2011). Biofortification has clear advantages over the technological enrichment since synthetic vitamins received from feed are converted into natural ones in hen body. Biofortification of eggs with vitamins is one of the most promising strategies to increase consumption of vitamins for population (M.S. Calvoa et al., 2013).

Keywords: biofortification, vitamins, carotenoids, poultry, eggs

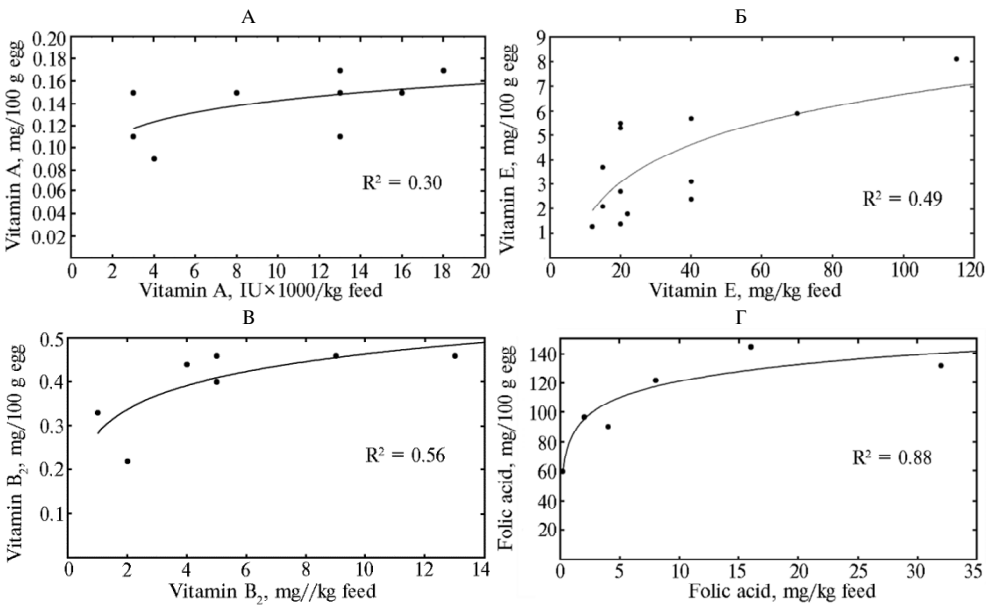
The problem of micronutrient deficiency among population is being addressed in several ways. One of the approaches that find the world acceptance is technological modification, in which vitamins or their mixtures are added to raw material used in producing food products (e.g. bakery flour) or directly to food products for mass consumption (1). Unfortunately, an unreasonable opinion that synthetic vitamins are poorly metabolized in the body can be found not only among people, but also in scientific papers. Therefore, in recent years, more at-

tention has been paid to biofortification (bio-addition) — the enrichment of livestock products via feed vitamin supplements. In this case, the vitamin, getting to the animal body passes through the biotransformation stages and is consumed by man in natural form. Although the laying hen need for most vitamins has been well studied and the optimal vitamin content in feed has been established, interest in the chicken egg as a perfect object to be used for improving its vitamin composition has increased in recent years, as the egg value can be increased in a natural way by enriching poultry ration with vitamins and minerals [2].

Chicken egg is a natural functional food of mass consumption which is used in nutrition of all population, so its biofiltration is of particular interest. According to the food nutritional tables, two large chicken eggs provide up to 20 % of the recommended daily protein intake, up to 30 % of vitamin B_2, up to 6 % of vitamin E, and 12 % of vitamin A [3, 4]. However, the real concentration of vitamins in eggs which were produced in different poultry farms and smallholder agricultures [5] depends on the vitamins in feeds and can differ essentially from the values indicated in the food chemical composition and nutritional tables [6, 7].

This review for the first time summarizes the modern findings on vitamin enhancing in the egg not only by enrichment of laying hen rations with vitamins, but also by herbal dietary components and UV irradiation of birds.

When vitamins content in the feed does not reach the norms recommended for egg-laying hens [2], the amount of vitamins in the egg is minimal (Fig.). An increase in most dietary vitamins in the poultry feed is accompanied by a gradual non-linear regression of its amount in the egg (see Fig.). It can substantially exceed the values given in the food nutritional tables used to calculate the intake of vitamins with a diet.



Vitamin A (A)**, E** (B)**, B_2** (C) **and folic acid** (D) **content in a sound egg depending on their dietary amounts** [5, 16-19, 25]**.**

Vitamin E. The effect of various doses of vitamin E in laying hens diets, from 20-60 mg/kg feed [8] to 100-200 mg/kg feed [9, 10] and above [11], is shown on the Figure (see B). According to various researchers [12, 13], an increase in dietary vitamin E from 10 to 150 mg/kg was accompanied by its concentration in the egg yolk. This increasing was going on up to 200 mg of α-

tocopheryl acetate per 1 kg of feed [14]. The vitamin E accumulation in the egg yolk increased up to 6.8-38.9 % with the addition of selenium (0.1 g/t).

Amount of vitamins in eggs of laying hens fed with rations enriched with vitamins (literature data)

Vitamins	Data of national tables of the chemical composition of food products [6, 7]	Ration	
		without additional feed enrichment	with additional feed enrichment
D	88 IU 310 IU[a]	25-80 IU [17, 20] 150-450 IU[a] [21]	180-1700 IU [17] 33.7-150 μg[a] [21, 22]
K	–	0.01-0.05 mg [20]	0.05 mg [17]
B_6	0.14 mg 0.46 mg[a]	0.11-16 mg [17, 20] 0.35-1,50 mg[a] [17]	–
Niacin	0.19-0.20 mg	0.07-0.15 mg [17, 20, 23] 0.05 mg[a] [17]	–
B_{12}	0.0005 mg 0.0018 mg[a]	0.0004-0.0010 mg [17, 23] 0.0018 mg[a] [17]	0.0016 mg [17]
Folate	7-32 мкг 0.022 mg[a]	0.009-0.078 mg [2, 17, 23] 0.12-0.17 mg[a] [17, 23]	33-75 μg [24, 25]
Pantothenic acid	1.3 mg 4 mg[a]	1.2-1.6 mg [17, 26] 3.5-12.5 mg[a] [17, 20]	1.9 mg [17]
Biotin	0.02 mg 0.056 mg[a]	0.016-0.030 mg [17, 20] 0.1 mg[a]	0.070 mg [17]
β-Carotene	–	0.014 mg[a]	0.52 mg[a] [18]
Lutein	–	0.3 mg/60 g	1.5 mg/60 mg [27] 0.8-2.7 mg[a] [5]
Cryptoxanthin	–	–	0.08-0.20 mg[a] [5]

N o t e. Dashes mean the absence of data in accessible literature; [a] — the content per 100 gm of egg yolk.

Vitamin E is absorbed in the small intestine, and the effectiveness of this process depends on the diet composition, the used dosage, age, sex, and other individual chicken characteristics. Vitamin E accumulates in the liver and adipose tissue, but it is not enough for continuous requirements. For example, the amount of vitamin E that transfers from the laying hen in one egg is more than the reserve of this compound in the liver [12, 15].

The amount of dietary vitamin E affects the eggs quality, protects polyunsaturated fatty acids (PUFA) from oxidative damage for 28 days of storage at room temperature [28] and promotes retinol and carotenoids preservation [12, 15, 29, 30] in the egg. Enrichment of chicken feed with ω-3 PUFA together with vitamin E prolongs the best before dates, reducing the lipids oxidation [31]. According to A. Barroeta [32], at low PUFA content in chicken diet (15 g/kg), 60 mg/kg α-tocopherol should be imposed to the diet for maintaining lipid stability, whereas at high PUFA content (30 g PUFA/kg feed), 200 mg/kg is required. Vitamin E is non-toxic, even its high doses do not cause hypervitaminosis, but excessive addition to feed should be economically viable [33]. Furthermore, high doses of vitamin E (10000-20000 IU/kg of feed) significantly reduced the vitamin A and carotenoid concentration in the egg yolk [19].

In addition to tocopherols, natural sources of this vitamin are used for enrichment the chicken feed. Supplementation of rice bran oil (RBO) containing 1.3 % tocotrienols which are a form of natural vitamin E to the hen feed for 7 days improved their content up to 0.62 mg/egg against the usual content of 0.11 mg/egg [34].

V i t a m i n D. As compared to others, vitamin D comes not only with food, but it can also form in the skin of humans and animals under the influence of ultraviolet radiation, and in fact can not be denoted as a vitamin. It is a prohormone that converted to 1,25-dihydroxyvitamin D, its hormone form in the organism. There are from 200 up to 2000 genes directly or indirectly responding to the vitamin D action. The range of its proven physiological effect is extremely wide and includes inhibition of cell division, stimulation of insulin synthesis, apoptosis, suppression of renin production, stimulation of production of catheli-

cidin, the peptide having an antimicrobial effect, by macrophages [35]. Vitamin D deficiency is linked to many socially important chronic diseases (cardiovascular, myocardial infarction, type 2 diabetes, autoimmune diseases, tuberculosis, bronchial asthma, atopic dermatitis, urticaria, prostate, breast, intestinal cancers), neurocognitive disorders, and depressions [35].

The vitamin D pool in human is determined by the combination of its synthesis in skin under the influence of sunlight and the consumption of two basic alimental forms of vitamin D, the ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). A decrease in blood vitamin D concentration is observed in 50-92 % of the working age population and children of our country, independently of seasons [36]. The causes of vitamin D deficiency are both in inadequate food consumption, and in low endogenous synthesis due to the Russian geographical location [36]. Recommended amount of vitamin D_3 in the human body has an important preventive potential for health. Food sources of this vitamin for human is scarce, in addition vitamin D is not contained in products of vegetable origin. The main sources are (in descending content order) cod liver, fatty fish, chicken eggs, liver, butter.

Accordingly, the perspectives for the biofiltration of chicken eggs with vitamin D become particularly important [37]. Vitamin D is mainly located in the yolk in the forms of cholecalciferol and $25OHD_3$. The vitamin D egg content raises by its increasing in chicken feed. It is more effective to increase the vitamin egg yolk content by adding vitamin D3 to chicken diet [22]. According to some authors, it is possible to produce eggs with 2-fold vitamin D_3 concentration (approximately 2-3 μg/egg) [38] by redoubling vitamin D_3 feed content compared to physiological norm. The D_3 content in hen egg yolk which received 6,000 IU (150 μg) or 15,000 IU (375 μg) of vitamin D_3 per 1 kg of feed varied from 9.1 up to 13.6 μg/100 g and from 25.3 up to 33.7 μg/100 g, respectively. While adding dietary vitamin D_2, the indicator was 4.7-7.0 and 13.3-21.0 μg/100 g of yolk [22].

Since 2009, in the EU, according to EU Regulation No. 887/2009 [39], for chickens fattening, in addition to cholecalciferol, the stabilized metabolite of $25OHD_3$ is allowed. It is the main circulating (transport) form of the human's vitamin. The maximum content of $25OND_3$ combination with vitamin D_3 per 1 kg of chicken feed should not exceed 125 μg (5000 IU). But it was found, that if the chicken feed contains vitamin D only in the form of $25OHD_3$, it may be completely absent in the form of cholecalciferol in the yolk [38].

Alternatively, to get enriched vitamin D eggs, irradiation of chicken with ultraviolet light or bio-addition were suggested. The eggs of hens irradiated with ultraviolet for 3 hours daily during 4 weeks and provided D_3-adequate diet (3000 IU/kg of feed) contained 2.5 μg of vitamin D (vitamin D_3 + $25OHD_3$), which is almost 5 times higher compared to its amount in eggs of hens which were not exposed to ultraviolet light (dose at a distance of 20 cm was 76 $\mu W/cm^2$) [40]. Curiously, endogenous synthesis occurs mainly in the legs of chickens, where the plumage is the smallest. The reliance of the vitamin D_3 and $25OHD_3$ content in the egg yolk on the time of daily UV irradiation was nonlinear. With a daily irradiation for 300 min, vitamin D_3 content was increased to 28.6 μg/100 g of egg yolk dry matter, but did not reach the plateau, whereas the amount of $25OHD_3$ was maximal even after irradiation for 60 min [41]. Vitamin D_3 concentration in egg yolk was three- to fourfold higher ($p < 0.001$) in the hens that were exposed to sunlight under outdoor and indoor/free-range farming than in those kept indoor [42]. The vitamin D_3 concentration in egg yolk in the free-range farming was 14.3 μg/100 g dry matter, in contrast to 3.8 μg/100 g. The vitamin D content in the egg yolk under mixed keeping conditions occupied an intermediate

position. The $25OHD_3$ content of egg yolk was also influenced by sunlight exposure, although it was less pronounced than that of the vitamin D_3 ($p < 0.05$).

Therefore, ultraviolet or natural sunlight irradiation of laying hens is a promising strategy to fortify eggs and chicken meat with vitamin D while at the same time providing a safe approach without risking an overdose of this vitamin.

Vitamin A and carotenoids. A 5.3-fold increase in the dietary vitamin A to the optimum content [2] resulted in an 1.3-fold increase in its content in the egg (see Fig., A).

The main carotenoids, which make natural egg yolk color, are lutein and zeaxanthin [43, 44]. Their content is up to 80-90 % of total carotenoids [2]. The maize-based diet of layer hens usually contains about 11.8 mg of carotenoids per 1 kg of feed (mainly in lutein and zeaxanthin), and a wheat-based diet contains 5.6 mg carotenoids per 1 kg [45]. Enhancing egg yolk color may result from adding dietary carrots, peppers, pumpkin, hips to 1 % of feed weight and herbal flour up to 5-6 %. Such enrichment allows for the carotenoids corresponding to 8-10 mg per 1 kg of feed [17]. Adding orange, yellow or purple carrots to the ration at 70 g per hen increased the yolk lutein more than 1.5-fold, and the yolk β-carotene more than 100-fold [46]. Eggs from hens, receiving a diet with cabbage *Brassica oleracea* var. *acephala* leaves (120 g per chicken for 24 hours) had a higher content of lutein, β-carotene, and orange xanthophyll violaxanthin [47]. Adding 5 or 10 grams of tomato powder per 1 kg diet increased amount of lycopene, β-carotene, lutein and vitamin A in the yolk, whereas malondialdehyde decreased [48]. The lutein content in egg yolk of Japanese quail increased significantly if the diet included 0.2 % of the calendula extract [49]. In the addition in fodder of 3 % chlorella powder, the amount of lutein increased from 0.20 mg per egg (13 μg/g yolk in the control group) to 0.43 mg per egg (27 μg/g yolk) [50]. Clearly, such egg carotenoid enrichment has fundamental importance in the production of organic foods. The example of obtaining a 'double biofortified egg' with an increased content of carotenoids by incorporating biofortified corn into the diet is described [51].

Besides the use of natural carotenoid sources for enhancing egg yolk pigmentation, different carotenoids including not specific for egg yolk are added to the poultry feed. Among them are Lucanthin (cantoxanthin) at a dose of 0.9-1.5 up to 70 mg/kg feed, and Lipocarotene, a mixture of β-carotene (0.9-1.8 g per thousand chickens) and lycopene (0.4-0.8 g per thousand chickens) [52]. Sometimes higher doses of carotenoids (up to 400 mg/kg of β-carotene, canthaxanthin and lutein) [53] are used. β-Carotene enrichment of chicken eggs at a dose of 200 mg/kg feed leads to rising pigment content in the yolk 37 times more, from 0.14 up to 5.2 μg/g [18]. Consequently, dietary carotenoids fed to laying hens leads to an increase in the total content of these pigments in eggs by an order as compared to the tables of chemical composition; as a result, such an egg can ensure the intake from 5 up to 10 % of an adequate consumption of carotenoids [43]. Emulsification of carotenoids provides their desired concentration when the used doses are 20-30 % lower [54]. Diet supplemented with 2.5 % spirulina powder (*Arthrospira platensis*) or 30 mg/kg Lucanthine was equally effective in enhancing egg yolk colour [55]. Increasing doses of dietary lycopene (0; 420 and 840 mg/kg) and α-tocopherol (0; 84; 164; 200; 284 and 364 mg/kg) was accompanied by an increase in lycopene ($p < 0.05$) and vitamin E ($p < 0.05$) in the egg yolk, whereas lutein and zeaxanthin concentrations remained constant [56]. The amount of egg yolk lutein depends on its dietary intake according to a saturation curve pattern, that is, the egg pigment first increased from 0.3 to 1.5 mg/60 g with increasing dietary lutein up to 375 mg per 1 kg, and then remained unchanged regardless of further rise of its dietary dose [27].

Lutein is the main carotenoid which prevents macular degeneration during ageing. Lutein of fortified egg yolk is absorbed better compared to isolated lutein or lutein contained in plants [57]. Daily consumption for 4.5 weeks of 1.3 yolks of chicken eggs, which provides 0.38 mg of lutein and 0.28 mg of zeaxanthin, lead to increased blood concentration of these carotenoids by 28-50 and 114-142 %, respectively [43]. At the same time, the carotenoid intake due to the yolks was equivalent to consumption of 60 g of spinach dishes or 150 g of corn dishes. As lutein is a specific carotenoid which concentrated in the retina yellow spot, dietary products enriched with bioavailable lutein can be considered as a factor that reduces the risk of age-related macular degeneration [26, 58].

B vitamins. Dietary B_1 provides its increase in the egg by about 25 %, and vitamin B_2 supplements can lead to a 1.7-fold increase in B_2 level in eggs (see Fig., B, D).

Women of childbearing age with a non-optimal folate status have risks of children being born with neural tube defects, and the clear linkage between folate concentration, homocysteine in the blood, and risk of cardiovascular diseases is shown [59]. Natural folic acid (pteroyl-L-glutamine) is found only in trace amounts. The 50-80 % of natural forms of this vitamin in foods are polyglutamates, the 5,6,7,8-tetrahydrofolates (H4 folates). Bioavailability of natural folate is lower than that of folic acid. This difference is partly due to the fact that folic acid can be absorbed directly, while folates (mainly polyglutamates) are preliminarily hydrolyzed by disconjugase to monoglutamyl phosphate. Due to its high stability, folic acid is the only form of folate, which is added directly to food products and is part of multivitamin mixtures for animals. Among the opponents of the technological vitaminization method of food, there is a view that enrichment with folic acid leads to an increase in the amount of unmetabolized folic acid in the human body. As mentioned above, biofortification, in the process of which biotransformation of folic acid in the chicken organism proceeds, rejects this argument.

The egg folate content dependence on folic acid addition to the barley diet (0-128 mg/kg during 21 days) had the shape of the saturation curve. The folate content reached maximum 32.8-42.2 μg per egg, when adding synthetic folic acid up to 2 μg/kg feed, and further remained constant [24]. According to other data, enrichment of eggs with folate up to 41.0±0.7 μg occurred at a dose of 4 mg folic acid per 1 kg of barley-based feed, and this value remained stable during 28 days of storage at 4 °C [60]. A twofold increase in the folate content per egg melange occurred when 3.5 mg of folic acid per 1 kg of feed was added [61]. Supplementation of hen diet with folic acid (from 0 to 32 mg/kg) for 12 weeks increased folate amount in the egg to 75 μg, or 2.3-fold [25]. In the egg, folate is in the form of 5-methyltetrahydrofolate and only 10 % is folic acid. In vivo experiments on rats showed that the folate of folate-enriched eggs of hens, which were fed with its dietary form, was well absorbed in the body [62]. The excess of folic acid in the corn- and soybean-based diets (50 mg per 1 kg) was accompanied by a decrease in feed intake by hens and a reduced hatchability [63].

A relationship between the vitamin content in the hen diet and in the laid eggs [64] found in all cases (see Fig.) shows that the amount of egg vitamins can not increase unlimitedly, but tends to a certain maximum value, when a further increase of dietary vitamins will not lead to an additional increase in their content in eggs (see Fig.). That is, the eggs are saturated with these micronutrients [16, 17]. Excessive dietary vitamins can decrease productive performance of laying hens [63].

It is obvious, that to achieve the maximum amount of each vitamin, special researches are required to determine its optimal content in the diet. In

this case, excessive consumption of some vitamins can disrupt the balance of others in the egg. Thus, excessively high vitamin A content in the diet (400,000 IU/kg) led to a decrease in vitamin E and carotenoids in the yolk [19, 65]. Significant decrease of the vitamin E concentration in the yolk was the result of simultaneous enrichment of hens' diet with vitamin E and β-carotene (200 mg/kg feed) [18]. An increase of the canthaxanthin level in the diet from 12 up to 24 mg per 1 kg caused an increase in γ-tocopherol amount in the egg yolk [66]. An increased amount of all dietary vitamins led to a simultaneous increase of folate, biotin and pantothenate, vitamins A, E, B_1, B_{12}, D, and $25OHD_3$ ($p < 0.05$) in eggs [67]. Simultaneous enrichment of chicken eggs with vitamins D, K and iron showed that the addition of 12000 IU of vitamin D_3 and 7.5 mg of vitamin K per 1 kg of feed for 20 days elevated the levels of these vitamins 4.6 and 4.8 times, respectively [68].

Given the functional links between vitamin in the body, the simultaneous intake of vitamins is more effective for improving body state [69], so the maximum egg enrichment to an amount that meets the criteria for enriched food products [70] seems very prospective.

Thus, through optimal vitamin enrichment of the poultry feed, eggs with a maximum content of vitamins and carotenoids can be obtained, which significantly increases their nutritional value. One egg can provide up to 40-50 % of the recommended daily intake of vitamins D, B_{12}, K, pantothenic acid, 30 % of vitamin E, 20 % of folate, about 10 % of vitamins A and B_2, and up to 30 % of adequate amount of lutein consumption. In this case, enrichment with vitamins meets the criteria for food production, i.e. from 15 up to 50 % in a portion. Biofortification of hen eggs with vitamins is one of the effective strategies providing population with vitamins.

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DEEP PROCESSING OF COLLAGEN-RICH POULTRY PRODUCTS FOR DIFFERENT USE

(review)

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Abstract

The competitiveness of meat processing technologies requires deep processing of protein containing raw materials including low-value wastes and by-products of meat processing. The connective tissues after animal and poultry meat processing can reach 16 % of initial carcass weight and hence the reasonable utilization of these resources is reasonable. Low-value by-products can be transformed to protein products via hydrolysis resulting in the preparations of isolated collagen-rich high-purity proteins with key functional and technological properties for food, feed, medical, and cosmetic industries. Chicken skin (J. Stachowiac et al., 2004), necks and bones (M.I. Kremnevskaya et al., 2016; P.F. De Almeida et al., 2013), trachea of chickens, ducks and ostriches (T. Jaroenviriyapap et al., 2009) were studied as secondary collagen-rich raw materials. The most common techniques of collagen extraction are acidic and high-temperature hydrolysis (K.A. Munasinghe et al., 2014), papain and pepsin hydrolysis (P. Hashim et al., 2014), alkalase and trypsin hydrolysis (Z. Khiari et al., 2014) and microbial fermentation (A.Yu. Poletaev et al., 2011; O.V. Zinina et al., 2013). Deep processing of secondary collagen-rich raw materials in the meat industry will reduce the existing deficit of food and feed protein, expand the assortment and increase the output of meat products and low cost digestible feeds, and improve the ecological situation. For pharmacology, short peptides are of interest, the regulatory function of which has been known for a long time and is used in medicine (A.D. Neklyudov et al., 2007) which could be produced by deep processing of animal and poultry carcasses. The importance of dietary collagen is also associated with imino acids with –NH groups (proline, hydroxyproline) which are necessary for tissue growth and development (S. Busche, 2011). Different techniques of processing collagen-containing raw materials allow to manufacture protein products with specified properties for use in food, feed and other industries.

Keywords: collagen-containing animal wastes and by-products, enzymatic hydrolysis, short-run high-temperature hydrolysis

Effective utilization of resources is one of the basic concepts of modern global agriculture. In this regard, opportunities for conversion of non-digestible animal-derived protein components such as collagen, elastin, and reticulin are being sought. Massive yield of connective tissue from animal carcasses may reach 16 %. Processing of collagen-containing substrates is predicted to save 10-20 % of the main raw material in manufacturing full-value meat products and up to 70-100 % of the main raw material in production of artificial covers and films. The use of collagen protein allows decreasing the cost of production, loss from thermal processing, and improves quality upon use of the low-grade meat for processing [1]. Most traditional technologies have already been used for a long time for production of protein stabilizer from the pork skin and beef vein,

liver and sub-product sausages, sulze, meat jelly, etc. [1]. Skin collagen forms transparent gels and may be used as gelling agents.

This review summarizes information on properties of collagen-containing raw materials, methods of its processing and their effect on the functional properties of collagen. Particular emphasis was made on the use of protein-based by-products obtained upon processing of poultry production and on properties of protein-containing substrates for food, pharmaceutical and feed industries.

Collagen structure and functions. Specific amino acid composition and spatial structure of collagen molecules define not only their physiological functions, but also technological features, in particular, stability at extraction, an ease of separation from other components, ability of restoration from solutions with formation of supramolecular structures, which extends the sphere of application of modified collagen products.

Tropocollagen consists of three polypeptide equal size chains forming a triple helix [2, 3] based on hydrogen bonds with numerous inter-chain cross-links between the amino acid (AAs) residues (each polypeptide chain contains nearly 1000 residues). Convergence of chains is ensured by regular distribution of glycine (each third position), being the only amino acid lacking β-carbon and lateral radicals. Collagen includes 19 AAs which, depending on structure of the lateral radicals, are divided into three groups: non-polar chemically inert AAs (glycine, valine, alanine, isoleucine, phenylalanine and proline), AAs having reactive groups (serine, threonine, tyrosine, methionine, oxyproline), and polar AAs dissociating as bases (lysine, oxylysine, arginine, histidine) or as acids (glutamine and asparagic acid).

Collagen in wastes of skin is rich in proline and oxyproline (nearly 20 %), glycine and alanine (over 50 %), practically lacking (or containing in small quantities, if insufficiently purified from other proteins) any aromatic, heterocyclic, and sulfur-containing amino acids [1]. Collagen is one of few proteins not only containing oxyproline, but also oxylysine. Thermal stability of tropocollagen units and fibrils increases with the increase of share of proline and hydroxyproline amino acids [4, 5]. The content of proline and hydroxyproline is significantly lower in fish, in particular cold-water fish, than in mammals [5].

The distinguishing features of the collagen are its unique mechanical properties, chemical inertness, amino acid composition, and ability to transform into low-molecule protein product, gelatin, upon prolonged heating in water, and in water-based solutions of acids and alkali. Various factors affect physical and chemical properties of collagen [6].

By its physiological effect, proteins of the connective tissue, including collagens, are similar to dietary fiber displaying clear cytoprotective activity, normalizing microbiocenosis of intestines, preventing dystrophic changes in epithelium and permeability of colon mucosa cell barrier, thus, reducing the likelihood of penetration of bacteria, toxins, and polymeric residues with allergizing effect. Collagen breakdown products (gluten, gelatin, etc.) own properties of the dietary fiber stimulating secretion of the digestive juice and intestinal motility, and favorably affecting good microflora [7]. Uncombined charged AAs groups in the collagen molecule, which are localized in polar zones having interlaced with hydrophobic areas, may link ions of heavy metals in the intestinal duct with further formation of the insoluble complexes, which can not penetrate, and are excreted from the body [5]. The ion absorption by hydrolyzed collagen in acidic (pH = 1.2) and alkali (pH = 7.8) environment comprises 71.00 and 82.00 %, respectively, for Pb^{2+}, 68.00 and 74.67 % for Cd^{2+}, 25.33 and 50.00 % for Cu^{2+}, and 81.2 and 91.00 % for Hg^{2+}. Absorptive ability increases with the increase of the serial number of element (and, accordingly, ion radius) with the maximum

for mercury ions. Linking mechanism has not yet been clearly identified, but it had been established that all proteins are characterized by its apparent ability for non-specific linking with metals by guanidine group of arginine, etc. Possibly, peptide chains break down upon fermentative processing of vein waste, due to which functional groups become easier accessible for reaction with metals.

By-products of collagen-containing raw material in poultry farming. Traditional technology of use of the connective-tissue proteins in meat product formulations had not led to the desired results (http://www.pandia.ru/text/category/vovlechenie/), since native components of the connective tissues have low organoleptic value and are functionally incomplete. Besides, because of bovine spongiform encephalopathy (BSE) and influence of Muslim traditions, the need for more safe and acceptable collagen sources is raised.

In Russia, nearly 20 % of poultry production is subjected to deep processing, thus yielding nearly 650 thousand tons of by-products per annum. Legs, skin, trachea, and bones are used for extraction of the collagen proteins in poultry, whereas product yield is lower than in case of cattle and pigs. No tryptophan and small amount of methionine was found in the connective tissue (tendons, cartilage, and skin) mainly consisting of the collagen; these proteins are characterized as incomplete and are recommended as dietary fiber for improvement of the intestinal motility and functioning of the digestive duct. Meat without visual connective tissue has lower biological value than meat with natural content of the connective tissue. This confirms feasibility of use of poultry skin in production of meat products.

By-products of poultry, in particular skin, are rarely processed and transformed into valuable products. Extraction of collagen, whether containing or not containing telopeptides, from the chicken skin had been described [8]. However, chicken skin may be used for production of the collagen substrates, which are highly valued due to its unique functional properties. Chicken skin contains 75 % of I type collagen (the strongest archetypical form is fibrillous triple helical protein) and 15 % of III type collagen (embryo derma collagen). Upon collagen extraction by pepsin or ethylenedyamine (after removal of fat by heating of the chopped chicken skin at temperature of up to 40-60 °C), the output product is rich in telopeptides. Therefore, poultry skin serves good alternative source of high-quality collagen.

Upon mechanical dissection of the bird's carcass or its parts, 27-40 % of meat and bone residue is produced, with bone percentage of 15-20 % and ash to protein ratio of 0.7 in the chicken bones. Interestingly, 25-30 % is attributed to complete proteins, at that, protein and mineral substances are mainly contained in bone tissue, and liquid and fat — in meat tissue [9].

Due to complex structure of chicken tracheas, which are also characterized by high content of muscle and connective tissues, separation of meat from the bones is practically impossible. Technology of 8-hour lasting hydrolysis of trachea and bone residues at temperature of 100 °C in presence of inorganic catalyzer had been developed. Maximum density of the produced collagen product is 123 g/cm^3. Advanced production technology of ready-cooked and smoked whole-muscle products allow for addition of brine in the raw material. Herewith, retention rate and duration of processing mostly depend on diffusion rate of the brining agents. To decrease such rate, mixtures of collagen and muscle fractions of protein products of meat and bone residues of the poultry carcass may be used in various proportions. Addition of the protein mixture allows obtaining higher yield and nutritional value of ready-to-use meat products, and allows improving its organoleptic characteristics and rheological properties [10].

Chicken, duck, and ostrich tracheas may be used as sources of collagen

and chondroitic sulphate [11]: it was noted that chondroproteins comprise from 49 to 73 % in the output products, chondroitic sulphate (mainly in form of chondroitine-4-sulphate) was identified by high-efficiency liquid chromatography (HELC) at 0.574-6.37 % level in terms of dry matter (mainly in ostrich, lower in duck, and the least in chicken). Hydroxyproline in collagen and chondroitic sulphate makes 66.19 and 84.38 %, respectively, or 4.04 and 7.40 % per dry matter. Collagen extracted from chicken buttock was compared with collagen extracted from the Achilles tendons in cows [12]. Jelly produced based on chicken leg collagen had been highly valued, thus highlighting the opportunity for production of high-quality gelatin from such type of raw material [13].

Processing methods and properties of protein products. Hydrothermal, acid, alkaline, and fermentative hydrolysis are used for extraction of the collagen and production of protein-containing substrates with high accessibility of the collagen from low-value raw materials. Each of the above-listed methods allows for production of the collagen both in solvable and in denaturized forms. Hydrothermal processing results in collagen denaturation with breaking of the specific configuration of polypeptide chains, and bonds within and between molecules. As a result, sizes and structure of the collagen fibers are changed. Full collagen hydrolysis takes place at temperature of 126 °C and lasts 3 hours. Strong acid hydrolysis results in complete destruction of tryptophan and in destruction and racemization of oxyacids, dicarbonic acids, and proline with production of D-isomers of several known amino acids, which are not metabolised by cell and may act as cell growth inhibitors. Alkaline hydrolysis causes racemization of the majority of amino acids and destruction of arginine, lysine, cystine, and cysteine. It results in production of the complex of defective toxic substrates. Fermentative hydrolysis with the use of proteolytical ferments lacks the drawbacks of acid and alkaline hydrolysis. Although such type of hydrolysis can be no more than 70-80 % complete, components produced due to the cleavage are physiological, easily penetrating in cell, and are engaged in metabolic process. Fermentative hydrolysis may also be performed with the use of live cultures of bacterial producers.

In USA, poultry processing industry is one of the fastest growing food industries [14]. In examining the contents of collagen in chicken bones and skin, the authors of the research had extracted collagen by **acetic acid**, lemon acid, alkali, with the use of two-stage hydrolysis by **acetic acid** and pepsin. Research results had shown high potential of chicken by-products as an alternative source of collagen for production of highly-valued products [14].

A multiple stage procedure has been developed for collagen extraction from chicken bones, allowing for removal of 87.5 % of mineral substances and 57.1 % of fat, provided loss of protein of nearly 18.6 % and hydroxyproline of 14.9 %. Collagen yield had comprised nearly 85 % of the initial volume, with of its quality and functional properties assessed [15].

The extraction procedure of the collagen from chicken legs by acetic acid at 4 °C within 24 hours with the use of papain and pepsin yielding 18.16 and 22.94 % collagen, accordingly, had been described [16)]. Produced collagen is rich in glycine, glutamine acid, proline, and hydroxyproline. Electrophoretogram of samples had shown presence of two α-chains (α_1 and α_2) and β-chain, i.e. main component of chicken leg collagen is collagen of I type. In both cases, samples were resistant to thermal denaturation at 48.40 and 53.35 °C, respectively.

One of perspective processing techniques of secondary protein-containing raw material is fermentative hydrolysis with the use of live microorganisms. Fermentative processing allows for practically complete preservation of all essential

amino acids. Herewith, use of ready-to-use fermentative preparations in the industrial scale may be expensive and costly, whereas introduction of live microorganisms allows decreasing the processing cost of collagen raw material [17, 18]. Effect of microbial cultures tissues is assessed by alteration of functional, technical, physical, chemical, and morphological properties, as well as by nutritional and biological values of the product. The effect of processing by microbial ferments on certain structural tissue elements, in particular collagen fibers had been confirmed by histological micro-structural studies [19].

Collagen is known for its high swelling ability, on which its functional and technical properties are based. Collagen proteins have unchallengeable features as compared to vegetable origin structure-forming agents, for instance carageenans and vegetable gums. Unlike vegetable origin hydrocolloids, properties of which are effected by concentration of culinary salt, food-grade phosphates, defrosting process, etc., collagens excellently preserve and display its functional properties in meat systems [20-22].

Areas of interest for pharmacology are short peptides (two amino acids and more, with molecule weight less than 10 kDa) which are the components resulted from meat processing. Their regulatory function is known for a long time and is used in medicine [23]. Dietary collagen significance is also associated with amino acids with –NH groups (proline, hydroxyproline) which are necessary for tissue growth and development [24]. Upon fermentative hydrolysis of collagen, low-molecule biologically active peptides could be extracted from secondary products of turkey processing. Protein-containing substrates and solid biomass may be produced from turkey heads followed by fermentative hydrolysis during various periods. Molecular weight distribution studied in turkey head hydrolysates by exclusive chromatography had shown that mixture of proteolytic ferments could produce great amount of low molecular weight peptides ranging from 555.26 to 2093.74 Da. These collagen peptides showed excellent solubility over a wide pH range (pH 2-pH 8) and were able to bind cholic acids. Enzyme cocktails for hydrolysis represent a potential new way to produce low molecular weight bioactive collagen peptides from poultry by-products [25].

Another modern technology for processing collagen-containing raw material, which is considered to be among the most perspective methods, is short-term hydrothermal hydrolysis. Dry collagen-containing proteins produced by this method from meat-and-bone residues and chicken legs are completely water-soluble. Digestibility of these products exceeds 95 % indirectly indicating the high protein assimilation. By physical and chemical properties, the collagen-containing proteins from chicken legs are favorably comparable to animal proteins from meat-and-bone residues due to an increase in weight proportion of collagen in dry protein by 13.8 % and fat decrease by 40.0 % [26]. Such results had shown possibility of domestic production of collagen-containing extracts in lieu of the imported additives of the animal proteins from pig and beef skins. Among the advantages of short-term high-temperature processing is a 100-fold decrease in time, double increase of protein concentration in broth, triple increase of the total output of protein and dry substances and increase of the protein yield from the chopped chicken legs by 24.3 % as compared to meat-and-bone residues at equal conditions (26).

Traditionally, no-waste technologies are aimed at processing of trash products for protein feed production. As we know, AAs are divided into essential and non-essential. It had previously been thought that animal body may independently synthesize non-essential amino acids, but this is clearly not confirmed. Non-essential amino acids (for instance, glutamine, proline, glycine, and arginine) play an important role in regulation of gene expression, cell signaling, an-

tioxidant activity, neurotransmission and immunity. Besides, glutamine and aspartate participate in maintenance of the integrity of small intestine and its digestive function [27]. Accordingly, animal feed formulations shall be balanced by essential and all non-essential amino acids, which shall be accounted for upon revision of "a perfect" protein conception [27)]. It had been shown that feeding of broiler chickens with low-protein diets lacking glycine and serine may decrease the rate of their growth and cause depression [28].

Not only feed additives, but also veterinary preparations may be produced based on protein-peptide compounds from low-value animal raw materials. Thus, Kolimak and Dinormin are designated for treatment of the immune deficit and gastrointestinal diseases in piglets [29, 30]. By effectiveness and economic indicators, such preparations outperform the imported analogues. Kolimak contains liophilic extracts of pig stomach, duodenal and pancreas, and has clear protective and medicinal properties at gastrointestinal disorders, such as antibiotic-associated diarrhea and gastroenteritis. The developed technology preserves active substances of protein origin in the extracts [31]. Low-value raw material can also be used for creation of functional products with rehabilitation, preventative, and medicinal effects. In addition to compounds responsible for regulatory functions, slaughter by-products contain antimicrobial peptides participating in protection from external factors. Dinormin is a preventative and medicinal preparation from extracts of immune competent organs in pigs (thymus, spleen, and mesenteric lymph nodes) containing significant amount of neuromediating amino acids (aspartic, glutamine acids, glycine). It is highly effective at treatment of immunodeficient diseases in animals due to the essential regulation of the immune system, allows increasing weight gains and improving survival rates of the livestock [32].

Feed additives and meat properties. It is evident that animal rising technologies affect quality of the meat and by-products. Detection and identification of proteins and peptides in animal and vegetable raw materials extend opportunities for their use [33]. Obviously, development of feed additive production based on deep processing of protein-containing raw material, as well as effective use of such additives to pre-form future properties of the meat products during animal's life requires deep knowledge of relationship between meat consumer and technological qualities, meat protein composition and how it is influenced by stress and free-radical oxidation. All these must be accounted in developing feed formulations.

A large number of polypeptides of two to 30 amino acids ensuring effective meat processing and quality of finished products are extracted from muscle proteins of cattle, chicken, and pigs. Compounds associated with meat tenderness and moisture-retaining property are well studied [34, 35]. Relationship between the heat shock proteins and meat quality (tenderness and moisture-retaining property) was established [36, 37]. Heat shock proteins (HSP) are highly-conservative and expressed in all cells of a body as a response to physical, chemical or biological stressing factors [38, 39]. Absolute majority of the pathological changes in functions of cells, tissues, and organs is followed by deviation from the normal protein profile of healthy body [40].

Slow-down in lipid peroxidation promotes better taste, texture, and nutritional value of meat products [41]. Consumer preferences most of all depend on color of chicken skin and meat [42, 43]. Broiler body may not synthesize pigments [44], and standard commercial diets based on maize and soybean grains do not ensure sufficient quantity thereof [45]. Therefore, pigments are usually added in feeds [46]. Majority of pigments used for a long time in poultry farming industry in many countries are synthetic. They are more stable and cheaper than natural

ones, but their safety is often questioned [42], and choice is often made in favor of healthy products rich in natural carotenoids [47-49]. Food-grade extract of calendula can improve growth, pigmentation, antioxidant ability and quality of chicken broiler meat [50]. Calendula extract contains mixture of xanthophylls with active components luthein and several zeaxanthins (xanthophylls of carotenoid group) which are considered safe since are present in edible plants. Amongst natural pigments, extracts of marigolds are mostly often used in poultry feeding [45]. Luthein may not only improve the color of chicken skin, but has very strong antioxidant properties [42, 51] which are important given the fact that antioxidant status is closely related to meat quality. However extract of dietary marigold in poultry feeding is mainly used for changing color of egg yolks. At the same time study of the effect of marigold extract on meat quality in broiler chicken had shown that natural carotenoid improves moisture-retaining property of meat. That is, more tender texture may be due to the increase of antioxidant activity or changing in moisture-retaining ability [52]. Intra vitam, antioxidant status affects animal and poultry health, and antioxidant potential of muscle tissues after slaughter significantly determines the meat quality. Lipid peroxidation by free radicals causes stress and increases contents of malonic dialdehyde, being the finished product of lipid peroxidation [53], which also deteriorates meat quality and changes its color. Accumulation of antioxidants, on the contrary, stabilizes color and increases meat shelf life [54]. Dietary antioxidants, such as vitamin E, may compensate unfavorable effect of oxidation stress on growth and broiler meat quality [55] and, accordingly, may improve economic effect of feed additives based on deep processing of protein-containing by-products.

Pale soft exudative (PSE) meat remains a serious problem for poultry processing industry. This defect is directly due to protein denaturation caused by rapid decrease of pH in early post-slaughter period at high temperatures [56, 57]. It had also been shown that stressing agents (increased stocking density, heating stress, and transportation stress) may result in PSE [58, 59]. This example indicate the necessity for accounting all factors of the economical effectiveness of feeding additives derived from deep-processed poultry by-products, which are intended for intra vitam formation of the technological properties of meat.

Thus, secondary by-products in poultry farming are safe and available sources of collagen and protein for food, pharmacology, and feed production industries. In Russia, deep annual processing of poultry yields nearly 650 thousand tons of by-products. Hydrothermal, oxidation, alkaline, and fermentative hydrolysis are used for extraction of the collagen from low-quality raw material. Dry collagen-containing proteins derived from short-term hydrothermal hydrolysis of meat-and-bone residues and chicken legs are completely water soluble. Over 95 % proteolysis indirectly indicates high digestibility of the produced proteins. To develop technologies for production of feed additives by deep processing of protein-containing raw material, as well as their effective use, requires proteomic study of poultry farming products focused on intra vitam formation of consumer and technological properties of poultry.

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CLUSTERBEANS *Cyamopsis tetragonoloba* (L.) Taub. — PROPERTIES, USE, PLANT GENETIC RESOURCES AND EXPECTED INTRODUCTION IN RUSSIA

(review)

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Abstract

Clusterbeans *Cyamopsis tetragonoloba* (L.) Taub. is annual leguminous plant of multipurpose use. In India it is known as a forage crop, green mass serves for animal feeding and as a green manure (K.V. Muradov, 1973). Young green pods are used as vegetable, and many vegetable guar varieties are cultivated (N.K. Dwivedi, 2009). Guar meal (husk and germ) is a high-protein ingredient for feeding cattle, broilers and fish, its antidigestive factors can be reduced by temperature treating and adding enzymes (M. Hussain et al., 2012). Guar powder Churi 40 contains about 40 % of protein, granular guar fodders Korma 50 and Korma 60 contain 50 and 60 % of protein. Guar gum extracted from endosperm of guar seeds is used widely in food, textile, paper, cosmetic and oil industries for its ability to emulsify and thicken liquids (D. Mugdil et al., 2014). The demand in guar gum is increasing in domestic and world markets. Plant genetic diversity of the crop is allocated mainly to India where guar had been growing from ancient times. In USA guar breeding has succeeded with improved varieties (W. Liu, 2003). Guar collection founded in the Institute of Plant Genetic Resources (VIR), Russia, by N.I. Vavilov lists more than 100 accessions. Germination of guar seeds from VIR collection after 40-year storage even at the room temperature remained high. The research data and the practice of guar growing in Russia testify that the crop could be successfully cultivated in the southern regions. Here, the main desired traits of the guar are short time for crop maturation and high gum viscosity.

Keywords: guar, guar meal, granular guar fodder, guar gum, galactomannans, genetic resources of clusterbeans, introduction

Guar *Cyamopsis tetragonoloba* (L.) Taub. is annual tropical legume. Like other beans, guar seeds contain a significant amount of protein, its green mass is used fresh and dry, the plants enrich soil with nitrogen. Guar gum [1], which is obtained from endosperm of seeds is of particular value and widely used in food, cosmetic, textile, paper, oil industry. In recent decades, from a little-spread tropical culture, guar is turning into one of the most popular in the world, which updates the task of its introduction in Russia.

The purpose of this review is to summarize information about the particular plant features and products, genetic resources preserved in world's collections and in Russia, and about the perspectives of cultivation of this crop non-traditional for Russia.

Botanical and ecogeographic description. Guar (cluster bean,

Indian acacia) — hewar *Cyamopsis tetragonoloba* (L.) Taub. of *Fabaceae* L. family, tribe *Indigofereae*. Synonyms: *Cyamopsis psoraloides* (Lam.) DC, *Dolichos fabaeformis* L'Herit., *Dolichos psoraloides* Lam., *Lupinus trifoliatus* Cav., *Psoralea tetragonoloba* L., *Cordaea fabaeformis* Spr. (http://www.theplantlist.org). The genus Cyamopsis comprises four species: *C. tetragonoloba* (L.) Taub., *C. senegalensis* Guill. & Perr., C. *serrata* Schinz., *C. dentata* (N.E. Br.) Torre [2]. J.B. Gillett divided the genus into three species: *C. tetragonoloba* (L.) Taub., C. *senegalensis* Guill. & Perr., *C. serrata* Schinz., without identifying *C. dentata* (N.E. Br.) Torre as a species; in his opinion, the genus *Cyamopsis* is closest to the tropical genus of the *Indigoferae* family [3]. H.A. Senn expected that the genus *Cyamopsis* originated from the genus *Indigofera* due to aneuploidy [4]. *Cyamopsis senegalensis* is regarded as the ancestral form of cultural *Cyamopsis tetragonoloba* (L.) Taub. species [5]. An argument in favor of such origin is the presence of galactomannan in seeds of *C. senegalensis* which is similar in structure and content to the guar galactomannan. Potentially *C. senegalensis* can be considered as a source of gums [6].

The place of guar origin is India, Pakistan, and probably Africa [2], but it has never been found in the wild. N.I. Vavilov considered that the center of guar origin and diversity was India [7]. Although, green guar beans are used in India as a vegetable, the Indian name of guar is “gau-aahaar” (“cow’s food”) [8], that is, traditionally the guar is primarily an ancient forage crop [9]. There is a belief that guar *Cyamopsis tetragonoloba* originated through transdomestication from an African species which were imported to India as horse fodder by Arabian traders [5]. The guar is mainly cultivated in the arid regions of northwestern India, the Thar desert territory, which covers the Indian states of Rajasthan, the southern part of Haryana, Punjab and north Gujarat, also the southeast Pakistan. In this area with 90-200 mm of rainfall annually, most of precipitations fall during the summer monsoon (from July to September). Pasturing is dominated in the region, and perhaps, guar for centuries served as forage for cattle, camels, horses, and sheep. Not long ago, it was announced about the finding of missing link between wild species of the genus *Cyamopsis* and the cultigene. In the Northwest India, the wild-type forms have been found in the domesticated guar crops. To date, 66 samples of the so-called adak guar have been collected. These plants are prostrate, have small inedible dehiscent pod, its seeds are characterized by a rest period. Further study is necessary to clear up the role of native forms in the guar origin and possibility of using additional genetic variation which was lost during domestication [10].

Guar, like many annual legumes, is self-pollinating plant (percentage of natural crossing is incidental) [11, 12]. Plants vary significantly in height being 50 cm to 1.5 m tall. Stem is solid and lignified by the maturing time. Taproots can access moisture in low soil depths, so that the plant can tolerate short-term drought. As to morphology, guar plants have basal branching or fine branching along the stem may be single stemmed. Leaves are trifoliate, pubescent or smooth. Flowers almost peduncles, with corollas from white to bright pink in color, the inflorescence is axillary raceme and born in clusters. Pods settle tightly forming groups (clusters), due to which English name of cluster beans originated. Pods are straight, slightly curved, from 4 to 14 cm long, containing 5 to 12 seeds. Mature seeds vary greatly in color (dirty white, pinkish-gray, gray-beige, brown, black), seeds are rounded-quadrangular, flattened, have a large spherical endosperm which contains a spare galactomannan polysaccharide. Diploid chromosome number is 14*n* [13]. Guar is a thermophile plant, undemanding about soil (growing on sandy soils and well drained clay soils). Like other legumes, guar refers to soil-improving crops. It is used in

crop rotation with cotton, sorghum, wheat [2]. Nowadays, guar is mainly grown for grain; its seed consists of embryo (40-45 %), seed coat (14-16 %) and a large endosperm accounting for 38-45 % of the total seed [14-16].

Application. In Indian breeding and genetics guide (1957), guar was listed as forage plant and vegetable crop "for poor people" [17]. Nowadays, in India guar is cultivated as livestock fodder, vegetable and technical plant and it is also used in Indian traditional and modern medicine [18, 19]. Young beans are eaten stewed and salted [20].

Guar green mass, which is used for cattle forage and as green manure, contains 15.56 % of crude protein and 15.74 % of crude fiber [21]. The best time to cut guar for green forage is the flowering phase and the milk ripeness of the beans. The attractivity of the guar green mass for cattle is increasing after grinding and pre-wilting. The recommended guar hay proportion in the diet of adult sheep may be up to 70 % [22]. In the arid regions of India, after harvesting beans, cattle and camels are grazing on the guar plant residues in the fields [23]. Guar is recommended as an alternative to water intensive crop alfalfa in a country with such arid conditions as the Arab Emirates [24].

The guar bean contains antinutritional substance, so when whole grain feeding, thermal treatment is required [25], and in poultry nutrition guar beans should not exceed 10 % [26]. The guar protein is well balanced in amino acid composition [27]. The protein amount in the embryos and spermoderm is 28.9-46.0 %, embryos and husks in the raw material for guar fodder account for about 25 and 75 %, respectively [28]. Guar meal Churi and granulated feed Korma (the exchange energy in the latter is 2022-2074 kcal/kg)are using in dairy cattle feeding. Guar meal Churi 40 % is obtained after splitting, refining, roasting, triturating and sterilizing. The feed contains 5-7 % fat, 5-10 % fiber (with humidity up to 10 %), the amount of raw protein (38-42 %) is higher than that in corn gluten fodder (http://guarprotein.com/). Guar meal digestibility is 76 % after heat treatment and 71 % without treatment. Thermally processed Churi food can replace soybean meal in the bovine ration. Korma 50 feed is obtained mainly from the embryos fraction (raw materials are purified without roasting). It contains 48-50 % protein and is used in cattle and poultry diet. Roasted Korma 60 contains up to 56 % protein with improved digestibility. Feed is recommended for animals and fish, replaces a more expensive soybean meal. Bio Guar Protein (Pro NX 60+ (Cyamopsis Biotech, India), the premium segment feed, is a roasted guar meal with a protein content of 60 % and higher, is destined for aquaculture, feeding salmon fish and shrimp (http://guarprotein.com). Producers of guar feed export it to many countries, including Russia. Guar seed feed is the cheapest source of vegetable protein for ruminants and poultry, its addition to diet reduces costs significantly. It is well eaten by cattle but can cause problems in monogastric animals [29]. Guar-based feeds increase fat content and milk yield in cows, positively affect diet digestibility, guar fodder may be used separately or as an ingredient of the compound feeds, but the transition to this feed must be gradual [30, 31]. The high amino acids content makes guar fodder a useful additive for broilers and laying hens, but the increase in its proportion in the poultry diet is limited by antinutrients such as trypsin inhibitors, gum residues and saponins. Roasting destroys trypsin inhibitors, resulting in their less amount in guar fodder compared to soybean meal [28]. Basically, the growth of broilers is slowed down by traces of gums, rather than seed antinutrients [26]. One of the methods to eliminate the negative consequences of eating guar fodder by the bird is the addition of the enzyme β-mannase directly to the compound feed, resulting in a decreased viscosity of the gum trace amounts [32, 33].

According to data 2014, 79 large industry non-integrated works operate in India, where the guar seeds are divided into a shell and an embryo [34] as sources of the protein fraction and the endosperm (split) containing galactomannan, the base of guar gum which is considered the most valuable processed product of guar seeds.

Guar gum. Locust bean (*Ceratonia silique* L.), guar (*Cyamopsis tetragonoloba*), tara (*Caesalpinia spinosea Kuntze*) and fenugreek (*Trigonella foenum-graecum* L.) are commercial gum sources of plant origin [35]. Polysaccharides in seeds of *Fabaceae* family species, with different types of endosperm development, are mainly galactomannans which consist of D-mannose and D-galactose and are localized in the endosperm cell walls. Theoretical interest arouse from polyfunctionality characteristic of such phyto-polysaccharides [36, 37]. They show a protective function, as well as serve as the energy reserve and are involved in regulation of the water balance during seed germination [38]. Due to the hydrocolloid properties, the galactomannans which contained in endosperm provides moisture retention, necessary for seed germination in drought conditions. Galactomannans of legume seeds differ in the ratio of mannose and galactose (M:G), in molecular weight and galactose group location on the mannose skeleton. In galactomannans, the mannose units form a chain consists of hundreds (1→4)-β-D-mannopyranose units with α-D-galactopyranose units, which are joined by 1→6 bonds and in different ways (depending on the plant species) are distributed along the main mannose chain.

Chemically, guar gum is a non-ionic polysaccharide the molecule of which is formed by a mannose skeleton with attached side galactose residues. The empirical formula of guar galactomannan is $(C_6H_{10}O_5)n$ [39]. The D-galactosyls are arranged in small groups of mostly two to four units, attached to contiguous D-mannosyl chains and separated mostly by two, or occasionally three, D-mannosyl residues [40]. The galactomannan molecule of guar gum has the largest molecular weight among water soluble natural hydrocolloids. Galactomannan content of guar seeds is up to 35 % of the seed dry weight [41].

Galactomannan is readily soluble in cold water with the formation of viscous colloidal solution, even in low concentrations, so guar gum is used as a natural thickener, sealant and stabilizer in many industries [8, 9]. Most people in developed countries consume guar gum daily in the form of food additive E-412 in dairy, meat, bakery products, ice cream, yogurt, sauces, etc. [41]. Guar gum as a product reached out to the international market in 1957. The main production of guar is concentrated in India. This country is the largest producer and exporter of guar gum in the world market. According to 2012 data, the market size of this product annually comprised from 1,000,000 to 1,600,000 tons [42].

India accounts for approximately 80 % of the world's guar gum production per year, followed by Pakistan (15 %), where the guar is grown on irrigated land, and the remaining 5 % is produced in the USA, Australia and South Africa [42)] Approximately 90 % of guar gum produced in India is exported, mainly to the US, China, Germany, Russia, Canada and Italy. The USA is the largest importer of this product (about a quarter of the world's consumed guar gum), and the main sphere of its use in the USA is the extraction of shale gas and oil. It is expected that in the coming years the demand for guar gum will grow by about 4 % annually due to the shale's boom in the USA and the popularity of natural thickeners in the food, cosmetics and other industries (https://marketpublishers.com/r/G6AA788F0A6EN.html).

As an additive for drilling fluids, guar gum possesses unsurpassed properties. During oil drilling wells, guar gum prevents loss of water from the viscous mud and well suspends bentonite clay. Guar gum of rapid hydration with

a viscosity of 6500 to 9000 units is in use in mining for hydrofracturing formation [43]. To obtain the quick hydration gum, the extrusion step [44] is introduced into the gum production process. A quick hydration gum forms a viscous gel in a few minutes (normal guar gum needs 2 hours).

In the guar gum manufacturing process, the seeds after husking are split into endosperm parts, between which the embryo is enclosed. After separation of the embryo and the hull, the so-called split is obtained during sorting. Guar split is a polished endosperm halves, in the water-insoluble cell walls of which galactomannan is deposited. To break down the cell walls and extract the gums, a fine grinding of the split is necessary. The water soluble fraction is 85 % of the split [43]. After processing, guar gum looks like a powder of cream color. The gum quality for food (additive E-412), cosmetic industry and other purposes depends on the production technology [41, 45].

Genetic resources of guar. Indian guar varieties and local guar plant populations have different morphological and agrobiological features depending on their use. Information about Indian guar varieties is given in an official publications [34, 46] and available online (http://agropedia.iitk.ac.in/content/guar-cluster-bean-varieties-india, http://www.seednet.gov.in). Forage species are tall, with good foliage, vegetable species are shorter; there are also universal varieties. The main center for guar breeding in India is the Rajasthan Agricultural Research Institute (RARI, Jaipur, Rajasthan). There, guar verities RGC-936, RGC-1002, PGC-1003, RGC-1017, RGC-1033, RGC-1038, RGC-1055, RGC-1066 etc. were developed for cultivation in this subarid state (http://raridurgapura.org/varieties-developed.htm). In the CCS of Haryana Agricultural University (http://hau.ernet.in, Hisar), guar selection is conducted for the increasing gum content in seeds (varieties HG75, HG182, HG258, HG365, HG563, HG870, HG20, HG884 are characterized by gum content from 29 to 32 %). In Haryana Agricultural University forage varieties HFG119 (with broad dark-green leaves) and HFG156 (tall, branched, disease-resistant) have been derived. Forage varieties Bungel, Agaita Guara, and RGC-936 are known. Some forage varieties (Durgapura Safed, Durgajay, Mara Guar, Uday, Lathi, Kranti-1031, RGC-1033, RGC-1038) are also grown for gums, so, they serve as dual-purpose varieties [47, 48]. In India, the guar is covering area over 2200 ha.

If in the states of Northern India (Rajasthan, Haryana, Gudirat and Punjab) guar is cultivated for gum and forage production, in the South India it is cultivated mainly for vegetable purposes. Young pods contain protein (3.2 g), carbohydrates (10.8 g), humidity (81 g), calcium (57 mg), fat (5 g), iron (4.5 mg), vitamin C (49 mg) for every 100 g of edible portion. In the state of Karnataka, in order to obtain young pods, guar is cultivated throughout the year. At the University of Agricultural Sciences (Dharwad), selection of vegetable guar to enhance bean yields and quality is ongoing. The aim is to distribute the vegetable guar cultivation in the north of the state to more arid regions [50, 51]. In vegetable guar selection, genetic diversity on qualitative and quantitative traits is preliminary estimated to select genotypes for hybridization [52-54]. One of the breeding programs included study of genetic diversity of 30 guar genotypes. The genotypes were clustered based on the feature similarity, and the hybridization programe for creation of varieties with improved qualitative and quantitative characteristics of beans was developed using inter-cluster distances and the percentage of trait contributions [55]. The most popular vegetable varieties are Pusa Navbahar, Pusa Sadabahar, Durgabahar, Kachan Bahar, Pusa Mausami. The most popular and widespread variety is Pusa Navbahar obtained in 1984 (its disadvantage is disease susceptibility).

The main purposes of guar breeding in India are early maturity, gum

yield, and diseases resistance. Despite the fact that guar plants are widely cultivated in India, the average crop yields here is not high because of monsoon fluctuations in the main cultivation zone and insufficient amount of certified seeds. Growth of guar demand necessitates task of expanding guar area on irrigated lands [46]. Most researches confirmed low genetic diversity of the crop in India. Thus, SSR analysis of 31 genotypes on 17 traits and cluster analyses showed low genetic variability among plants. Within the guar breeding program for the southern states of India, 42 genotypes were evaluated on 12 traits; cluster analysis gave visual picture of four groups separated irrespectively of their geographic origin [57]. Other researches also failed to identify correlations between genetic diversity and geographical location of samples [58, 59].

On the contrary, in Pakistans local lines and varieties, the clustering of the tested samples corresponded to their geographic origin [60]. Chemical and physical mutagenes were used to extend range of genetic diversity of guar plants [61, but economically important traits were not found in the mutants.

Plant genetic resources in India are preserved in the National Bureau of Plant Genetic Resources (NBPGR, New Delhi). Annually more than 600 samples are sown for maintaining and studying to identify donors of valuable traits [62]. In Pakistan, guar is explored in the National Agricultural Research Center (NARC, Islamabad).

The USA is the third major guar producer in the world, after India and Pakistan. The first sample was registered in the USA in 1910, cultivation began in the 1940s. Guar was tested in the Arizona state as forage crop (haymaking and even pasture), also agricultural technology, the character of growth and susceptability to diseases were studied [63, 64]. Since 1950, after the guar gum discovery, researches continued at an experimental station in Oklahoma [65] in order to increase the seed yield and resistance to the major guar diseases. Brooks (1964) was the first variety improved in the USA. It replaced initial varieties such as Texsel and Groehler, then Hall and Mills verities were derived. In 1971-1979, Kinman variety in tests produced 17 % more seed than Brooks, and became the most popular [66]. Esser variety, released simultaneously with Kinman, has high resistance to diseases and later ripening. Lewis variety had more beans on the main stem than Kinman in yield tests during 1980-1983, and produced seed yield higher than that of Kinman and Esser [67, 68]. Santa Cruz variety (1982) was distinguished by drought tolerance [69].

Varieties of American breeding have a range of morphological features that distinguish them from Indian varieties, and are based on samples which has passed a long period of adaptation and breeding in the United States. From 1998 to 2007, in the researches which were supported by Halliburton PC (USA), 50 lines and guar species showed variation of the M:G ratio in seeds from 1.6:1 to 2:1 [43]. Selection for the maximum proportion of galactose to mannose resulted in Matador and Monument varieties. Matador (2005) is characterized by a numerous side shoots and maximum seeds. Monument (2010) is early maturing, nonbranching, having high position of the first fertile node and fits for mechanized harvest. The rights to use these commercial varieties are owned by Texas Tech University (Lubbock) and Halliburton [70]).

One thousand and fifty guar samples obtained through the introduction station and 33 breeding lines were deposited in the National Center for Genetic Resources Preservation (Fort Collins, Colorado) [71]. To date, there are about thousand accessions of guar collection in the United States, 500 samples have been deposited in Fort Collins, and an active collection of 410 samples is conserved in the Plant Genetic Resources Conservation Unit (Griffin, Georgia). In total, there are 355 samples from India, 32 samples from Pakistan, two from

Iran, by ones from South Africa, Senegal, Sudan, 10 species and breeding lines from the USA, as well as the African wild guar species *Cyamopsis senegalensis* Guill. Perr. and C. *serrata Schinz.* [72]. Guar can be successfully grown in Georgia state [72]. Alternative guar distribution to Wisconsin and Minnesota (more northern and wet states) were considered in addition to Arizona, Oklahoma and Texas. Dwarfish varieties of American breeding meet the early maturing requirements which are necessary for these areas [73].

In Australia, the guar varieties, which were periodically introduced from India and the USA, are studied on various traits (yield, gum production, etc.) [74-76]. There is the government program to support guar growing and processing gums [77]. In Italy feasibility and economic reasonability of guar farming is concerning [78, 79]. In Argentina, an agroclimatic zoning methodology was developed to determine the potential areas for guar growing [80]. In Egypt, the guar is suggested as a green fertilizer for the sandy soils remediation [81].

Guar seeds were first imported by the All-Union Research Institute of Plant Industry (VIR) to the Soviet Union from India, including 8 samples collected in Punjab state during expeditions of V.V. Markovich in 1927-1929 and 6 samples from Pune, Maharashtra state, introduced in 1931). Experiments on guar cultivation were carried out at the southern experimental stations of the VIR. At the Sukhum Experimental Station in 1928 (humid subtropics), in the sowing, seeds were feeble. At the Maikop experimental station (the Republic of Adygea), in the years with low rainfall, the filled seeds were matured. At the Kharkov experimental station, the plants grew poorly, and the seeds could not be obtained [82]. In 1956-1966, after collection replenishment, mainly with Indian accessions from different areas, as well as accessions from the USA and Australia, the total number of guar samples in VIR collection had exceeded 100 accessions. While studying VIR Central Asian branch collection (Tashkent), the samples were differed in early maturity characteristics, in some areas they developed well, but in others the guar plants were damaged by *Fusarium* fungi and viruses. VIR scientists recommended guar cultivation in Uzbekistan for gum manufacturing [82]. In the USSR, guar was also studied in Turkmenistan. There, 59 samples of different geographical origin, including 50 samples from India, 2 samples from Pakistan, 2 samples from the USA, and 5 samples from Australia, were studied in the arid zone (1963-1970, The Central Botanical Garden) and in the sowing (1971-1980, Institute of Botany of the Academy of Sciences of the TSSR) [21, 83]. The aim was to assess the prospects of guar using as an annual forage crop. High seeds yield had breeding varieties from India and the USA (18.6-24.3 c/ha). Raw protein in seeds ranged from 25.8 up to 26.6 %, and galactomannan ranged from 29.6 up to 33.5 %. Growing period from seed germination to the end of fruiting season in the south of Turkmenistan under spring sowing was 85-111 days with a maximum green biomass yield of 589 c/ha. In Turkmenistan, because of the lack of annual legume capable to produce a significant green mass, guar was recommended as a protein-rich forage crop for the arid zone. However, it was unclaimed and not widely spread because of wrongly established sowing time as it was studied in 1966 in Sukhumi and the Leningrad region [84].

Guar seeds that were stored for 40 years at +4.5 °C (Kuban gene bank) had high sowing qualities with laboratory germination of 92 to 94 % [85]. Field germination of seeds stored at room temperature was high too, i.e. 48 of 50 samples dated 1977-1980 produced seedlings in the Krasnodar Territory in 2017.

Perspectives of guar cultivation in Russia. Based on comparative climatic characteristics of India and the USA with the Southern Russia conditions, the opportunities of growing early maturing guar varieties in some regions have been established. The suitability for cultivation of this crop deter-

mines the sum of effective temperatures > 10 °C which should be about 3400-3500 °C. According to summer temperatures and other climatic parameters, the agrarian regions of the North Caucasus and Crimea are less productive than India, but similar to the USA, where the guar is successfully grown in the Southern states. The vegetative growth guar cycle needs 350-500 mm of rainfall. Consequently, the flat part of the Stavropol and Krasnodar Krai is well provided with natural moisture to cultivate the guar. In the Crimea and the Rostov region, the additional irrigation is advisable. The optimal sowing dates are when the temperature of the arable soil layer passes through 20 °C [86].

In the Ust-Labinsk region of the Krasnodar Krai, guar growing was optimized and breeding was carried out. As a result, breeding material with valuable characteristics (seed productivity, lower bean attachment height, etc.) was obtained. The seed yield of the best samples in the variety tests has exceeded 24 c/ha. Experiments revealed factors limited crop yields (soil temperature and humidity at sowing, alternaria fungi and bacterial rot damage) [87].

In 2014-2015, under introduction of four Indian guar samples from the VIR collection, the seeds yield in the Rostov region was 16.4-19.5 c/ha, while the plants were infected with bean yellow mosaic virus and pea enation mosaic virus [88]. Further testing of the best samples was carried out in different ecogeographic conditions at the southern VIR stations. The optimal sowing dates in Krasnodar Krai are form the end of the first decade to the beginning of the second decade of May at a soil temperature of 20-24 °C. The early maturing guar lines have been identified which ripen steadily in the Kuban and the Volgograd region with a grain yield of more than 18 c/ha and green mass of 600 c/ha. Crude protein per absolutely dry matter was 33.59 % [85, 89]. Under southern Russia conditions, physiological maturity of guar seeds is reached within 100-130 days after sowing. The quality of the gum obtained from such seeds satisfies the requirements.

Thus, guar, which has been known for a long time as fodder, green manure and a source of food protein, now become one of the most important technical crops because of the growing need for guar gum in the food, cosmetic, and petroleum industries. India, Pakistan and the USA are the main producers of guar and its products. Conditions in some south areas of Russia are also suitable for industrial cultivation of this crop, which actualize its introducing and breeding. The experience in studying and cultivation of this crop in the USSR and Russia has been accumulated, and VIR collection maintained genetic resources of guar plants from the main areas of its origin and cultivation which can be used for introduction and as donors of valuable traits. The priority in breeding varieties for Russia is improved productivity, early maturity and disease resistance.

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ECO-PHYSIOLOGICAL AND BIOCHEMICAL BASES OF THE GREEN CRYO-FEED FORMING IN YAKUTIA

(review)

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Abstract

Food grown in Yakutia can be used as a supplement to the diet of many farm animals in Siberia, the Far East and the European North. Understanding mechanisms of cold adaptation in plants, being of general biological significance, also makes basis for use of this practically unlimited natural forage resource. Here, we summarized ecological, physiological and biochemical aspects for the formation of nutritional value of autumn-vegetating herbaceous plants which were frozen with natural cold have been considered. These plants are highly nutritious, autumn/winter fattening food (green cryo-feed), for herbivores in the extremely harsh conditions of the North. In the article an overview data on plant response to low temperatures and their adaptation is provided (T.I. Trunova, 2007; L.V. Gusta et al., 2013; K. Miura et al., 2013). As a result of generalization of long-term authors' investigations (A.Ya. Perk et al., 1987; K.A. Petrov et al., 2010; A.N. Ilyin et al., 2015) and other publications, the general theory is developed of the mechanisms of resistance of plants and animals to prolonged hypothermia in the conditions of permafrost (cryolithozone) in Yakutia. It is assumed that adaptation of plants to long-term low temperature stress have closely connected with their main source of energy (lipids, unsaturated fatty acids), which plays a major role in the formation of high nutritional value of autumn-vegetating plants which were frozen with natural cold in the permafrost zone. Cold hardening of perennial herbaceous plants in the cryolithozone of Yakutia is evidently due to cell accumulation of primary and secondary carotenoids with the most pronounced antioxidant properties (B. Demmig-Adams et al., 2006). Green cryo-feed provides vital activity of herbivores, including livestock (the Yakut horse, reindeer, etc.), under conditions of prolonged and extremely cold winter. Green cryocorm technology allows to provide animals with protein, fatty oils, carbohydrates and vitamins throughout the whole wintering period. As a result of our experiments have been shown highly nutritional value of green cryo-feed for feeding of livestock, for example for herd horse breeding, that allows to recommend the cryo-feed for wide introduction into agricultural practices in regions where unlimited resources of cold could be used.

Keywords: Yakutia, adaptation to cold, green cryo-feed, nutritional value

Cold resistance means ability of the plants to resist low positive temperatures, frost tolerance (frost resistance) means tolerance to temperatures below zero during winter. Winter resistance, including frost resistance, means complex resistance to unfavorable factors of winter season, namely frost, thaws, ice crust, asphyxiation, heaving, rotting, etc. [1-4]. In the past decades, frost resistance was considered as ability of the plants to prevent formation of ice inside cells and to enhance resistance to intracellular ice under the effect of negative temperatures [5-7]. Chill resistance (ability to resist relatively short-term effect of

negative temperatures) is also important [2].

The Republic of Sakha (Yakutia) is among the coldest regions of the world. Winter temperatures may fall below –60 °C. Nevertheless, Yakut horses and other herbivorous animals survive here at open air all year around. Evolution origins of Yakut horses and genetic basis of their adaptation still remain unresolved [8], most probably these horses have appeared after migration of the Yakut people few centuries ago, and represent one of the most vivid examples of fast adaptation to extreme conditions of criolithozone. In our view, high-energy biologically active substances accumulated in organs of green cryo-feed in the autumn-winter period play the key role in regulation of adaptation to the extreme conditions of criolithozone. Understanding mechanisms of cold adaptation in plants, being of general biological significance, also makes basis for use of this practically unlimited natural forage resource. Food grown in Yakutia can be used as a supplement to the diet of many farm animals in Siberia, the Far East and the European North. It should be noted that Yakut breed of horses is unique by high concentration of essential fatty acids in its meat playing an important role in human metabolism [9-11].

In this review we have considered environmental, physiological, and biochemical aspects for formation of the nutritional value in naturally frozen autumn-vegetative Northern plants which serve highly-nutritional autumn-winter fattening feed (green cryo-feed) for herbivores in extremely harsh conditions of their habitation.

Reaction of plants to low temperature exposure. Plant winter resistance has been investigated for a long time with frequent discussion of the reasons of destruction from frost (loss of heat, vessel rupture, dehydration, formation of ice, acidification and strengthening of cell fluid, attainment of the specific temperature minimum, and etc.). Research results of the physiological aspects of plant adaptation to low temperatures are provided in more detail in a series of study overviews [6, 11-18].

There are three types of cell death in freezing [5], i.e. due to rapid formation of intracellular ice initially focused in cytoplasm and further in vacuole; due to dehydration and deformation resulted from intracellular ice formation; due to formation of inter- and intra-cellular ice. “Winterkill” type depends of the physiological state of plants, of their readiness for wintering. Damage of plants upon formation of ice, mainly intracellular ice, results in dehydration and mechanical deformation of cytoplasm, and finally in structural rupture of protein and lipid components of membranes due to enzymatic hydrolysis of phospholipids and due to formation of phosphoric acid. The root cause of damage and loss of cold-resistant and heat-loving plants due to low temperature exposure is transformation of liquid crystal membranous lipids into a solid gel. Phase change in lipids results in the increased size of membranous pores, which is both accompanied by their semi-permeability loss and inactivation of sugar and K^+ ion active transport systems, as well as by faster water discharge from cells and, thus, causing killing of a plant [19].

Adaptation of plants to low temperature stress. Formation of physiological mechanism of resistance to unfavorable wintering conditions involves rest and hardening stages [20]. This mechanism prevents formation of ice inside a cell under the effect of negative temperatures and ensures higher resistance to intercellular ice that reduces dehydration and mechanical deformation of protoplast. Cryo-resistance is realized at various levels, from organismic to molecular, provided that cryo-resistance mechanisms in woody and herbaceous species have certain characteristics. Thus, at the end of vegetation period as a day becomes longer all buds of woody species at mid and high latitudes

go into the state of deep physiological rest that allows them overcoming the unfavorable period. As apart from woody species, herbaceous species do not go into the state of physiological rest, but their growth is suspended under the effect of low temperatures (0-5 °C). Not only low positive temperatures, but also the light is required for hardening. At that, hardening ability is different in winter cereals at various ontogenesis stages: the closer is generative stage, the lower is the hardening ability [21, 22].

Cold hardening of herbaceous plants significantly changes metabolism, whereby I.I. Tumanov [20] had distinguished two consecutive hardening phases. To this end, it occurs only in case when first negative temperatures (second phase) effect after low positive ones (first phase). Cryo-protector formation process is mostly expressed during the first hardening phase. Cryo-protectors are low- and high-molecular compounds: carbohydrates, lipids, proteins, etc. Due to cryo-protectors a cell gets more protection against formation of intracellular ice and dehydration. The most intensive increase in sugars and lipids, main nutritional substrates of respiration in plants, is noted. Increase of sugars promotes water retention in a cell in unfrozen state, which prevents denaturation of proteins [6]. Besides, cold hardening significantly increases concentration of lipids, phospholipids, and unsaturated fatty acids that provided for decrease of temperature of transformation of membranous lipids from a liquid crystalline state into the gel-like state [23-26]. Such temperature is lower than freeze point in frost resistant plants, and is above 0 °C in nonresistant plants.

Free fatty acids (FFA), not included in membranous lipids, may influence on the activity of mitochondria. Transfer of mitochondria into a low-energy state in frost-resistant winter cereals was noted about 30 years ago by V.K. Voynikov at al. (27, 28). Upon such transfer, FFA content in mitochondria had increased 2-3 times, and within a half an hour the seedlings had retained the temperature which was 7-10 °C higher than air temperature. All such effects were not found in summer cereals. Upon hypothermia and in control, FFAs were mainly presented by C_{16}-$C_{20:}$ accounting for 84 % of the total fraction, whereat unsaturated acids comprised the most part of the total amount of fatty acids amongst C_{16}-C_{20}. Phospholipase A_2 activates upon reduction of temperature, thus, resulting in accumulation of FFA and changes in the nutritional processes in a cell. Fatty acids in mitochondria become not only the main oxidation substrates but also the most important regulator, i.e. the disconnector of respiration and phosphorylation, facilitating transformation of energy of respiratory substrates into the heat upon hypothermia [29, 30]. First of all, it is noted by activation of the alternative oxydase, plant uncoupling mitochondrial protein PUMP and stress protein CSP 310 causing thermogenesis and local temperature increase. It allows plants to gain time for adaptation change of metabolism, e.g. change of the content and structure of membranes, transportation of required metabolites through membranes, synthesis of cell dehydration stress proteins (anti-freeze, chaperons, dehydrins and disconnectors) and etc. [27, 28, 31, 32].

The second hardening phase in herbaceous plants is characterized by significant structural cell changes. Intercellular ice is formed in autumn vegetative species under the effect of negative temperatures, which prevents formation of ice within cells. In this period, there are two main adaptation mechanisms: stronger discharge of free water from plant cells through the membranes and protection of cell components from dehydration consequences. Water is discharged through membranes due to an increase in concentration of unsaturated fatty acids [6, 33]. Changes in phospholipids also affect the membrane features and increase its permeability for water, whereat rapid reduction of phospholipids upon freezing results in stronger water discharge in intercells and protects a cell

from intracellular ice formation [34].

Green cryo-feed. In mid latitudes air temperature often falls till –20...–40 °C. Permafrost zone lays to the north, where air temperature is even lower. The main point of seasonal vegetation growth and development dynamics in the cryolithozone is that termination of growth processes and going of perennial plants into the state of deep physiological rest with simultaneous sharp reduction of photosynthetic activity coincides with the second part of August — beginning of September and coincides with the period of maximum soil respiration intensity and the most formation of seasonally-thawed stratum. After-grass and many autumn-vegetative herbaceous species, which are subjected to hardening, remain green till late autumn and in such state move under the snow. It should be noted that in cryolithozone of Yakutia, as well as in other Siberian regions with similar climate, such unfavorable events as rotting, asphyxiation and heaving due to the lack of recursive warming widely occurring in the regions with soft climate are kept to a minimum at beginning of winter season.

Main and specific aspects of climate in Central and North-Eastern Yakutia is extra-continental conditions with low amount of precipitations (200-250 mm), strongly shortened (up to 80-120 days) frost-free period due to late and early chill, significant temperature fluctuations within a year (up to –60 °C in winter and up to +40 °C in summer), unusual dryness of cold air, as well as availability of eternal frost. Thus, green cryo-feed — wintergreen herbaceous species, which serve winter-grazing food for most animals — is widely used in Yakutia [35, 36].

Study of growth and development characteristics [37], as well as nutritional value of herbaceous plants in Yakutia [38-41], had shown that here cereal-sedge plant formation is often subjected to mechanical damages (pasturing, domestic mowing, etc.) and long-term flooding. Vegetation of damaged and underwater plants starts later, they often can't go fast enough the entire growth and development cycle, and, going under the snow, preserve significant part in a green frozen state, in which case, green mass passes cryo-preservation in a form of fattening feed [41].

Nutritional value of green cryo-feed. Study of the extensive material had shown that during winter wintergreen parts of wild herbaceous plants always preserve high concentration of nutritional and biologically active substances [39-42], which is important for Northern agriculture, especially for feed production. First zoo technical studies confirming the effectiveness of use of the green plants of late and summer-time sowing, being preserved with the use of the natural cold, have been conducted in Khakassia in 1947 at Krasnoyarsk experimental station [43, 44]. In furtherance, patented green cryo-feed making method was developed and tested in conditions of the Central Yakutia [45, 46]. It involves late summer sowing terms (July 8-20) of the released variety of annotinous cold-resistant herbaceous plants — oat (*Avena sativa* L.), rape (*Brassica napus* L.), pea (*Pisum sativum* L.) that perform well under the effect of temperatures of up to –7 °C. Naturally frozen plants go under snow in green state. Afterwards, they are gathered and used as cattle feed.

Autumn in Yakutia is characterized by the most favorable weather conditions for improvement of thermal resistance of autumn-vegetative herbaceous plants. Predominant meteorological elements involve lots of bright sunshine days required for photosynthesis and chilly nights allowing suspending the consumption of carbohydrates for respiration. According to long-term average data, period in the Central Yakutia with temperatures suitable for passing of the first hardening phase (daily temperatures reaching +10...+15 °C, and nighttime temperatures reaching –1...–2 °C) coincides with the II–V pentads of September [47,

48]. It is the period when ability of plants to overcome first negative temperatures due to cold hardening is gradually formed.

We have conducted comprehensive ecophysiological and biochemical studies for cold-resistance in the released local-breed annual and perennial Yakut species of herbaceous plants: common oat (*A. sativa* L.) Nyurbinsky variety and awnless brome (*Bromopsis inermis* Leys) Ammachaan variety. Cereals planted in conditions of the Central Yakutia were subjected to natural cold hardening during the second half of September. In pursuance thereof, awnless brome was sown during the period optimal for the climate region (end of May – beginning of June), afterwards, during the heading commencement phase (II decade of July), plants were cut to promote lying of new vegetative shoots. Alternatively, common oat was sown in later periods (in the mid of July) significantly shifted as compared to commonly accepted terms (end of May —beginning of June). Growing shoots of awnless brome and common oat were subjected to natural hardening by low positive temperatures (from 0 to +5 °C). At beginning of October, naturally frozen plants went under the snow during the stem elongation phase. These studies allowed examination of effect of the first and second cold adaptation phases on herbaceous plants of Yakut cryolithozone.

In high latitudes, adaptation of winter-resistant plants to low temperature is due to adaptation of photosynthetic apparatus (PSA), i.e. shift of assimilation optimum CO_2 to lower temperatures with an increase in CO_2 assimilation rate [49]. Hardening cells of frost-resistant plants are rich in photosynthesis products since photosynthetic intensity in such plants (as apart from non-resistant species) at near-zero temperatures significantly exceeds the respiratory activity [49-53]. It results in accumulation of the large amount of sugars playing multifunctional role at low-temperature adaptation in plants [6].

Field studies had shown significant increase of carotenoids of violaxatine cycle in all studied species of perennial cereals in the Central Yakutia at beginning of autumn chilling simultaneously with growth of the amount of chlorophyll. The same pattern was observed in horsetails in the cold pole region, provided that here quantity of carotenoids had increased 1.2-3.0 times as compared to summer values upon simultaneous increase of the content of oxygen-containing substances and reduction of the amount of chlorophylls compared to xanthophylls and carotenoids. In general, vegetables were characterized by low content of chlorophylls and carotenoids which is due not only to the extremely severe conditions in the region, but also to specificity of plants of *Equisetaceae* family. Close to winter, an increase in the amount of secondary carotenoid, rhodoxanthin, was observed in the studied horsetail species. Probably, in such circumstances, rhodoxanthin plays the more important antioxidant role than violaxatine cycle, which is unable to operate at constant low temperatures [39].

Accordingly, one of properties of the cold hardening of perennial herbaceous plants in the cryolithozone of Yakutia is clearly high concentration in the cells of primary and secondary carotenoids with most expressed antioxidant properties. Carotenoids protect light harvesting complex of PSA from excitation energy surplus at high intensity of light [54] which, in conditions of autumn low positive temperatures, allows plants to timely complete the first phase of cold hardening. The later results in accumulation of the large amounts of high-energy compounds, important plastic substrates and bioactive substances such as sugars, proteins, fatty acids of bulk lipids and antioxidants (polyenoic fatty acids, vitamin C, β-carotene, lutein complex, etc.) [6, 39-41], which significantly improve nutritional value of autumn-vegetative and wintergreen herbaceous plants in cryolithozone.

Therefore, production technology of the green cryo-feed allows satisfy-

ing the need of animals in protein, fatty oils, carbohydrates and vitamins during the entire wintering period. It includes late-summer sowing (mid-end of July) of the most frost-resistant annual fodder crops and their mixtures (oat, oat + pea, oat + rape) with harvesting green mass approximately within 60-70 days (cutting in windrows in September, pressing and warehousing in October) [45, 46]. Cold-preserved perennial sown and natural meadow grass may be used for the same purpose. Wintering of Yakut horses adapted for free year-round pasturing is also possible in such circumstances [55, 56].

Preeminence of cryo-feed was established upon comparative zootechnical assessment of after-grass productivity in essential grass stand and late sowing of oat for making of cryo-feed at wintering of horses [57]. The best digestibility of nutritional substances in winter grass mass of oat as compared to grass stand after-grass is due to high content of carotene and provitamin E in plants preserved by the natural cold during vegetation. Feed capacity of 1 ha with the cryo-feed at wintering of adult animals had comprised 129-142 days per head. To this end, supportability of exchange energy with a view to 100 kg of body weight reached 32.6 mJ, which exceeds the standard value by 14.5 %, consumption of the digestible protein comprised 242.4 g, which exceeds the standard value by 31.0 %. During inspection of a mare breeding stock fed by cryo-feed in December-January, a 30-day outcome of born live foals made 72 % that is 12 % higher than that in the control group. In general, economic effectiveness comprised 2.18 rubles per 1 ruble of costs [57]. High effectiveness of oat winter pasturing from December to February (85 days) was revealed for young Yakut horses. In the trial group, body weight gain in animal, as compared to the control group fed by hay in combination with wintering at natural pastures, comprised 16.9 kg per heads. Cost of 1 dt of the body weight in young animals in the trial group was 51.2 % lower than in the control one. Accordingly, use of frozen oat crops sown during summer is effective, absolutely harmless for young horses, and ensures gaining of body weight during the coldest winter months. Studies had shown changes of several biochemical blood parameters of young animals signifying improvement of protein, fat, and carbohydrate exchange in the trial group, which allows such method for wide use in horse herd farming [58-60].

It appears that specific role of green cryo-feed FFA in regulation of adaptation in the herbivores to cold climate in cryolithozone of Yakutia is associated with effect on thermogenesis in mammals under the long-term low-temperature stress, fulfilling functions of the main substrate for oxidation in mitochondria and disconnecting respiration and phosphorylation [29, 30].

The studies allowed us defining for the first time the general theory of resistance mechanisms in plants and animals to long-term hypothermia in conditions of Yakut cryolithozone [61-65].

Thus, adaptation of plants to long-term low-temperature stress is closely related to main energy sources — lipids and polyenoic fatty acids playing the main role in formation of the high nutritional value in green cryo-feed, the naturally frozen plants in cryolithozone. Green cryo-feed protects vital activity of herbivorous animals, including agricultural species (Yakut horse, reindeer, etc.), in conditions of long-term extremely cold winter. For agricultural production in cryolithozone, it is important to apply special resource and energy saving technologies adapted for local properties.

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ASSOCIATION OF POLYMORPHIC TYPES OF STEAROYL-CoA DESATURASE GENE (*SCD1*) WITH ECONOMICALLY VALUABLE TRAITS IN RUSSIAN POPULATION OF AYRSHIRE COWS

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Abstract

In modern animal breeding genes involved into biochemical and physiological pathways and having SNP polymorphisms in coding sequences (exon) leading to amino acid replacements in proteins, and in regulatory elements effecting transcription, are considered as promising marker genes. Significant attention is given to study of relations between polymorphisms in lipid metabolism genes such as leptin (*LEP*), thyroglobulin (*TG*), diacylglycerol O-acyltransferase 1 (*DGAT1*) with milk and beef productivity traits in cattle. Little is known about stearoyl-CoA desaturase gene polymorphism and its associations with cattle traits, particularly in Russian populations. Enzyme stearoyl-CoA desaturase plays an important role in fatty acids metabolism. For dairy cattle fat and protein content as well as live weight and rate of weight gain which make an impact on animal puberty age are among the most important traits. In our paper polymorphism of single nucleotide substitution (SNP) rs41255693 in *SCD1* gene has been revealed along with analysis of its link with economically important traits in first lactation cows (n = 201) of Ayrshire breed belonging to OAO PZ Novoladozhskii of Leningrad region. SNP in rs41255693 region is located at fifth exon of *SCD1* gene and characterized by amino acid Ala-Val substitution (allele $C > T$) and revealed by FauI restriction endonuclease. Allele *C* is characterized by the presence of restriction site. High frequency of allele *C* (0.858) and genotype *CC* (0.731) has been observed in the population under study. Allele *T* is rare with frequency of 0.142, and *TT* genotype has been detected in only 3 animals (frequency 0.015). In order to evaluate animal breeding value (BV) comparison with contemporaries has been employed showing deviation of each animal productive trait from that of contemporaries. Animal BV was calculated by the following traits: milk yield for 305 and 100 lactation days, percentage of fat and protein, yield of fat and protein, weight at birth, at 10, 12 and 18 month age, live weight at first service and at first lactation. Beyond, age of first service, first calving and days open were also taken into account. Association between genotypes of *SCD1* gene and such parameters as weight gain, animal development and reproductive traits (age at first service, age at first calving and days open) has not been established. Nevertheless, it has been demonstrated that data concerning *SCD1* gene polymorphism allows for QTL (Quantitative Trait Loci) high fidelity mapping for such traits as milk fat and protein content. Cows of the population under study having *CC* and *CT* genotypes exceeded with statistical significance the cows having *TT* genotype in BV milk yield for 305 days ($p < 0.01$), BV milk yield for 100 days ($p < 0,02$), yield of fat, kg ($p < 0.001$), and yield of protein, kg ($p < 0.001$).

Keywords: allele, *SCD1* genotype, Ayrshire breed, cattle, live weight, days open, calving, mutation, screening

Nowadays, genome-wide association study (GWAS) in various bovine breeds was unable to identify quantitative trait loci (QTL) universally associated

with desired economic useful traits. Usually, it is because of genotypic (bred-specific) influence, animal adaptation to environment, feeding and keeping conditions [1, 2]. SNPs (single nucleotide polymorphisms) related to genes, which are involved in control of productivity and quality of livestock products, could facilitate a prediction of cattle potential. Genes involved in lipid metabolism are of special interest because the fat content of milk has significantly higher heritability coefficient than the total amount of milk produced (0.60 vs. 0.18). Milk production is more often considered as combination of total milk yield, protein percentage and fat percentage. Systems governing mammal lactation are involved in body metabolism and immunity, which participate in animal adaptation to the environment [1]. Thus, one should consider association of a particular gene not only with milk production but also with all economically valuable traits.

Lipid metabolism of productive animals is of high importance. A total of 107 genes, 240 proteins, and 80 metabolites are involved in its regulation. Stearoyl-CoA-desaturase (SCD, EC 1.14.99.5) catalyzes desaturation of palmitic and stearic acids to monounsaturated fatty acids, the palmitoleic and oleinic acids, in mammal apidocytes [3]. SCD deficiency causes reduction of fat contents in tissues, an increased sensitivity to insulin and, consequently, accelerated metabolism. It was observed that gene *SCD1* knockout causes metabolic attenuation, atrophy of oil glands, intrusion of epidermal lipid barrier, disorder of thermal control and cold resistance [4, 5]. SCD ferment displays protective antiapoptopic effect by indirect regulation of triglyceride accumulation in cells [6].

Bovine *SCD1* gene is located on chromosome 26 and consists of 6 exons and 5 introns [7]. Information on associations of various SNPs, both intronic and exonic, of *SCD1* gene with economically valuable traits in various breeds is ambiguous. Thus, genome-wide association study of productive and reproductive traits of Holsteins [8] and northern red dairy cattle [9] did not find such association for *SCD1* gene. In other studies [10], with the use of two-stage association analysis of 50000 SNPs for identification of genome areas associated with certain fatty acids in cow milk, it was shown that only in two genes, *DGAT1* on chromosome BTA14 and *SCD1* on chromosome BTA26, such polymorphism strongly influences the unsaturated fatty acids ($C_{4:0}$-$C_{18:0}$) and medium-chain fatty acids in bovine milk fat. Association of *SCD1* gene with milk level of unsaturated fatty acids had also been noticed [11]. In the search for associations of 51 SNPs in 37 candidate genes with 47 fatty acids in milk, positive correlation between *T* allele of *SCD1* gene (SNP rs41255693) and amount of $C_{20:0}$ and $C_{22:1\ cis\text{-}9}$ in milk fat had been discovered [12]. It is fat composition, as well as proportion of saturated and unsaturated acids, which defines dietetic value of milk. Based on published data, it could be assumed that several SNPs in *SCD1* gene could be considered as potential markers upon forecasting the qualitative composition of milk. It should also be noted that a breed itself, due to inter-breed differences of genes, polymorphism of which is associated with milk production, is of particular importance. There are studies indicating on variation between breeds in milk composition, i.e. in percentage of saturated and unsaturated fatty acids,. Herewith, negative genetic correlation between proportion of the unsaturated fatty acids and fat is seen [13].

Several breeds show high polymorphism of *SCD1*, which suggests location of this gene in the regions of imprints of natural selection. Japanese researchers had detected high polymorphism in exon 5 of the *SCD1* gene, with 8 SNPs revealed in this region in Japanese Black beef cattle [14]. As the same time, only 3 of these SNPs were found in Jersey and Holstein cattle [15] and amongst 12 Italian livestock breeds [16].

Various SNPs in *SCD1* gene are known to be associated with cow milk yield and composition. In Holstein cows of Chinese selection, evaluation of 5

SNPs both in intronic and exonic areas (g.6926A>G, intron 3; g.8646A>G, intron 4; g.10153A>G, g.10213T>C and g.10329C>T, exon 5) showed that the genotypes heterozygouse for each of these SNPs had validly higher 305-day lactation yields and production of milk fat and protein [17].

This paper is the first to report the influence of polymorphism in exon 5 of the *SCD1* gene on economically valuable traits in a population of Ayrshire cows of Russian selection. These findings deepen the knowledge of biosynthesis of milk fat and could facilitate selection for improved dietetic milk quality in the studied population.

Our subjective was to examine *SCD1* gene polymorphism for SNP rs41255693C>T (NC 007327.6, position 2144708, https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=41255693) and its association with economically valuable traits of Ayrshires, the type Novoladozhskii selected in Russia.

Techniques. For DNA extraction, blood samples of first-calf Ayrshire cows (n = 201) (OAO PZ Novoladozhskii, Leningrad Province, 2015) was taken from tail vein in K3EDTA vials and stored at –20 °C. DNA was extracted by phenol method [18].

PCR-RFLP (restriction fragment length polymorphism) was used for genotyping. Polymerase chain reaction (PCR) was conducted in 10 μl of reaction solution (67 mM Tris-HCl, pH 8.6; 2.5 mM MgCl2, 16.6 mM NHOH, 0.125 mM of each dNTP, dATP, dGTP, dCTP, dTTP, 0.5 μM of each primer, 50-100 ng genomic DNA, and 2.5 IU Taq DNA-polymerase (SibEnzume LTD, Russia) with the primer pair F — 5′-CCT-AAG-CAG-CAG-ACC-ACT-AG-3′, R — 5′-TGG-GCT-CAA-CGT-CAC-CTG-3′ (Eurogen CJSC, Russia) at an amplifier Thermal Cycler T1000 (Bio-Rad Laboratories, Inc., USA) according to the following protocol: initial denaturation for 1 min at 94 °C; 35 cycles: 30 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C; final elongation for 10 min at 72 °C. For determination of animal genotypes, the obtained amplificates were treated by endonuclease FauI (SibEnzyme LTD, Russia). The reaction mixture was incubated at 55 °C for 2 hours followed by electrophoretic separation of fragments in 3 % agarose gel containing 0.1 rg/ml ethydium bromide. The signal was recorded using gel documentation system Gel Imager-2 (Helicon Company LLC, Russia). GeneRuler Ultra Low Range DNA Ladder (Fermentas, Lithuania) was used for sizing DNA restriction fragments.

Economic characters of dairy cows were taken from the electronic database IAS Selex of OAO PZ Novoladozhskii. Pedigree value (PV) of cows was estimated using SGS-VNIIGRG software [19]. The following parameters were taken into account: PV for 100- and 305-day lactation yield; percentage of milk fat and protein; milk fat and protein yield; PV for body weight at birth, at 10, 12 and 18 months of age; body weight at first insemination and after first calving; age at first insemination and first calving; service period.

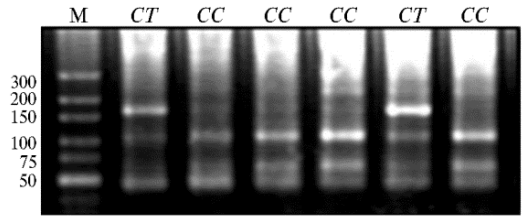
Statistical processing of data was performed using AtteStat software (http://www.studmed.ru/programma-attestat-1205_1778bebd8f9.html) and Microsoft Excel. Genotype and allele frequencies were quantitated. Deviations of these parameters from the Hardy-Weinberg equilibrium were estimated by χ^2 criterion. Means (M) and deviations from mean values ($\pm m$) were calculated. Statistical significance of the differences in mean values of economic characters between animal groups with different genotype was determined by Student's t-test [20].

Results. Nowadays, Ayrshire breed is widely spread in Russia, with 25 % of the total livestock concentrated in Leningrad region. Ayrshire cows are characterized by high milking, with high milk fat and protein content [21]. In our study, we selected a group of first-calf cows, type Novoladozhskii, owned by OAO PZ Novoladozhskii. For many years, in this farming unit, which remains

the leader by milk productivity in Ayrshire dairy cattle in Russia, the genetic potential of milk productivity in herd grows both from generation to generation and from year to year [22]. The herd is improved by engagement of pedigree resources from Scandinavian countries and nearly 80 % of the livestock has Finnish origin. Keeping type is free stall barns, animals have free access to silage and haylage all year round, concentrates are calculated individually depending on productivity of each cow.

So far as full realization of animal genetic potential for performance is ensured due to feeding and keeping conditions, gene alleles would most probably influence on productive traits. According to records at the beginning of 2016, milk productivity of first-calf cows for 305 days was 7619 kg with fat content of 4.25 % and protein content of 3.50 %; body weight was 563 kg. Milk of Ayrshire cows is highly appreciated for its technological properties, namely milk fat quality, and is recommended for production of cheese and butter [23].

Practically, dietetic milk properties and their improvement attract attention of stock-breeders due to healthy diet issues. A more detailed study of SNPs associated with genes of lipid metabolism in a population of Ayrshires which differ from other dairy breeds in high milk fat content allows for better understanding mechanisms of milk fat biosynthesis and regulation. Results of such studies allow identifying animals with the most valuable genotypes, which, in turn, allows breeders to improve effectiveness of selection.



FauI restriction of PCR-amplified fragment of stearoyl-CoA-desaturase gene *SCD1* (SNP rs41255693) in Ayrshire breed: M — molecule weight marker GeneRuler Ultra Low Range DNA Ladder (Fermentas, Lithuania; from 10 to 300 bp), genotype *CC* — fragment sizes of 105 and 58 bp, genotype *CT* — 163, 105 and 58 bp (OAO PZ Novoladozhskii, Leningrad Province, 2015).

The figure shows electrophoretic separation of fragments resulted from restriction of the obtained PCR products. After treatment of 163 bp amplicons with FauI endonuclease, the presence of restriction site defined *C* allele, whereas the absence indicated *T* allele. Fragments of 105 and 58 bp corresponded to *CC* genotype, 163, 105 and 58 bp corresponded to *CT* genotype, and 163 bp corresponded to *TT* genotype.

Comparison of the obtained electrophoregrams revealed prevalence of certain SNP rs41255693 alleles and genotypes for the gene *SCD1* across the studied population of Ayrshires. Homozygotes *CC* and *C* allele were identified most frequently (0.731 and 0.858, respectively). Only three animals (0.015) had genotype *TT*, and frequency of *T* allele was 0.142. H_o for heterozygotes *CT* was 0.254 at H_e of 0.243 ($p = 0.05$). The sample had not displayed any significant deviation of the actually observed genotype frequencies from those theoretically expected according to Hardy-Weinberg law ($\chi^2 = 0.338$).

In the studied sample, *CC* and *CT* genotypes appeared to be the most numerous and took precedence over animals with *TT* genotype by a number of indicators: PV for 305-day lactation yield ($p \leq 0.01$), PV for 100-day lactation yield ($p \leq 0.02$), yield of milk protein, kg ($p \leq 0.001$), and yield of milk fat, kg ($p \leq 0.001$) (Table). *TT* genotype was rare, and animals of such genotype were inferior to other cows by milk productivity indicators, and had low growth rate and longer service period. It should be noted however that such data was obtained from small sample, i.e. further studies are required for making final conclusions.

Economic characters of Ayrshires depending on genotypes for stearoyl-CoA-desaturase *SCD1* gene (*M*±*m*, OAO PZ Novoladozhskii, Leningrad Province, 2015)

Parameter	Genotype		
	CC (*n* = 147)	*CT* (*n* = 51)	*TT* (*n* = 3)
PV for 305-day milking yield	–20.25±23.47[a]	11.76±37.76[b]	–289.66±92.31[c]
PV for 100-day milking yield	–4.02±6.38[d]	–0.11±10.87[e]	–58.00±23.11[f]
PV for milk fat content (%)	0.007±0.008	0.010±0.010	–0.060±0.10
PV for milk protein content	–0.006±0.005	0.004±0.008	–0.060±0.040
Fat yield, kg	315.89±4.17[g]	325.22±6.77[h]	260.35±13.02[i]
Protein yield, kg	265.14±3.21[g]	273.90±5.10[h]	215.36±11.86[i]
PV for BW at birth, kg	0.29±0.25	–0.10±0.38	0.43±2.64
PV for BW at 10 months of age, kg	0.50±1.80	5.47±2.81	–12.80±18.40
PV for BW at 12 months of age, kg	0.79±1.89	4.50±3.01	–8.43±11.30
PV for BW at 18 months of age, kg	–0.72±2.69	7.26±4.34	–10.76±14.27
PV for BW at the 1st insemination, kg	–1.23±1.78	–0.34±2.58	1.10±4.48
PV for BW after first calving, kg	–1.56±1.13	0.43±1.54	–6.00±4.04
Age at first insemination, months	17.18±0.13	16.90±0.24	17.07±0.09
Age at first calving, months	26.37±0.14	26.35±0.31	25.82±0.72
First lactation, service period, months	106.78±5.26	111.35±8.30	156.75±36.72

N o t e. *C* allele corresponds to the presence of restriction site for endonuclease FauI in 163 bp amplicons, *T* allele corresponds to absence of such site. PV — pedigree value, BW — body weight.

[a, c; b, c] Differences are statistically significant at $p \le 0.01$.

[d, f; e, f] Differences are statistically significant at $p \le 0.02$.

[g, i; h, i] Differences are statistically significant at $p \le 0.001$.

We had not found reliable association of SNP rs41255693 in the gene *SCD1* with such traits as growth and development, reproductive properties (age at first insemination, age at first calving, and service period).

Frequency of gene alleles is one of the main characteristic of a breed or population. Evaluation of SNP rs41255693 in the gene *SCD1* across bovine breeds showed that differences in alleles and genotypes depend on the goal of selection for primary use. Thus, frequency of *T* allele in Brown Swiss cows comprised 0.15 [24]. With increase of Swiss breed proportion in Kostroma cows, their frequency of *T* allele had decreased from 0.611 to 0.231 [25]. In Holstein breed, animals of *TT* genotype have not been found while frequency of heterozygous genotype comprised 0.618 [26]. The prevalence of *T* allele increases in cows of meat and meat-and-milk breeds (e.g. 0.217 for Kalmyk breed vs. 0.321 for Mongolian hogorogo breed) [26]. Low frequency of *T* allele is observed in populations of milk cattle with high milk productivity, which is in line with our findings.

A number of papers show positive association of SNP in exon 5 of the gene *SCD1* with fat yield and milk fat percentage in Holsteins and Jersey cows [27], as well as with total milk fat percentage in Brown Swiss breed [28]. Holstein-Friesian breed of Polish selection displays positive influence of SNP g.10329C>T (exon 5) on of milk protein content ($p \le 0.05$) [24]. Polymorphism in exon 3 of gene *SCD1* of dairy breeds, the Black-and-White and Ayrshire, was characterized by relatively low frequency of allele variant *A*, upon a decreased fat percentage in milk of Ayrshire cows [29].

There are studies in which no associations between *SCD1* gene and body weight have been identified [14, 30, 31], that is also confirmed by our data.

There was a report on negative impact of SNP T878C in *SCD1* on reproductive traits of Holstein cows that declines profitability in milk herds [32]. We did not found association between *SCD1* gene and reproductive properties of the examined population of Ayrshire cows, which could be explained by both the breed specificities and the external factors, e.g. feeding and keeping conditions.

Totalizing all above, it should be noted that most studies had revealed association between the gene *SCD1* polymorphic variants and milk composition in terms of saturated and unsaturated fatty acid content and total fat yield. It therefore may be said that realization of genetic potential could ensure produc-

tion of high-quality milk. This could also be promoted by proper feeding and keeping as factors maximizing phenotypic manifestation of a genotype.

Thus, we found out SNP rs41255693 polymorphism in the gene *SCD1* of Ayrshires from a population of Russian selection. In the population, *CC* homozygotes had prevailed with the frequency of 0.731 regardless of significant number of *CT* heterozygotes (0.254). The frequency of *TT* genotype found only in three cows was 0.015. High *C* allele frequency (0.858) was seen, whereas *T* allele was rare (0.142). Animals carrying *TT* genotype are few and differ from other groups in low growth rate and longer service period. *TT* genotype is also validly inferior to *CC* and *CT* genotypes with regard to PV for 305-day milking yield ($p \leq 0.01$), PV for 3100-day milking yield ($p \leq 0.02$), and the yield of milk protein and milk fat ($p \leq 0.001$). No valid relationship had been established between SNP rs41255693 in the gene *SCD1* and body weight gain in cows from the birth until the age of first lactation. So *CC* and *CT* genotypes are desirable, of which *CT* heterozygotes have better milk productivity and good growth rate. Selection for desired gene *SCD1* genotypes could significantly improve the effectiveness of breeding in the studied population. In this, molecular methods will facilitate revealing necessary genotypes at earlier age and accelerate formation of a herd with higher productivity potential.

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GENOMIC EVALUATION OF BULLS FOR DAUGHTERS' MILK TRAITS IN RUSSIAN BLACK-AND-WHITE AND HOLSTEIN CATTLE POPULATION THROUGH THE VALIDATION PROCEDURE

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Abstract

The rapid development of molecular genetic methods in animal breeding over the past ten years has given rise to an increase of the selection intensity at the population level. Expansion of the economically useful traits spectrum of dairy cattle allowed increasing the opportunities for breeding to improve the cows' health and for studying the essence of the metabolic synthesis of milk components. The purpose of this study was to verify the effectiveness of genomic forecasting in the development of the concept of dairy cattle genetic assessment in the regional and national aspects. The study for bulls' estimations in Russian Black-and-White improved by Holstein and Holstein breeds by simulation of breeding process using 124 herds of the Moscow and Leningrad regions was carried out. The effectiveness of genomic prediction as compared to the parent averages (PA) and the estimated breeding values (EBV) of sires have been shown. The selection of testing bulls based on the genomic information corrects PA and is to refine EBV that is further obtained by using progeny. Repeatability of genomic EBV was obtained through validation of parentage and genomic information for 100 sires with data for at least 300 daughters. This dataset lay down the core of the newly created Russian regional reference group of dairy cattle. For calculating the additive relationship matrix 1050 ancestors was used. For genomic relationship matrix, 39818 nucleotide polymorphisms were taken into analysis. Based on the REML, BLUP SM, GBLUP methods the procedures to assess of animals were carried out. The average annual genetic trend for milk production traits in the studied populations from 1987 to 2006 was +60 kg, +2.5 kg, +1.5 kg by milk yield, milk fat and milk protein, respectively. It was found that the repeatability of genomic estimates was ranging from 0.371 to 0.606 for milk production traits, which on average exceeded the PA value by 0.147. The accuracy of the evaluation obtained by progeny tested bulls ranged from 0.879 to 0.900 that was higher than the genomic prediction by 0.405 units. The principles of creating the reference population based on the analysis of multi dimension scaling and genetic distances were studied. The distinction between two regional populations (Moscow and Leningrad regions) was F_{st} = 0.0025. The decay of the linkage disequilibrium between the markers at distances up to 1000 kb is shown. In distance from 5 up to 70 kb the linkage level was get the maximum values from 0.20 to 0.54. In the framework of the metabolic pathways study for the milk components synthesis genetic parameters and mean least square estimates

were obtained for the extended milk composition: lactose ($h^2 = 0.18$), dry matter ($h^2 = 0.10$), solids-not-fat ($h^2 = 0.19$), milk freezing point ($h^2 = 0.06$), somatic cells score ($h^2 = 0.10$) and milk urea ($h^2 = 0.04$). The values of additive genetic variances have been get indicating the objective possibilities of using them in Russian dairy cattle breeding sector. To obtain reliable whole-genome associations a further replenishment of the database of the cows' milk component will be carried out using additional spectra. The complex studies have grounded approaches to the use of genomic estimations, the principles of reference population extension and widening list of features for the quantitative and qualitative milk composition assessment.

Keywords: genomic breeding value, milk production, reference population, linkage disequilibrium, heritability, milk components

Development of modern techniques and approaches requires rethinking of the existing pedigree practice in livestock farming. Within the scope of breeding programs, animal breeding system becomes a priority since it largely depends on the selection pressure and on breeding value assessment tools by such categories as parents of sires, as well as parents of high-producing cows. Complex testing of animals in terms of their productivity, breeding record, total impact of the genetic markers across the entire genome, and progeny characterization are the integral part of the work with domestic stock.

The project for genome assessment in dairy cattle, which had started at the beginning of the XXI century, had opened new genome era in selection [1, 2]. First genome assessment had been obtained for bulls of Holstein and Jersey breeds [3, 4]. Nowadays, at least 32 countries participate in genomic multi-trait across country evaluation (GMACE). However, only in 11 of these countries a forecast of the genome pedigree value by milk performance has the official status for use in dairy cattle selection programs [5-7].

Practical efficiency of genomic selection depends on many factors, the main these are size of the reference population, contribution of information about each genotyped animal and its progeny in a series of generations, genetic variability of the analyzed signs (heritability), genome scanning density, distribution of the quantitative trait loci (QTL) and linkage disequilibrium (LD) between markers [8]. However, rather different objectives of development of the genome value estimation algorithms arise upon obtainment of such parameters, in particular by number of species in the reference population (for instance, over 1 million heads in USA). Thus, APY algorithm is used for formation of the population nucleus out of the animals tested by offspring quality and young (for breeding) genotyped animals and presupposes inversion of the smaller genome matrix. For Holstein breed the optimal number comprises at least 12 thousand heads depending on the efficient population size [9]. Issues of prompt access to information and time spend by computer for the procedure also appears to be the priority: from 2 hours upon use of the APY algorithm to 1 month upon using of the standard approach to obtainment of the genome relationship matrix (GRM) type G^{-1} [10].

Besides, studies are being conducted for identification of separate causal nucleotides (point mutations) for qualitative traits (QTN), which along with numerous known non-coding polymorphic replacements (single nucleotide polymorphism, SNP) may improve the accuracy of the genome forecast. The research is aimed at optimization of SNP number with high variability share of causal variants for construction of the GRM, enabling to obtain information on the large number of genotypes and 100 % approximation of the assessment accuracy [11]. Thus, causal SNPs validly associated with economically useful traits in dairy cattle have been detected on chromosomes 5, 6, 9, 14, 15, and 20. Polymorphisms located close to or inside the genes *DGAT1* (BTA14), *GHR* (BTA20), *ABCG2* (BTA6) display the most genetic dispersion by milk production indicators [12-16]. At the same time, regions with genes *FAM181A*, *SLC24A4* and

NKX21 have also been found on chromosome 21 (47-59 million bps) for poorly inherited traits (age of first calving service period) [17]. It should be noted that milk and meat characteristics (protein fraction, fat acids) indicating on close relationship with several QTL regions in the genome have been obtained during study [18, 19]. This research is of high relevance for understanding how biosynthesis of primary structural elements occurs and what metabolic ways regulate expression of quantitative traits in animals.

Economic indicators of fat and protein content and the number of somatic cells are of the most interest to control the milk composition during selection. Recently, special emphasis is placed on the herded animal health indicators, which may be indirectly determined by composition of the feedstock [20]. Introduction of additional assessment parameters of the quantitative and qualitative composition of milk (traces of acetone and β-hydroxybutyric acid, urea content) shall have positive effect on realization of the genetic value in animals by normalization of the metabolic process (acidosis, ketosis), improvement of fertility, extension of productive use, and improvement of the quality of products obtained [21].

Genetic analysis of inheritance of lactose level, dry matter (DM) content and milk solids-not-fat (SNF) in cow milk had shown perspectives of these traits in selection: for lactose $h^2 = 0.281$-0.340, for DM $h^2 = 0.298$, for SNF $h^2 = 0.20$-0.30 [22, 23]. Genotype determinacy (h^2 from 0.072 to 0.130-0.220) had also been found for urea concentration in milk characterizing the balance between the energy value of a diet and protein consumption by an animal [24]. Other individual parameters such as milk freezing point (FP) allow assessing milking procedure in terms of excessive contents of water in milk. Due to significant influence of paratypic factors, e.g. age of animal, month of year, lactation stage, herd size, milking system) on the FP, heritability value did not exceed $h^2 = 0.120$ [25, 26; A. Costa et al., 2017, personal communication]. In-deep analysis of milk fractions would contribute to a better understanding of animal metabolism in order to find mechanisms of its genetic regulation.

Creation of the genome assessment system is one of the integration priorities of the Russian livestock breeding into the global structure for improving the competitiveness of the national breeding material. This problem may not be solved by simple reproduction of foreign models due to significant differences in environmental factors (feeding, keeping, used technologies, and climate conditions), genetic structure of population (genetic variability, LD), methods and ways of measurement of the economically valuable traits. Recently, there is accumulation of breeding records, and extension of the Russian reference population of Russian Black-and-White improved by Holsteins and Holstein breeds, as well as approbation of modern genotype assessment methodologies in animals. Best world practices in this domain may be effectively incorporated upon attainment of comparable information.

Genome forecasting methodology had been practiced in Moscow and Leningrad regions with high results on productive parameters and pedigree work in dairy stock farming. The article displays the results of selection modeling in sires by validation of the genomic breeding value forecasts for the first time implemented on the Russian Black-and-White improved by Holsteins and Holsteins. It was shown that within the scope of the regional component, genetic assessment values shall be corrected by the ancestor values, which in its turn are adjusted by the large number of SNP markers. Our findings describe additive variability on additional milk composition indicators which reflect qualitative and quantitative parameters of milk production.

Purpose of this study was estimation of the genomic breeding values for

milk production traits in core population of Russian Black-and-Whites improved by Holsteins and Holsteins based on full-genome data.

Techniques. Study was conducted in populations of Russian Black-and-Whites cattle improved by Holsteins and Holsteins in Moscow Province (MP) and Leningrad Province (LP). Fifty sires from each region (1987-2006 years of birth) were genotyped using biochip Illumina Bovine SNP50K v2 BeadChip with a total of 54609 bovine SNPs (Illumina, USA). The sample included bulls with at least 300 first-calf daughter cows, with known milk yielding for 305-day lactation, milk fat fraction (weigh%, MWF) and protein fraction (weigh%, PWF), milk fat (MF) and milk protein (MP). This sample was the core reference population used to simulate validation of bull sires by ancestors (based on breeding records), offspring quality, and full-genome data.

Estimated breeding values (EBVs) were calculated via BLUP Sire Model (BLUP SM) method according to earlier approved mixed type model [15]. The database summarized the information on 77375 first-calf cows from 124 pedigree herds, including 35190 heads from 77 herds in MP, and 42185 heads from 47 herds in LP. Parents' average estimates were recalculated by removing EBV values for 10 randomly selected sires from each regional population, 20 heads in total; direct genome value (DGV) was obtained by GBLUP (genomic BLUP) method [27]. The validated sample consisted of 68175 heads (31485 heads from Moscow region, and 36690 heads from Leningrad region). Variation-covariation components were assessed by the restricted maximum likelihood (REML) method using the population constants of heritability, i.e. 0.180 for milking, 0.221 for milk fat fraction, 0.177 for fat weight, 0.173 for milk protein fraction, and 0.142 for protein weight. EBV in sires was calculated with the use of software of BLUPF90 family, including REMLF90 [28].

Total number of ancestors in breeding records of sires comprised 1050 heads. Calculation of the genetic distances between populations (F_{st}) and multidimensional scaling (MDS) with assessment of the linkage disequilibrium between markers were performed with the use of Plink 1.07 and Plink 1.9 package software [29]. After quality control of genotyping, 39818 SNPs were analyzed. For determination of LD values, calculations were made for each chromosome with further averaging in sliding window of 1000 kilobase pairs (--chr ... --r2 --ld-window 1000 --ld-window-kb 1000 --ld-window-r2 0) [30].

Milk composition database obtained by infrared spectrometry of milk samples (a CombiFoss F+ device, FOSS, Denmark) from 7784 cows of 7 pedigree herds (Moscow Province, 2016) were analyzed to study the metabolic ways and regulation of milk component synthesis. Selection and genetic parameters for quantitative traits (lactose content in milk, %; dry matter content, %; fat free milk solids — FFS, %) and qualitative traits (freezing point of milk, °C; somatic cell score, CSC; urea concentration, mg/100 ml) were evaluated. Average values were assessed by method of least square (MLS) using Statistica 7.0 software. The interactions considered were herd × month of a control event, herd × age (number of lactations), and herd × influence of the sire.

Results. We have compared the animals by the years of birth to estimate breeding value (EBV) with regard to regional location and genetic grouping, including for the purpose of prediction of the selection effect and effectiveness of the selected sires from different generations. This comparison showed incremental growth of the genetic potential in bull groups regardless of the region of origin and the number of assessed offspring.

In Moscow region, average annual genetic trend estimates were +37 kg for milk, +1.7, and +0.8 kg for milk fat and protein, whereas in Leningrad region these values were more than 2 times higher, +84, +3.3 and +2.3 kg, respectively.

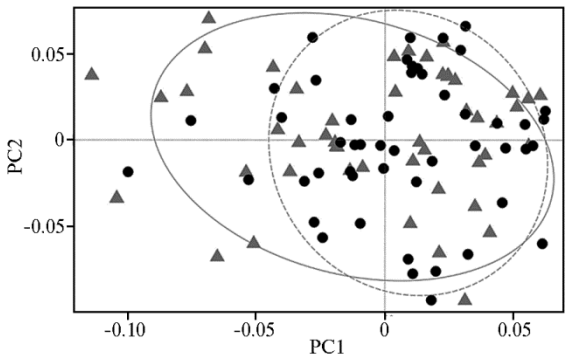

Fig. 1. Spatial distribution of bull sire populations of Russian Black-and-White improved by Holstein and Holstein breeds from households in Moscow (▲) and Leningrad (●) regions by principal components PC1 and PC2.

We suppose that such growth was mainly due to massive and intensive use of pedigree Holsteins in both these regions. In-depth MDS analysis found that the regional cattle populations as a whole, through the example of sires, did not genetically differ from each other (Fig. 1). Herewith, fixation index (F_{st}) of 0.0025 completely confirmed the identity of animals by origin from the Black-and-White Holstein root.

Allocation of linkage disequilibrium between SNP markers showed the polymorphism heritability at a distance from 0-5 to 60-70 kbp with r^2 = 0.201-0.544 for MP and r^2 = 0.199-0.518 for LP. Moderate relationship with the linkage from 0.151 (MP) to 0.153 (LP) had been observed at 110-120 kbp distances, which potentially indicates on possibility for equally effective QTL mapping in dairy cattle populations of different origin (Fig. 2). Minimum LDs were denoted at 1000 kbp distance between markers (r^2 = 0.080).

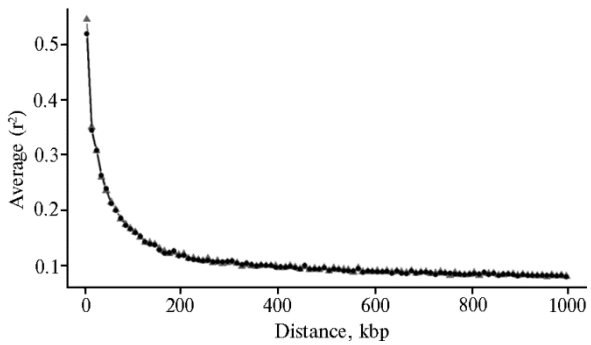

Fig. 2. Allocation of linkage disequilibrium between SNP markers at 1000 kbp distance for populations of bull sire populations of Russian Black-and-White improved by Holstein and Holstein breeds from households in Moscow (▲) and Leningrad regions (●).

We validated the estimated genomic breeding value as compared to other estimates (Table. 1) and found average repeatability of 0.343 for breeding record, 0.490 for genome, and 0.894 for offspring. This ensured excess of the direct genome value (DGV) as compared to PA estimates by +0.147 units, or by 14.7 %. Comparison for PA and DGV were made using EBV estimates by Spearman's correlation coefficient. EBV accuracy was calculated based on estimation of variance components of the genetic variability by REML methods. Maximum precise values for genomic estimates have been obtained for milking (0.606), milk protein (0.535), weight fraction of protein (0.468), and milk fat (0.468). By fat content of milk, predictive estimates were minimal. In our opinion, this may be due to differences in the direction and intensity of selection of Russian Black-and-White improved by Holstein, and first of all, Holstein cattle in Moscow Province (for milking and percentage of fat and protein) and in Leningrad Province (for milking and total dairy products). One can speak about the effectiveness of predictive genomic estimates for clarification of the breeding value by parent averages (PA) and adjustment of the estimates by quality of offspring, provided insufficient number thereof. The obtained predictive estimates have allowed for short validation of the economically useful traits in heritability patterns of populations of Russian Black-and-White improved by Holstein and Holstein breeds from the central regions. For practical use of genomic prediction in breeding, it is important to develop selection criteria for reference population, to unify related cattle groups within an information system, to assess

effectiveness of information obtained for a set of traits, to validate the estimates and to select young bulls with desirable genomic prediction for controlled mating.

1. Validation of the estimated breeding values of Russian Black-and-White improved by Holstein and Holstein breeds of servicing bulls (Moscow and Leningrad Regions) **Validation of the estimated breeding values of bull sires of Russian Black-and-White improved by Holstein and Holstein breeds** (Moscow and Leningrad provinces)

Trait	PA	DGV	EBV
Milk yielding for 305-day first lactation	0.495	0.606	0.900
Fat fraction (weight%)	0.272	0.371	0.897
Milk fat	0.208	0.468	0.899
Protein fraction (weight%)	0.403	0.468	0.896
Milk protein	0.338	0.535	0.879

N o t e. PA — parental averages, DGV — direct genome pedigree value, EBV — estimated breeding value. For PA and DGV, calculation of repeatability was made based on Spearman's correlation coefficient, for EBV calculation of the accuracy was made based on variation components by REML method.

In order to find additional parameters for quantitative and qualitative characterization of the milk composition, we have determined genetic interlinkages and variability of a number of indicators (Table 2). Although some of them were due to paratypical factors, study of the genetic component may enable to identify complex regulation mechanisms of such traits both at population and at genome levels.

2. Genetic parameters and MLS assessment for additional indicators of milk composition in cows of Russian Black-and-White improved by Holstein and Holstein breeds (Moscow and Leningrad provinces)

Indicator	MLS	Indicator					
		lactose	DM	SNF	FP	SCS	Urea
Lactose	4.65±0.01	$0.18^{н}$	–0.07	0.24	0.09	–0.43	–0.07
DM	12.31±0.06	–0.08	$0.10^{н}$	0.24	–0.13	0.08	–0.19
SNF	8.99±0.02	–0.03	0.48	$0.19^{н}$	0.17	0.04	–0.23
FP	–0.586±0.003	–0.36	0.19	0.41	$0.06^{н}$	–0.03	–0.34
SCS	3.9±0.1	–0.73	0.07	–0.01	0.25	$0.10^{н}$	0.03
Urea	27.9±0.4	–0.16	–0.15	0.19	–0.53	–0.06	$0.04^{н}$

N o t e. DM — dry matter, SNF — solids-not-fat, FP — milk freezing point, SCS — somatic cell score; diagonally — heritability (^{н}), below diagonal — genetic correlations, above diagonal — phenotypical correlations.

Heritability of the traits in extended milk compositions significantly varied. Percentage of lactose (0.18), SNF (0.19) and SCS (0.10) ranges were close to standard values. DM and FP displayed low additive variability (0.10 and 0.06, respectively), with low urea content, which, at our opinion, was due to the lack of diet balance as per energy value (deficit) and protein (excess), and limited observations. Because of significant influence of paratypic factors, we failed to establish valid differences in inter-group variability between daughters of sires. Close genetic correlation was found between the lactose content in milk and the counts of somatic cells (r_g = –0.73), which may be due to use of lactose by bacteria. Moderate correlations were between dry matter and solids-not-fat (r_g = 0.48), as well as between milk freezing point and solids-not-fat (r_g = 0.41), concentration of urea (r_g = –0.53) and concentration of lactose (r_g = –0.36). When number of somatic cells in milk increases, its freezing point decreases (r_g = 0.25), which may evidence negative influence of pathogenic microflora on physical properties of milk. These studies would go on and would be aimed at extension of the database by control daily indicators for years 2013-2017.

Thus, estimated breeding values (EBV) in sires by single nucleotide polymorphism (SNP) are more effective than parent averages (PA). Population and genetic analysis had confirmed the validity of use of the unified reference sample for determination of EBV due to the identical origin of animals included in population core. The most tight linkage between the markers had been observed at a

distance of 5 to 120 kbp with minimum $r^2 = 0.15$, thus ensuring stable transfer of the genetic information for a number of quantitative trait loci at a breed level, regardless of the population affiliation. Additional indicators of milk composition may also be indicative of animal health and quality of products, which may, in its turn, extend the selection opportunities. Analysis of full-genome associations might allow for creation the map of metabolic pathways of milk biosynthesis in cows of Russian Black-and-White improved by Holstein and Holstein breeds.

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CHARACTERISTICS OF ALLELE POOL OF THE ROMANOV SHEEP BREED FOR THE PRION PROTEIN GENE ASSOCIATED WITH GENETIC SUSTAINABILITY TO SCRAPIE

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Abstract

The Romanov is a unique indigenous sheep breed of Russia, belonging to the group of Northern short-tailed sheep. The breed is known all over the world, due to out-of-season breeding ability, phenomenal fecundity (up to 10 lambs) and unsurpassed quality of sheepskins. Presently the gene pool of the breed is actively involved in creation of new types of modern prolific sheep and it is considered as an important genetic reserve for the sheep breeding of the future. Diseases resistance is the most important selection trait in sheep. One of the diseases which can cause serious economic loses is spongiform encephalopathy of sheep, also known as scrapie. Scrapie is a fatal neurodegenerative disease of sheep and goats, belonging to the class of transmissible spongiform encephalopathies (TSE), which also includes bovine spongiform encephalopathy (BSE). Three polymorphisms in amino acid codons 136 (A/V), 154 (R/H), and 171 (R/Q/H) of the *PRPN* gene are associated with resistance or susceptibility of sheep to classical scrapie. Depending on the *PRNP* genotype, there are five classes of genetic sustainability to Scrapie (G1-G5). The ARR allele is desirable regarding the resistance to scrapie. However, discovery of atypical scrapie (Nor98) showed a possibility of transmitting BSE to animals of different sustainability classes, including G1 (ARR/ARR genotype). It is shown, that L/F amino acid substitution at position 141 provides resistance to atypical scrapie. The aim of our work was to study the allele pool of the Romanov sheep by the *PRNP* gene, associated with sustainability to both classical and atypical forms of scrapie. The material for the work was tissue samples of 364 clinically healthy Romanov animals including three modern populations of the Yaroslavl region and one population introduced for breeding in the Kamchatka. Genomic DNA was isolated using the Nexttec columns (Nexttec Biotechnologie GmbH, Germany). Identification of the alleles in the codons 136 (A/T/V), 141 (L/F), 154 (R/H) and 171 (Q/R/H/K) was performed by pyrosequencing on the PSQ96MA device (Quiagen, USA). We found four alleles, 136/154/171 — ARR, ARQ, AHQ and VRQ, and nine haplotypes of PRNP as ARR/ARR, ARR/ARQ, ARR/AHQ, ARQ/ARQ, AHQ/ARQ, AHQ/AHQ, ARR/VRQ, VRQ/AHQ and ARQ/VRQ, relating to all five classes of genetic sustainability to the classical Scrapie. The allele of wild type ARQ (the frequency from 0.704 to 0.933) and the genotype ARQ/ARQ (sustainability class G3) were the prevalent. In all the studied groups, a desirable ARR allele was identified with frequencies varied from 0.022 to 0.089 and averaged 0.066. The undesirable VRQ allele was found in three of the four groups, while its frequency was relatively low — from 0.011 to 0.022. The study of the *PRNP* polymorphism by four codons 136/141/154/171 revealed the presence of five different alleles — ALRR, ALRQ, ALHQ, VLRQ, AFRQ and ten genotypes. We detected an animal carrying a sensitive to the atypical scrapie allele F at position 141 of *PRNP* (genotype VLRQ/AFRQ) with the allele frequency of 0.001. The

results will be applied in the development of breeding programs for Romanovs, as well as in strategic planning of conservation of the genetic diversity of this unique Russian Northern short-tailed sheep.

Keywords: prion protein gene (*PRNP*), allele pool, the Romanov sheep, genetic sustainability, scrapie

The relevant objective of modern biological science is conservation of a unique genetic pool of Russian autochthone breeds with their harmonious integration into the selection process, considering world trends in animal husbandry [1-3]. Livestock products obtained from such breeds are of interest for nutritional and process industries. Romanov is unique Russian authentic sheep breed, representing one of the offspring of northern short-tailed sheep [4, 5]. The originality of the Romanov breed is attributable to the combination of unsurpassed wool sheepskins qualities with phenomenal fecundity (up to 10 lambs) and polyestricity [6]. History of breed creation originates from the XVII century, it is first mentioned in 1802 [6]. A hundred years ago, coarse-wool sheep breeding, which was conserved in seminatural peasant farms, not only fill the peasants' needs in wool and meat, but also gave products for export [7]. At the beginning of the last century, recognized zootechnical scientist P.N. Kuleshov, who was concerned about the reducing number of sheep population in Russia, suggested that sheep areas should be outlined, among which the "sheep region of the short-tailed sheep with Romanov's in the center" [5] was placed first. At present, the gene pool of Romanov sheep is considered as an important reserve for the creation of new herds, lines, types of multiparous sheep for different use.

Transmissible spongiform encephalopathy of sheep, also known as scrapie, is one of the diseases which can cause serious economic damage to sheep breeding. Scrapie is a fatal neurodegenerative disease that affects sheep and goats, and relates to the transmissible spongiform encephalopathies (TSEs). This group also includes bovine spongiform encephalopathy, BSE. The cause of TSE is the presence of infectious pathogens, prions, which do not have nucleic acids and, apparently, are composed entirely of modified protein (PrPSc). Normal cellular PrP (PrP^C) is converted into PrP^{Sc} through a post-translational process, which results in a high content of β-sheets [8]. It has been established that the scrapie resistance in sheep is mainly conditioned by polymorphism of prion protein gene *PRNP* which encodes the normal PrP^C. Three mutations in amino acid codons 136 (A/V), 154 (R/H) and 171 (R/Q/H) are associated with resistance or sheep susceptibility to classical scrapie [9-13].

Five amino acids (AA) encoded by three notable codons 136/154/171 explain the formation of 15 possible genotypes on *PRNP* [14, 15]. The haplotype $A^{136}R^{154}R^{171}$, marked as ARR, is desirable in terms of sustainability to scrapie. Depending on *PRNP* genotype, there are five classes of genetic sustainability to scrapie, from G1 to G5 according to the sustainability decreasing. The most preferred genotype ARR/ARR is related to the G1 class. In the past 25 years, among thousands of genotyped G1 sheep, no cases of classic scrapie have been registered [16, 17]. However, the successful transfer of BSE prions to sheep of the ARR/ARR genotype by intracerebral inoculation showed that the resistance of this genotype toward the TSE agents was not absolute [18]. Two cases of encephalopathy in sheep of ARR/ARR genotype, with clinical symptoms similar to classical scrapie were diagnosed in Gearmany [15].

Animals of ARR/AHQ, ARR/ARH, and ARR/ARQ genotypes are genetically resistant to scrapie. In order to avoid susceptibility of progeny to this disease, they can be used only under controlled combinations of parents (G2). Individuals with genotypes ARQ/ARQ (wild type), ARQ/ARH, ARQ/AHQ, AHQ/AHQ, ARH/ARH and AHQ/ARH (G3) have low genetic resistance, but, when mating with G1 animals, produce resistant offspring. The genotypes

ARR/VRQ (G4) and VRQ/AHQ, VRQ/ARH, VRQ/ARQ, VRQ/VRQ (G5) are susceptible to scrapie and should be excluded from reproduction. It has been shown that other amino acid polymorphisms may influence the resistance to prion protein and BSE, in particular at positions 101, 112, 143, 172, 175 and 176, most of which show frequencies lower than 5 % [19]. Octapeptide-repeat polymorphism, that is, different number of repeated N-end sequence of eight amino acids, P(Q/H)GGGWGQ, was reported. The repeat number in cattle, sheep and goats varies both between and within species, ranging from two to five [20, 21]. Polymorphisms of *PRNP* in three positions in the promoter region (C5354A, T5382C and C5622G) were revealed, in this, two latter polymorphisms can affect significantly the transcription factors [22, 23].

The discovery of so-called atypical scrapie showed the possibility of transmitting BSE to animals of different resistance classes, including G1 (ARR/ARR genotype). For the first time, an atypical scrapie was found in Norwegian sheep in 2003 and was named Nor98 [24]. High incidence of atypical scrapie was further identified in Germany and France [25]. In 2005, in Great Britain, 37 % of scrapie-affected sheep was accounted for atypical cases [26]. Atypical and classical scrapie forms differ in number of important features. Atypical cases are characteristic for a later age (4 years and older), often in the infected herd only a few scrapie-positive sheep can be identified [27]. As compared to the classic scrapie, an atypical form only in rare cases (or not at all) can cause neuropil vacuolation or the presence of immunohistochemically detectable PrP^{Sc} in brain [24]. Abnormal PrP in atypical cases is characterized by higher sensitivity to enzymatic cleavage in comparison to the classical form [28]. It is shown that at Nor98 atypical infection, the role of the fourth amino acid in the AA codons 136/141/154/172 at position 141 (L/F) increases. Most cases of atypical scrapie had been identified among animals with low sensitivity to classical scrapie (classes G1-G3) [28-30].

With the detection of strong genetic resistance of the certain sheep *PRNP* genotypes to classical scrapie and the identification of the nature of atypical scrapie [24], it became possible to use *PRNP* polymorphism as an additional criterion in breeding programs for achieving a balance between genetic diversity of populations and prevention of scrapie diseases.

A study on the limited sample of Romanov sheep showed a low genetic resistance to the classical scrapie [31]. However, the surveyed herds were mostly of secondary origin and were formed based on a limited number of lines. Consequently, the obtained results do not allow us to characterize the breed allele pool. To date, resistance of Romanov sheep to the atypical scrapie has not been studied.

Animals from the Yaroslavl gene pool herd can be the most vivid model reflecting the entire genetic diversity of the Romanov breed allele pool. For verification of the hypothesis that the allele pool of the secondarily naturalized herds of this breed, formed by delivery of limited number of lines and undergone selection pressure of other factors, is not typical for the breed as a whole, these sheep must be studied.

This is the first report on genetic polymorphism of the Romanov sheep (*Ovis aries*) populations in the AA position 141 of the *PRNP* gene and their susceptibility to the atypical prion protein Nor98.

The aim of this paper was to study the allele pool of Romanov sheep on prion protein gene, which is associated with resistance to classical and atypical scrapie, on the historical territories of the breed origin and in the places of its secondary naturalization.

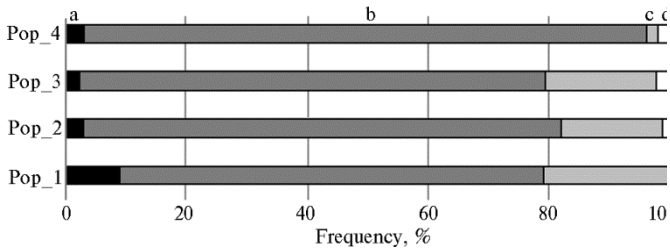
Techniques. The biomaterial was tissue samples (ear biopsy, blood) of 364

pre-healthy sheep of Romanov breed archived in 2013-2016. There were three populations from the Yaroslavl region, Pop_1 (n = 16, Avangard LLC), Pop_2 (n = 98, Agrofima Farmer LLC) and Pop_3 (n = 46, Zarechie LLC) and one population introduced for breeding in the Kamchatka Territory, Pop_4 (n = 52, OAO Kamchatagropromservis).

DNA was extracted using Nexttec columns (Nexttec GmbH, Germany) in accordance with the manufacturer's recommendations. The PCR was performed by the method of N.A. Zinovieva et al. [32]. The reactions were carried out according to the protocol of E.A. Gladyr et al. [33]. The alleles in the codons 136 (A/T/V), 141 (L/F), 154 (R/H) and 171 (Q/R/H/K) was identified by pyrosequencing on a PSQ96MA device (Qiagen, USA).

The data statistical processing was carried out using PSQ96MA SNP Software v.2.0, Microsoft Excel, and GenAlEx 6.501.

Results. Genotyping showed the presence in Romanov sheep of four haplotypes (ARR, ARQ, AHQ, VRQ) and nine genotypes on *PRNP* (ARR/ARR, ARR/ARQ, ARR/AHQ, ARQ/ARQ, AHQ/ARQ, AHQ/AHQ, ARR/VRQ, VRQ/AHQ, ARQ/VRQ) of all five cases of genetic resistance to classical scrapie. At the same time, there were differences in the distribution of haplotypes and genotypes between groups. The wild type ARQ haplotype was the most common with the frequency of occurrence from 0.704 in Pop_1 to 0.933 in Pop_4. In all groups, there was a desired ARR haplotype the frequency of which ranged from 0.022 in Pop_3 to 0.089 in Pop_1 and averaged 0.066. The undesired haplotype VRQ was found in three of the four groups except of Pop_1, at relatively low frequency, from 0.011 (Pop_2) to 0.022 (Pop_3) (Fig.).



Distribution of *PRNP* haplotypes ARR (a)**, ARQ** (b)**, AHQ** (c)**, and VRQ** (d) **associated with resistance to classical scrapie in Romanov sheep populations:** Pop_1 from Avangard LLC, Pop_2 from Agrofima Farmer LLC, Pop_3 from Zarechie LLC (Yaroslavl Province); Pop_4 from OAO Kamchatagropromservis (Kamchatka Territory).

The wild type ARQ/ARQ genotype of class G3 was the most frequent in all the populations (Table). Probably, it is due to the long-term pure breeding aimed solely to affirm unique adaptive and productive qualities. The frequency of ARR/ARR genotype which is the most resistant to the classic scrapie, was very low. A portion of animals carrying G2 genotypes differed 8 times and more between herds even within the same region. Apparently, it was related to the predominant use of ram producers of different lines.

In general, the studied Romanov sheep was characterized by relatively low frequencies of genotypes of the genetic classes G4 and G5 which cause a high risk of classic scrapie. Such genotypes were detected in three of four surveyed populations with a frequency of 2.2 to 4.1 %

We were the first to estimate the genetic status of the Romanov breed in terms of predisposition to the infection with atypical prion protein Nor98. The assay identified five different haplotypes, ALRR, ALRQ, ALHQ, VLRQ and AFRQ, and ten genotypes on *PRNP* (136/141/154/171), ALRR/ALRR, ALRR/ALRQ, ALRR/ALHQ, ALRQ/ALRQ, ALHQ/ALRQ, ALHQ/ALHQ, ALRR/VLRQ, VLRQ/ALHQ, VLRQ/ALRQ and VLRQ/AFRQ. The frequency of haplotypes and genotypes for four codons mostly had profiles which were the same as those for codons 136/154/171. Only in Pop_4, one animal carried allele F associated with sensitivity to atypical scrapie (VLRQ/AFRQ genotype) at position

141 of *PRNP*, so that this allele frequency was 0.001. It is important to underline that allele F^{141} was identified in the genotype VRQ/ARQ (class G5) in combination with the haplotype VRQ, the most susceptible to the classical scrapie.

Genotype distribution on prion protein gene *PRNP* (G) and on the classes of resistance to classical scrapie (C) in Romanov sheep

Genotype 136/154/171	Frequency									
	Pop_1		Pop_2		Pop_3		Pop_4		В среднем	
	G	C	G	C	G	C	G	C	G	C
					Class G1					
ARR/ARR	–	–	0.010	0.010	–	–	–	–	0.003	0.003
					Class G2					
ARR/ARQ	0.143	0.208	0.061	0.061	0.022	0.022	0.058	0.058	0.093	0.123
ARR/AHQ	0.066		–		–		–		0.030	
					Class G3					
ARQ/ARQ	0.446	0.792	0.592	0.888	0.609	0.934	0.865	0.904	0.566	0.852
AHQ/ARQ	0.316		0.265		0.282		0.039		0.258	
AHQ/AHQ	0.030		0.031		0.043		–		0.028	
					Class G4					
ARR/VRQ	–	–	–	–	0.022	0.022	–	–	0.003	0.003
					Class G5					
VRQ/AHQ	–	–	0.031	0.041	–	0.022	–	0.038	0.008	0.019
VRQ/ARQ	–		0,010		0,022		0,038		0,011	

Note. For description of populations, read *Techniques* section. Dashes mean the absence of the genotype in the populations.

As known, the susceptibility of sheep to classical scrapie directly depends on certain non-synonymous single nucleotide polymorphisms within prion protein gene *PRNP* located on chromosome 13 [34-36]. Modern molecular technologies make it possible to study genotype in the first days of animal's life and in order to early control over the spread of hereditary defects. Conservation, use and development of gene pools of local breeds for their effective integration in modern livestock husbandry with a view to obtain new breeding forms and to increase the range of native breeds are also in progress. The aim of most sheep breeding programs is to monitor a breed gene pool by gradually replacing genotypes which are characterized by hyper susceptibility to scrapie, using rams of ARR/ARR genotypes. Accumulation of the haplotype ARR in the breeds and populations is necessary for preventive protection against the classical pathogenic prion that causes scrapie. This fact is confirmed by research carried out in Canada in 2008-2012 on 184 Romanov sheep, which showed the plasticity of the breed gene pool and made it possible to bring the frequency of ARR haplotype and ARR/ARR genotype to 0.592 and 0.359, respectively, by selection [37]. At the same time, the high frequency of wild haplotype in aboriginal and domesticated breeds, which was noted in some papers [38, 39], remains an actual problem.

Thus, our data made it possible to evaluate the genetic resistance to classical and atypical scrapie in allele pool of Romanov sheep bred in the territories of historical origin and in the locations of introduction. The average frequency of undesirable $V^{136}R^{154}Q^{171}$ haplotype and F^{141} allele associated with susceptibility to atypical scrapie was 0.011 and 0.001, respectively, that is a positive factor for the further improvement and conservation of Romanov sheep breed. In the studied populations, a clear shortage of animals with G1 genotype was revealed and the frequency of haplotype ARR resistant to classic scrapie averaged 0.066. This creates the prerequisites for strategic breeding programs to accumulate genotypes resistant to pathogenic prion in the gene pool of Romanov sheep breed. Breeding for genetic resistance to scrapie and increasing proportion of the carriers of ARR allele and ARR/ARR genotype are necessary to prevent classical and atypical scrapie in Russian populations. The allele pool of the entire Romanov breeding stock must be studied to estimate genetic diversity of this unique northern Russian sheep.

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STUDYING THE STRUCTURE OF A GENE POOL POPULATION OF THE RUSSIAN WHITE CHICKEN BREED BY GENOME-WIDE SNP SCAN

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Abstract

A population of the Russian White chickens, bred at the gene pool farm of ARRIFAGB for 25 generations using individual selection, is characterized by resistance to a lowered temperature in the early postnatal period and white colour of the embryonic down. In 2002-2012, breeding was carried out by panmixia, and by now a new population of the Russian White chickens has been formed on the basis of the surviving stock. Comparison of the genetic variability of this population and the archival DNA of representatives of the 2001 population using microarray screening technology will help to assess the population structure and the preservation of the unique characteristics of its genome. The material for the study was DNA extracted from 162 chicken blood samples. Two groups of the Russian White breed were studied, the 2001 population and the current population. Genome-wide analysis using single nucleotide markers (SNP) included screening by means of the Illumina Chicken 60K SNP iSelect BeadChip microarray. Quality control of genotyping, determination of the population genetic structure by multidimensional scaling (MDS), calculation of linkage disequilibrium (LD) and allele frequency in the groups were carried out using PLINK 1.9 software program. The construction of a cluster delimitation model based on SNP genotypes was carried out using the ADMIXTURE program. According to the MDS analysis results, the current population can be divided into four MDS groups, which, when compared to the data of the pedigree, adequately reflect the origin of the studied individuals. The representatives of the ancestral population were genetically similar to the MDS3 group of the current population. Using the F-statistic of the two-way analysis of variance, a significant effect of the group, chromosome, chromosome in the group, and the distance between SNP markers on LD (r^2) values was observed. In the 2001 group, the maximum r^2 and the high incidence of LD equal to 1 were observed for all chromosomes, with a distance between SNP markers being 500-1000 Kb. There was also the greatest number of monomorphic alleles in this group. Based on the SNP analysis, we may conclude that the current Russian White chicken population is characterized by the disintegration of long LD regions of the ancestral population. Modelling clusters using the ADMIXTURE program revealed differences between the current population groups determined by MDS analysis. The groups composed of individuals included in MDS1 and MDS2 had a homogeneous structure and differed from each other at K = 4 and K = 5. The MDS4 group formed a genetically heterogeneous cluster different from the MDS1 and MDS2 groups at K of 2-5. The MDS3 group was phylogenetically close to the 2001 population (at K of 2-5). In general, the analysis of the current gene pool population of the Russian White chickens showed its heterogeneity while one of its groups (MDS3) was similar to the ancestral population of 2001, which in turn is characterized by a large number of monomorphic alleles and a high frequency of long LD regions. Thus, SNP scanning allowed evaluating the genetic similarity of individuals and the population structure of the Russian White chicken breed. Understanding the genetic structure is

an important point in the panmictic breeding and tracking of historical changes in the molecular organization of the genome of a gene pool population with a limited number of animals.

Keywords: population structure, genetic diversity, SNP genotyping, Russian White breed of chickens

Modern methods do not find noticeable application in in-depth studying domestic chicken native gene pool. At the same time, conservation and usage of beneficial qualities of their non-commercial breeds remains an important scientific and economic task. Gene pool poultry can be used in biotechnology and as a model for studying biological processes and identification of genes (genetic markers) associated with economically useful traits [1-4].

The white-feathered **Russian Whites,** a chicken breed with primary use for eggs, is being maintained in the Gene Pool Farm of the All-Russian Research Institute of Genetics and Breeding Farm Animals (ARRIGBFA) since 1953 and initially had a linear structure [1]. Two lines, No. 10 and No. 16, differed in adaptability to lower temperatures in the early postnatal period [1]. Another experimental group of this breed was characterized by the white color of neoptile, and entire population was kept at low temperatures for 25 generations [1]. Russian Whites breeding has been based on individual selection of parents. Until 2002, the population was reproduced within lines No. 10 and No. 16, and then, until 2012, the chickens were kept at a commonly accepted temperature and bred by panmixia method, as a result of which the linear breed structure was lost. Based on the surviving poultry, a new population of Russian Whites was formed, features of which were white down of one-day old chicks and ability of adaptation to lower temperatures (22-23 °C) compared to commonly accepted for this age (30-33 °C). At present, keeping at low temperatures is not applied [1].

Improvement of small population for desirable traits is impossible without an assessment of population genetic structure. Mini- and microsatellite molecular markers and other methods of DNA polymorphism study, widely used earlier [2-7], recently are given way to the numerous single nucleotide polymorphism (SNP). Thousands of SNPs allow genotyping of the whole genome and make it possible to associate the found marker variations with quantitative traits. SNP scanning is a highly effective genetic analysis tool that can identify structural features of the population, which can be used in breeding [8-11]. A combination of molecular genetic data with mathematical models enhances the accuracy of animal breeding value prediction for selection and management efficiency, which accelerates genetic progress in breeding populations [12, 13]. While breeding small populations, there is a prevalence increasing of extended haplotype areas, including regions of homozygosity (ROH), steadily passed on to the offspring over generations [14-16], which tends to reduce genetic diversity in a small population [17, 18].

Multidimensional scaling (MDS analysis) is a widely used tool to assess the differentiation of the studied groups (populations, breeds) [19]. Suggested methods are based on the predetermined structure of analyzed groups and calculation of genetic distance between individuals by using the algorithm for phylogenetic clustering [20]. Bayesian clustering models have been developed. They include genotyping in tens of thousands of loci and, as in STRUCTURE and ADMIXTURE software, can regard Hardy-Weinberg equilibrium and linkage disequilibrium (LD).

With the help of genome wide SNP genotyping, we first revealed a subpopulational structure of the modern Russian Whites chickens from the ARRIGBFA collection, possessing unique genetic material of domestic and foreign breeds, and found the differences of the studied poultry groups from the original population.

Our aim was to show the possibilities of whole genome SNP scanning for characterization of the genetic structure features in a small chicken population of domestic origin and the dynamic changes in its molecular architecture by a comparison of the current population of the Russian White breed with the population of 2001.

Techniques. DNA was extracted from blood samples collected from Russian White chickens (*Gallus gallus*) of ARRIGBFA bioresource collection (Genetic Collection of Rare and Endangered Chicken breeds, St. Petersburg-Pushkin). Two groups were analyzed: the population of 2001 (n = 6, unrelated individuals from two lines) and the modern population (n = 156). The SNP analyses included screening of 162 DNA samples with the Illumina Chicken 60K SNP iSelect BeadChip microchip (Illumina, USA). The quality of the genotyped SNP loci was monitored using PLINK 1.9 software [23]. In addition, DNA samples with a genotyping quality of SNP loci more than 90 % evaluated using GenomeStudio software (Illumina, USA) were selected for analysis. Hardy-Weinberg error (HWE) limits were set ($P \leq 0.0001$). SNPs, which were in linkage disequilibrium (--indep-pairwise 50 5 0.5) in the PLINK 1.9 software were deleted. To eliminate gender effects, SNP markers located on sex chromosomes were excluded. Population genetic structure was detected by MDS analysis with PLINK 1.9 software. Allele frequency and linkage disequilibrium in groups were also calculated using PLINK 1.9.

Multivariative analysis of variance (ANOVA) was carried out in the RStudio program [24]. The effects of group, chromosome, their interaction, and SNP interval on LD value were estimated by the linear model [10]:

$$r^2_{ij} = \mu + BL_i + Gga_j + (BL \times Gga)_{ij} + bSNP_{int} + e_{ik},$$

where r^2_{ij} is pair LD value, μ is the overall average LD, Bl_i is the effect of i group, Gga_j is the effect of the chicken chromosome j (chromosomes 1 to 28), SNP_{int} is the effect of interval between SNP markers, which was determined as the distance between markers (the number of nucleotides pairs), b is the regression constant.

Clustering based on SNP genotyping was carried out using ADMIXTURE software [25].

Results. Depending on SNP genotyping, the current population was conditionally divided into MDS groups and compared to the pedigree data and to genotyping of the ancestral population of 2001. Location of the points, as resulted from multidimensional scaling (Fig. 1), was influenced by low frequency monomorphic alleles and minor allele frequency (MAF). This complicated estimation of the variability of the remaining markers. Preliminarily, a restriction level of 0.1 has been chosen for MAF filtering to exclude all monomorphic alleles and minor alleles with a frequency under 10 %.

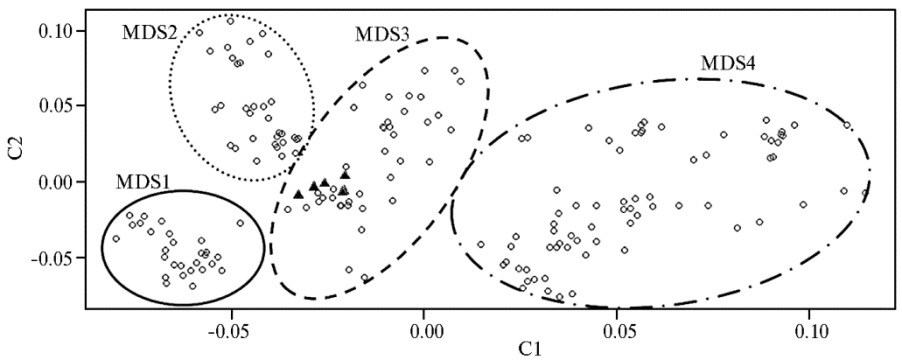

Fig. 1. Clustering (MDS1-MDS4) **of Russian White chickens based on SNP genotyping:** ○ — individuals of current population, ▲ — stored individual DNA samples of 2001; C1, C2 — coordinates (Genetic Collection of Rare and Endangered Chicken Breeds, ARRIGBFA, St. Petersburg-—Pushkin).

Modern population of Russian Whites could be conditionally divided into four clusters (MDS1-MDS4). Clusters MDS1 and MDS2 were separated along the C2 axis, MDS1, MDS2, MDS3 were separated from MDS4 along the C1 axis. The MDS1 cluster included mainly individuals descending from rooster No. 99. The MDS2 cluster grouped the offsprings of roosters No. 98 and No. 99 (Table 1). The MDS4 cluster predominantly comprised birds the ancestor of which was rooster No. 97. The MDS3 cluster consolidated the descendant of rooster No. 58 and the intermediates close in origin to the other clusters.

1. Distribution of Russian White chickens of current population for MDS clusters depending on ancestor roosters (Genetic Collection of Rare and Endangered Chicken Breeds, ARRIGBFA, St. Petersburg—Pushkin)

Father rooster, No.	Ancestor rooster, No.	Cluster				Total
		MDS1	MDS2	MDS3	MDS4	
981206	98	0	10	3	0	13
981205	98	0	16	0	1	17
981501	98	0	0	5	0	5
991803	99	0	14	0	0	14
991203	99	16	0	1	0	17
970905	97	0	0	0	12	12
971103	97	0	0	0	16	16
970907	97	0	0	0	15	15
971601	97	0	0	0	13	13
581706	58	0	0	9	0	9
481701	58	1	0	8	7	16
639		0	0	9	0	9
Total		17	40	35	64	156

Individuals from the selected clusters were grouped to study their genetic features (Table 2). Structure of the groups was estimated based on the presence and extension of genomic regions with linkage disequilibrium detected with SNP markers. The maximum average LD value was found in the 2001 population. The number of monomorphic alleles in this group also appeared to be the highest. The MDS3 group held a central position together with the 2001 population, but unlike it, had a minimal number of monomorphic SNPs and a significant number of minor alleles with a frequency of less than 10 %. In the 2001 group, the maximum values of r^2 and the high frequency (0.24) of linkage disequilibrium equal to 1 were found for all chromosomes with a significant distance between SNP markers (500-1000 kb). The overall calculated LD value per chromosome was high both in the current and in the ancestral population and varied from 0.150±0.006 to 0.587±0.006.

2. Characterization of ancestral population 2001 and current gene pool population of Russian Whites on the base of MDS clustering with SNP marker loci (Genetic Collection of Rare and Endangered Chicken Breeds, ARRIGBFA, St. Petersburg—Pushkin)

Indicator	Group				
	MDS1	MDS2	MDS3	MDS4	2001
Total genotyped SNP	57636	57636	57636	57636	57636
Including:					
loci with high genotyping quality (> 0.90)	43224	43224	43224	43224	43224
loci with monomorphic alleles	9176	8157	1507	5393	19833
loci with minor alleles (MAF ≤ 0.1)	5943	7800	10443	8200	3827
HWE (P ≤ 0.0001)	949	1021	1244	1543	0
LD (*M*±SEM)	0.272±0.001	0.241±0.001	0.193±0.001	0.197±0.001	0.506±0.001
LD frequency = 1 at distance between SNP 500-1000 kbp	0.07	0.03	0.02	0.02	0.24

N o t e. MAF — minor allele frequency, HWE — the number of SNPs not satisfying the Hardy-Weinberg equilibrium test (at P ≤ 0.0001), LD — linkage disequilibrium, *M* — average mean LD, ±SEM — standard mean error.

Additional multivariate dispersion analysis using the F-test (Table 3) showed significant (P < 0.0001) effect of groups, chromosomes, distances between SNP markers and the chromosome in the group on LD (r^2). The group and the distance between SNP markers exerted the greatest influence.

One of the effective methods for detecting differences between groups and

breeds of animals is cluster analysis of the admix models [25, 26]. While clustering in the ADMIXTURE program, the cross-validation coefficient (CV) was estimated to determine the optimal K value. The minimal error was observed at K = 12 (Fig. 2). The MDS1 and MDS2 groups had a homogeneous structure and did not differ at K = 2, partially differed at K = 3 and showed a significant difference at K = 4 and K = 5. The MDS4 group formed a genetically heterogeneous cluster which differed from MDS1 and MDS2 at K from 2 up to 5. The MDS3 group was more homogeneous and close to the 2001 population with a K value of 2 to 5.

3. Influence of MDS group, chromosome and interval between SNP markers on linkage disequilibrium (r^2) **in the population of Russian White chickens** (Genetic Collection of Rare and Endangered Chicken Breeds, ARRIGBFA, St. Petersburg—Pushkin)

Factor	DF	SS	MS	F	P
Group	4	3244	811.0	11947.40	$P < 0.001$
Chromosome	27	178	6.6	96.93	$P < 0.001$
SNP distance	1	244	243.8	3592.35	$P < 0.001$
Group × chromosome	105	289	2.8	40.61	$P < 0.001$

N o t e. DF — the number of degrees of freedom SS — sum of squares, MS — mean squares, F — Fisher distribution.

As is known, the traditional poultry breeding with individual records includes selection of parents for productivity, selection for own productivity, and selection of unrelated pairs. A panmictic breeding, as it was in current population of Russian Whites, it is difficult to determine the origin and genetic variability of the resulting offspring. The characterization of genetic variability solves the problem of determining the structure of population and makes it possible to estimate the dynamic changes in its molecular architecture.

In modern Russian White chickens, as descendants of 2001 population, a specific drift of genes could occur. Note, that the members of the population 2001 (2 individuals of line No. 16 and 4 individuals of line No. 10) took a position in the central part of distribution area of the same modern cluster MDS3 (see Fig. 1). Although the number of the ancestral individuals was small, their genotyping can be considered in data processing, since, due to a significant number of SNP markers used in population analysis, an increase in the number of analyzed individuals is not required for reliable estimates [27-29]. Unrelated individuals of different lines were very close to one another, because both lines descended from one rooster.

However, the genotypes of unrelated individuals which were involved in the analysis do not fully reflect the genetic breed diversity in 2001. Not all minor alleles of the population 2001 were accounted because of limited biomaterial available (6 individuals). Perhaps, some monomorphic alleles in the population were minor. At the same time, a part of minor alleles and all the monomorphic alleles were eliminated during MAF filtration in MDS analysis and could not affect the pattern of distribution and, consequently, the conclusions about the genetic proximity or remoteness of individuals and populations. In the preliminary analysis, the complete exclusion of minor alleles did not affect MDS clustering of the population. Removing SNPs that were in linkage disequilibrium (--indep-pairwise 50 5 0.5) did not change the cluster locations while varying criteria for filtering SNPs. Our findings have shown that the common origin has the most impact on the relative MDS distribution.

The presence of unique haplotypes is the essential characteristic of a population [19, 29]. Extension of genomic regions with linkage disequilibrium detected with SNP markers is considered the main structural feature of the studied groups [8]. A large number of markers in linkage disequilibrium was a distinctive feature of Russian Whites ancestral population, which influenced the LD

value. Long-distant linkage disequilibrium for significant number of SNPs found in unrelated animals indicates rather a limited number of ancestors involved in breeding. This is confirmed by other studies of commercial poultry lines [8, 17]. The modern population of Russian Whites is characterized by breakdown of long-range LD areas and a reduced frequency of the ancestral population haplotypes.

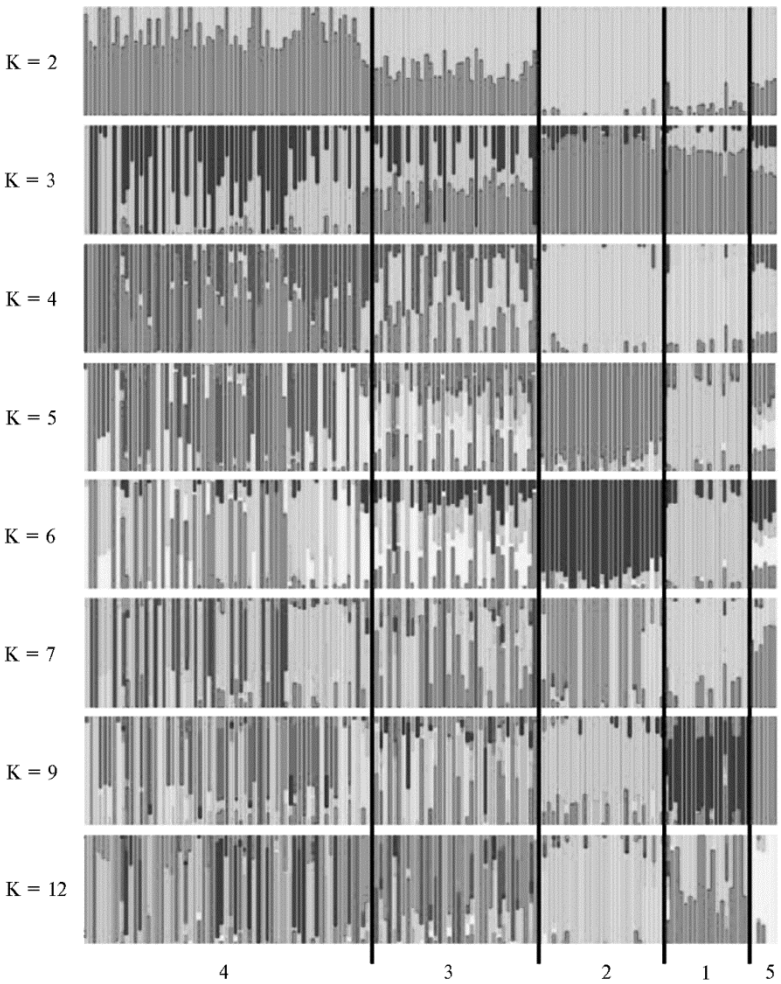

Fig. 2. Population variability on SNP markers in Russian White chicken breed as calculated using ADMIXTURE software: 1, 2, 3, 4 — MDS1, MDS2, MDS3 and MDS4 clusters of modern population, 5 — members of population 2001. K is the number of ancestral populations (Genetic Collection of Rare and Endangered Chicken Breeds, ARRIGBFA, St. Petersburg—Pushkin).

Thus, the heterogeneity of the modern gene pool population of Russian White chickens is based on their origin due to different ancestor roosters. A group (MDS3) was found which has the greatest similarity to the ancestral population of 2001. A distinguishing feature of the latter is a significant number of monomorphic alleles and a high frequency of long-range LD areas. In the modern population, the minor allele frequency increased and the LD values decreased. In general, SNP scanning makes it possible to identify the structural ties in breed based on the genetic similarity between individuals, which is especially important in panmictic breeding of small breeds when the number of animals is limited. The comparison of modern population and its ancestral population makes it possible to trace historical changes in the molecular organization of the Russian White breed

genome. Gene pool populations, the genetic variability of which has been formed for a long time, are a valuable source of biodiversity. Characterization of their genetic features is relevant as allows us to use the best animal qualities in breeding. In this paper, we report on important genetic characteristics of a small breed of domestic chickens. This information can be farther used for managing, conserving and using valuable genetic resources, and also for monitoring the dynamic changes in the molecular organization of genome for the limited gene pool population.

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STUDY OF PHENOTYPIC AND GENOTYPIC FEATURES OF REINDEER POPULATIONS OF THE NENETS BREED

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Abstract

Reindeer herding is the leading branch of animal husbandry in the Nenets Autonomous Okrug (NAO). Currently, reindeer herding is being conducted mainly by traditional methods, which are based on the assessment of the phenotype. For further development of reindeer herding and the increase of its productivity, it is necessary to improve the level of breeding work by using molecular genetic information. The aim of our study was to investigate the phenotypic and genetic features of the reindeer Nenets breed in the conditions of the NAO. To examine morphological features, the measurements of the exterior characteristics (height at withers, chest depth, chest width, chest girth, wrist girth, body length, loin width, head length, body weight) were made at three farms of the NAO: ERV (ERV, $n = 28$), Indiga (IND, $n = 34$) and Ilebts (ILB, $n = 25$). Based on the data obtained, the body built indices were calculated: massiveness, blockiness, bone, chest index, lengthiness and head. For genetic characteristics, the analysis of nine microsatellite (NVHRT76, RT9, NVHRT24, RT30, RT1, RT6, RT27, NV21, RT7) loci was carried out. Statistical processing of data was performed using the R software and R packages adegenet and diveRsity. For most of the exterior features, a significant advantage of IND and ILB over ERV was observed, respectively: height at withers — 99.2±0.42, 99.7±0.53 and 97.0±0.46 cm ($p < 0.01$); chest depth — 43.4±0.31, 42.6±0.32 and 43.7±0.46 cm; chest width — 27.7±0.35, 27.5±0.45 and 26.6±0.27 cm; chest girth — 125.6±0.69, 126.6±0.80 and 119.0±0.77 cm ($p < 0.01$); body length — 108.3±0.54, 107.7±0.81 and 102.2±0.32 cm ($p < 0.01$); wrist girth — 11.8±0.09, 11.9±0.11 and 11.21±0.05 cm; head length — 33.3±0.38, 33.9±0.51 and 32.1±0.18 cm; body weight is 104.6±1.17, 106.4±1.24 and 83.5±1.08 cm ($p < 0.01$). IND and ILB also exceeded ERV ($p < 0.01$) in massiveness, chest and lengthiness indices. As compared to the indicators of the 1970s, there was an increase in massiveness and chest index in IND and ILB, and bone and lengthiness in all the three groups studied. According to the results of principal component analysis (PCA), the similarity between IND and ILB was observed for both the morphological and genetic characteristics, which was manifested in the formation of overlapping arrays on the PCA-plot, while the ERV formed a relatively isolated cluster. Pairwise genetic distances confirmed the greater similarity between IND and ILB (F_{st} — 0.018 and D_{Jost} — 0.017). All the three populations were characterized by a deficit of heterozygotes ($F_{is} > 0$, CI 95 %). Allelic richness (A_r) varied from 6.17±0.499 (IND) to 6.78±0.494 (ILB). Thus, it was shown that the reindeer populations that are bred in the NAO have morphological and genetic differences. In addition, the populations with different morphological characteristics were not close genetically. Morphological features in the studied populations were within the breed standards, or exceeded them.

Keywords: reindeer breeding, morphological features, genetic diversity, microsatellites

Reindeer herding is the leading branch of animal husbandry in the northern regions of the Russian Federation. Indigenous peoples use reindeer for food, clothing and transport under harsh climatic conditions, to which other species of farm animals are not adopted, [1]. One of the leading places in the domestic reindeer population is the Nenets Autonomous Okrug (NAO). According to the pedigree records of January 1, 2015, about 170 thousand deer were bred in 23 farms [2] in the territory of the NAO. During economic reforms and intensive industrial development of the Far North territory, the Nenets saved reindeer husbandry [3]. By selective breeding, they created a large group of domestic reindeers with clearly expressed morphological and economically useful traits that animals steadily pass to their progeny. In 1985, the Nenets breed, among the other reindeer breeds was recorded in the State Register of livestock breeds, USSR. The deer of this breed are characterized by strong body built and medium size. This is the largest breed population of domestic reindeers on the North of European Russia and above the Urals, in the lower of Ob' and Yenisei rivers. Since the 1930s, massive zootechnical studies of Nenets deer in the Western Siberia resulted in improved animal productivity and morphometric parameters and exterior characteristics [4].

Unlike the other branches of livestock husbandry, pedigree breeding of reindeers is carried out mainly by traditional methods, which are based on visual estimates of productive, primarily meat, qualities and allow indirect assessment of the reproductive system. For a long time, the estimated of deer variety within and between populations was based on a phenotypic evaluation of animal morphological features characterizing their shape and appearance [5]. However, it is not enough for studing a population.

Development of reindeer herding and an increase in animal productivity are associated with the improvement of breeding work through modern selection and breeding methods. One of them is the use of microsatellite sequences, also known as short tandem repeat (STR), for genetic characterization of reindeers. Microsatellites are highly polymorphic, evenly dispersed throughout a genome and characterized by Mendelian inheritance. They are widely used in validation and control of animal origin, in estimation of the degree of inbreeding, breed purity, biodiversity, genetic differentiation and genetic structure of breeds of the main species of farm animals [6-10]. Microsatellite markers have been successfully used in studying genetic diversity [11, 12] and introgression of domestic and wild reindeer populations [13].

This is the first principal component analysis of Nenets breed reindeers in the territory of the NAO based on morphological and genetic data. The findings showed the presence of at least two groups in Nenets breed, which was also confirmed by comparing pairwise genetic distances among research populations.

The objective of our study was to investigate the phenotypic and genetic features of the reindeer (*Rangifer tarandus*) Nenets breed in the conditions of the Nenets Autonomous Okrug.

Techniques. Exterior features of the Nenets does reindeer aged 3.5-8.5 years were recorded in three farms of the NAO in 2016, i.e. APK Cooperative Farm ERV (ERV population, $n = 28$), Indiga (IND, $n = 34$), Ilebts (ILB, $n = 25$). Height at withers, chest depth and chest width were assessed using a Leidten measuring stick; chest girth, wrist girth, body length were estimated using measuring tape; loin width and head length were measured with the Wilkins' compass; body weight was recorded by weighting using a dynamometer DPU-5-2 for 500 kg (LLC Plant Testing Devices, Russia). Body mass index were calculated including massiveness, blockiness, boniness, chest index, lengthiness, big-headedness. The data obtained during inspection of three reindeer herding farms were summed up by age groups and the average values were calculated. For comparison, body in-

dexes of the Nenets breed recorded in the 1970s, were used [14].

For genetic characterization, DNA was isolated for ear tissue samples using Nexttec™ 1-step DNA Isolation columns (Nexttec Biotechnologie GmbH, Germany) as per manufacturer's recommendations. Polymorphism of 9 microsatellite loci (NVHRT76, RT9, NVHRT24, RT30, RT1, RT6, RT27, NV21, RT7) was assessed on DNA analyzer ABI3130xl (Applied Biosystems, USA) according to the previously developed protocol [15]. Allele sizes were determined in GeneMapper 4.0 software (Applied Biosystems, USA) followed by converting to numerical expressions, based of which the genotype matrix was formed in Microsoft Excel format. As control group for access of genetic diversity, wild reindeer samples (n = 32) collected in the course of expeditions to Western Taimyr were used.

Statistical data processing was carried out using R 3.3.3 software [16]. The calculation of indicators for Principal Component Analysis (PCA) based on microsatellite sequences was carried out with R adegenet software [17]. To visualize the results, the R ggplot2 package was used [18]. The expected (H_e) and observed (H_o) heterozygosity, inbreeding coefficient (F_{is}), allelic diversity (A_r) and genetic distances were calculated using R diveRsity [19]. Also, using this package, the degree of genetic populations differentiation was estimated based on the pairwise values of F_{st} [20] and D_{Jost} [21]. In the tables and text, the arithmetical mean values (M), standard errors (±SEM), and coefficients of variation (Cv) are given.

Results. Comparing the exteriors (Table 1), it can be noted that the ILB and IND reindeer-doe exceed the ERV reindeer-doe in height at withers by 2,75 and 2,27 cm ($p < 0.05$), respectively. The difference between IND and ILB reindeer-does was unreliable. All values comply with the breed standard of 94.5-100.5 cm [4]. In the studied reindeer-doe groups, the chest depth did not have significant differences. The chest width of IND was 1.06 cm more than that of ERV ($p < 0.05$). The IND and ILB reindeer-doe chest girth behind the shoulder was larger than that in ERV by 6.61 and 7.62 cm ($p < 0.01$), respectively. A similar trend was observed in the body length value, which was higher for IND and ILB than for ERV by 6.11 and 5.53 cm ($p < 0.01$), respectively. The last two indices for ERV were within the norm, while IND and ILB exceeded the breed standards for the chest width (114.5-124.0 cm) and the body length (99.0-107.0 cm) [4]. Loin width estimates were available only for IND and ILB and differed not reliably. In three farms, the wrist girth values ranged from 11.21±0.05 to 11.88±0.11 cm that complied with the breed standard (10.0-12.0 cm). Differences between IND and ERV ($p < 0.01$), and also between ILB and ERV ($p < 0.01$) were statistically significant. The head length of reindeer does in ERV population was less than that of IND and ILB ($p < 0.01$). ILB and IND does were 22.94 and 21.16 kg heavier ($p < 0.01$), respectively, compared to ERV animals thus showed superiority in body weight. The difference in body weight of IND and ILB animals (1.78 kg) was not statistically significant.

Body built indexes gave a better understanding of the exterior (Table 2). A higher animal massiveness in IND and ILB populations compared to ERV ($p < 0.01$) testified for the better torso development. This parameter of ERV animals was comparable to NAO data of the 1970s (NEN'70) (see Table 2). Blockiness is a convenient indicator for body weight assessment. Its differences among reindeer does from surveyed farms were not statistically significant ($p < 0.05$) and did not differ from the average for NEN'70. Boniness in ERV population was lower ($p < 0.05$) compared to IND and ILB, that pointed at more coarse skeleton. It should be noted that this index exceeded the value of NEN'70 in all tested breeding population. The chest index, reflecting a degree of animal chest development was higher in IND and ILB than in ERV, but significant differences were found only between ILB and ERV ($p < 0.05$). It should be noted that this index in

ERV was lower than that of NEN'70. The stretch index of IND and ILB also exceeded that of ERV (at $p < 0.01$ and $p < 0.05$, respectively). The head length indexes of studied populations differed not significantly.

1. Comparative estimates of exterior and body weight of reindeer (*Rangifer tarandus*) Nenets does aged 3.5-8.5 years (Nenets Autonomous Okrug, 2016)

Population	*n*	*M*±SEM	*Cv*, %	min-max	Range of variability
Height at withers, cm					
ERV	28	96.97±0.46	2.5	93.0-104.0	11.0
ILB	25	99.72±0.53	2.7	95.0-106.0	11.0
IND	34	99.24±0.42	2.5	95.0-106.0	11.0
Chest depth, cm					
ERV	28	43.66±0.46	2.6	41.7-46.3	4.6
ILB	25	42.64±0.32	3.8	38.0-46.5	8.5
IND	34	43.35±0.31	4.2	39.0-47.0	8.0
Chest width, cm					
ERV	28	26.59±0.27	5.3	24.0-29.6	5.6
ILB	25	27.54±0.45	8.1	22.0-34.0	12.0
IND	34	27.65±0.35	7.4	22.0-31.0	9.0
Chest girth, cm					
ERV	28	118.96±0.77	3.4	110.5-126.0	15.5
ILB	25	126.58±0.80	3.2	120.0-134.0	14.0
IND	34	125.57±0.69	3.2	118.0-134.0	16.0
Body length, cm					
ERV	28	102.21±0.32	1.7	99.0-106.0	7.0
ILB	25	107.74±0.81	3.8	102.0-117.0	15.0
IND	34	108.32±0.54	2.9	102.0-114.0	12.0
Loin width, cm					
ERV	–	–	–	–	–
ILB	25	11.88±0.20	8.4	10.0-15.0	5.0
IND	34	12.07±0.20	10.0	9.0-14.0	5.0
Wrist girth, cm					
ERV	28	11.21±0.05	2.6	11.0-12.0	1.0
ILB	25	11.88±0.11	4.6	11.0-13.0	2.0
IND	34	11.75±0.09	4.2	11.0-13.0	2.0
Head length, cm					
ERV	28	32.12±0.18	2.9	31.0-34.0	3.0
ILB	25	33.86±0.51	7.5	30.0-39.0	9.0
IND	34	33.25±0.38	6.6	28.0-39.0	11.0
Bogy weight, kg					
ERV	28	83.46±1.08	6.8	75.0-107.0	32.0
ILB	25	106.40±1.24	5.8	96.0-121.0	25.0
IND	34	104.62±1.17	6.5	88.0-121.0	33.0

Note. ERV, IND and ILB — populations of farms ERV, Indiga and Ilbets; *n* — sample size, *M* — arithmetic mean, SEM — standard error, *Cv* — coefficient of variability, min-max — min and max values. Dash means data absence.

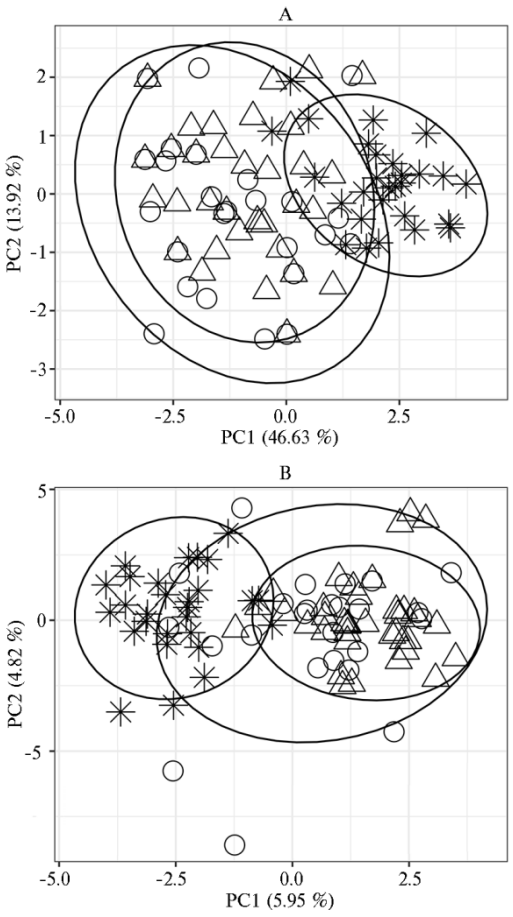
2. A comparison of exterior and body weight in current populations of reindeer (*Rangifer tarandus*) Nenets does aged 3.5-8.5 years and those of 1970th (Nenets Autonomous Okrug, 2016)

Body index	ERV (*M*±SEM)	ILB (*M*±SEM)	IND (*M*±SEM)	NEN'70 (*M*)	Breed standard [4], %
Massiveness	122.74±0.91	126.97±0.73	126.57±0.67	123.0	118.7-123.8
Blockness	116.40±0.73	117.59±0.88	115.99±0.67	117.7	–
Boneness	11.57±0.06	11.91±0.10	11.84±0.08	10.8	10.8-12.1
Chest index	60.96±0.77	64.67±1.12	63.86±0.88	62.8	59.4-65.5
Lengthiness	105.46±0.50	108.08±0.83	109.20±0.60	103.9	–
Head length	33.14±0.21	33.98±0.53	33.51±0.38	34.4	–

Note. ERV, IND and ILB — current populations of farms ERV, Indiga and Ilbets; NEN'70 — average data for NAO in the 1970th [4]. *M* — arithmetic mean, SEM — standard error. Dash means data absence

Based on the comparative analysis of morphological variability of the does from three farms (Fig. 1, A), it was established that the IND and ILB animals were in the same cluster, that is, had similar exteriors. The most of ERV does were clustered separately of the above-mentioned populations, indicating a different morphological type. Genetic analysis by microsatellites (see Fig. 1, B) showed similar results. The IND and ILB animals formed a common array, while the ERV does formed a relatively isolated cluster. PC1 and PC2 were re-

sponsible for 5.95 % and 4.82 % of the genotypic variability, respectively. In general, genetic analysis revealed a greater difference of ERV from other groups than morphological analysis.



Principal component analyses (PCA) **of morphological** (A) **and genetic** (B) parameters of **raindeer (*Rangifer tarandus*) Nenets does aged 3.5-8.5 years:** △ — population of Indiga farm (IND), ○ **— population of Ilbets farm** (ILB), ✳ **— population of ERV farm** (ERV). Circles mark 95 % confidence interval of clussterisation (Nenets Autonomous Okrug, 2016)

Calculation of pairwise F_{st} values (Table 3) showed the lowest genetic distance of 0.018 ($p > 0.05$) between IND and ILB. The difference between ERV and the other two populations was more significant, 0.101 for IND and 0.056 for ILB. When comparing farm populations to wild animals, ERV (0.041) and ILB (0.042) populations were the closest to the latter. IND was characterized by a greater remoteness from the wild form (0.078).

The F_{st} values are widely used and applicable for comparing findings reported by other researchers. However, recent studies have shown a limited use of this indicator to assess the degree of differentiation between populations with high intrapopulation heterozygosity [23], including reindeer populations [24]. Calculation of D_{Jost} pairwise values is the most suitable method for such purposes [21].

For domestic populations, the D_{Jost} values were similar to the pairwise F_{st} values, but for the wild population D_{Jost} was higher. While F_{st} indicated that genetically IND and ILB are closer to the wild population than to ERV, the D_{Jost} showed that the distance between ERV and other domestic populations is less than that of wild animals.

The indices of the observed heterozygosity of three populations did not differ significantly from each other ($p < 0.05$) and were in the range from 0.551±0.046 for ILB to 0.567±0.043 for

3. Indicators of pairwise genetic distances F_{st} and D_{Jos} for domestic reindeer (*Rangifer tarandus*) Nenets does (Nenets Autonomous Okrug, 2016)

Population	ERV	ILB	IND	WLD
ERV	0,000	0,054	0,107	0,060
ILB	0,056	0,000	0,017	0,116
IND	0,101	0,018	0,000	0,187
WLD	0,041	0,042	0,078	0,000

N o t e. ERV, IND and ILB — current populations of farms ERV, Indiga and Ilbets, WLD — wild population. F_{st} is given in the low table part. D_{Jost} is in the upper part.

IND (Table 4). Also, significant differences for the expected heterozygosity were not observed. The inbreeding rate (F_{is}) in all studied groups was positive ($p < 0.05$), which indicates a lack of heterozygotes in the populations. Perhaps

this is the result of target selection and limited exchange of genetic material with other herds. The indices of allelic diversity varied from 6.173±0.499 for IND to 6.788±0.494 for ILB and did not differ significantly. Compared to the wild population, domestic reindeer were characterized by lower heterozygosity and allelic diversity.

4. Indicators of genetic diversity of reindeer (*Rangifer tarandus*) Nenets does breed (Nenets Autonomous Okrug, 2016)

Population	*n*	H_o (*M*±SEM)	H_e (*M*±SEM)	F_{is} (95 % CI)	A_r (*M*±SEM)
ERV	28	0.563±0.058	0.706±0.047	0.193 (0.061-0.323)	6.430±0.650
ILB	25	0.551±0.046	0.742±0.027	0.257 (0.163-0.358)	6.778±0.494
IND	34	0.567±0.043	0.718±0.030	0.201 (0.088-0.322)	6.173±0.499
WLD	32	0.622±0.056	0.796±0.023	0.223 (0.102-0.330)	8.280±0.666

N o t e. ERV, IND and ILB — current populations of farms ERV, Indiga and Ilbets; *n* — sample size, H_o — observed heterozygosity, H_e — expected heterozygosity, F_{is} — coefficient of inbreeding, A_r — allelic diversity; *M* — arithmetic mean, SEM — standard error, CI — confidence interval.

Thus, reindeer does, bred in the Nenets Autonomous Okrug, had morphological and genetic differences. Animals from Indiga farm (IND) and Ilebts farm (ILB) for number of basic body parameters (height at withers, chest width, chest girth, wrist girth, body length, head length) and body weight exceeded the does of ERV farm (ERV). The coefficient of variation for all studied parameters were within 10 %, which shows a high degree of population consolidation, and by some indicators (chest depth, chest width, body length, wrist girth and head length) the ERV reindeer does were the most homogeneous. Exterior measurements in all populations either were within the breed standard, or exceeded standards (for example, for body length, chest girth in the IND and ILB populations). On boniness and extension, there was an increase in indices compared to those of 1970th (NEN'70). There was an increase in the massiveness in ILB and IND compared tp the breed standard, while in ERV the values were within the norm and did not differ from NEN'70. Microsatellite analysis revealed that the IND and ILB animals formed a common massif, while ERV animals clustered as an isolated group. It is shown that genetically different groups were characterized by different phenotypic features. Our findings are indicative of the need for a complex morphological and genetic analysis of reindeer populations. The in-depth information about the reindeer population structure will allow development of a strategy for management and further monitoring of the species.

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REPRODUCTIVE FUNCTION IN PUREBRED ARABIAN STALLIONS AS RELATED TO THE LEVELS OF CHEMICAL ELEMENTS IN MANE HAIR SAMPLES

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Abstract

In modern animal husbandry, along with the improvement of reproductive technologies, it is becoming increasingly important to restore the natural fertility of livestock, in particular breeding producers. Reproductive function is one of the most sensitive that reacts to changes in biogeochemical and environmental parameters. In this regard, research aimed at studying effects of the level of chemical elements in the body on reproductive function in stallions is rather important. The parameters of sperm quality depending on the level of chemical elements in hair sampled from mane were studied. In this work we used biomaterial of purebred Arabian stallions (n = 50) of the same biogeochemical province. Reproductive qualities of stallions were evaluated in relation to the pool of chemical elements rated by concentration in the mane hair. All animals were grouped according to the levels of individual elements in hair (i.e. up to percentile 25, within the interval of percentiles 25-75 and above percentile 75). Also the quality of fresh and cryopreserved semen was estimated. Hair profiles was determined for 25 elements (Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, I, K, Li, Mg, Mn, Na, Ni, P, Pb, Se, Si, Sn, Hg, Sr, V, Zn) by inductively coupled plasma atomic emission and mass spectrometry (ICP-AES and ICP-MS). Ejaculate was assessed for volume, concentration, total spermatozoa number, number of spermatozoa with progressive motility and semen viability. The validity of the differences was verified by Mann-Whitney U test. It has been found out that the mane hair analysis can be used to detect reduced fertility in stallions. Increase in average values of Sr to 4.19±0.12, Se to 0,559±0.015, B to 21.55±1.14 μg/g in hair results in decrease in number and activity of sperm and filtrate volume. Activity and survival of stallion spermatozoa after thawing was in inverse correlation with Pb and Sn pool in the body, as determined by element level in the mane hair. With the increase in the concentration of these elements to 0,806±0206 and 0,051±0.008 mg/g, the sperm activity declines after thawing by 3.8-7.7 % and survival by 26.4-29.5 %, respectively. Animals with copper, silicon and vanadium in hair less than that of percentile 25, had the most active sperm. The survival rate of fresh sperm was associated with V concentration in hair being the highest in animals with low levels of this mineral. Thus, the assay of mane hair mineral profile can be used in monitoring for timely correction of stallions' fertility.

Keywords: purebred horses, Arabian breed, stallion, profiles of mineral elements in the horse hair, reproductive function, sperm quality

At the present time, both new reproductive technologies and methods to restore the natural fertility of livestock, including horses, are relevant. In addition to the endogenous factors (parental diseases, infections, hereditary diseases), which can cause reproductive health disorders, the conditions of biogeochemical province and environmental parameters [1] hold a significant position, as well as mineral provision of animals. Chemical elements are important ecophysiological agents which determine the morphofunctional characteristics of the reproductive system [2-4].

Elements which are directly affecting the qualitative characteristics of semen can be divided into several groups: essential macroelements (Na, K, Ca, Mg, etc.), trace elements needed for normal sperm functioning (Zn, Cu, Mn, Co, Se) [5], and toxic elements (Pb, Cd, Hg, etc.) which are extremely harmful and dangerous even in small amounts [6-8].

Horse semen evaluation before the breeding period does not allow us quickly and efficiently to level individual deviations in the contents of chemical elements through the optimization of animal feed. Because of the relative labor intensity and injurious risk of sperm taking, an early assessment of its elemental status using some other biomaterial is preferable. Examination of the elemental hair composition is quite widespread in medicine [9]. This noninvasive method makes possible to assess animal health status, to exclude pathologies caused by heavy metals intoxication, and to detect disorders in animal nutrition [10].

This paper is the first report on the connection between some chemical element contents in the stallion's mane hair and sperm quality and cryoresistance. We found an inverse relationship between the strontium, selenium, boron accumulation and ejaculate volume, the quantity and motility of spermatozoa, and also between lead and tin content and the motility and viability of spermatozoa after thawing. Animals with a low copper, silicon and vanadium hair content had the highest sperm motility.

Our aim was to study reproductive function of purebred stallions depending on their mineral status, as estimated by mane hair elemental analysis.

Techniques. The research was carried out on 4-5 year old purebred Arabian stallions with body weight of 410.5±25.3 kg (Terskys Stud farm No.169, Stavropol'skiy Krai, 2016-2017). Animal keeping and experiments were in accordance with the instructions and recommendations of the USSR Ministry of Health Order No. 701 of July 27, 1978 “On the introduction of amendments to the Order of the Ministry of Health USSR of 12.08.77 No. 755” and “The Guide for Care and Use of Laboratory Animals” (National Academy Press, Washington, D.C. 1996). All efforts had been made to minimize animal suffering and to reduce the number of samples in the study. The selected experimental group contained clinically healthy stallions (n = 50). Mane hair, fresh and cryopreserved sperm served as a biomaterial. The samples of cryopreserved sperm were obtained from the Biosphere Collection “Cryobank of genetic resources” (All-Russian Research Institute for Horse Breeding).

To estimate reproductive qualities depending on the pool of chemical elements, the content of which was determined in mane hair, the stallions were grouped by the concentration of elements in the hair: to the 25th percentile, within the 25th to 75th percentiles, higher than the 75th percentile. Percentile intervals were based on previous reports on human biosubstrates, where the physiological norm in the population was identified as the 25th to 75th percentiles [11. Daily food ration of the test animals during 2 months prior to the biomaterial collection contained 75.3 g of Ca, 52.8 g of P, 14.3 g of Mg, 1211.8 mg of Fe, 128.1 g of Cu, 7.2 mg of Co, 604 mg of Mn, and 7.4 mg of I.

The elemental composition was analyzed by inductively coupled plasma

atomic emission and mass spectrometry (ICP-AES and ICP-MS). A microwave digestion system, model SW4 Speedwave (Berghof, Germany) was used for the digestion of samples. In the obtained ash, 25 elements (Al, As, B, Ca, Cd, Co, Cr, Cu, Fe, I, K, Li, Mg, Mn, Na, Ni, P, Pb, Se, Si, Sn, Hg, Sr, V, Zn) were determined using an Elan 9000 mass spectrometer (Perkin Elmer, USA) and an Optima 2000V atomic emission spectrometer (Perkin Elmer, USA).

Semen was collected using an artificial vagina in response to a mare in heat (All-Russian Research Institute of Horse Breeding, ARRIHB) (not less than 5 ejaculates from each animal) every 48 h. The sperm indicators of the first two ejaculates after the period of sexual dormancy were not used in the processing. Each ejaculate was initially evaluated by the following indicators: volume, concentration, total number of spermatozoa (TNS), total number of progressively motile sperm cells (TNS PM), and sperm survival at 2-4 °C. The samples were diluted with a lactose-chelate-citrate-yolk (LCCY) medium in a volume ratio of 1:3. The sperm were frozen in liquid nitrogen vapors using the ARRIHB technology. Frozen sperm were stored in liquid nitrogen at 196 °C. The thawing was carried out in water bath at 40 °C. After thawing cryopreserved sperm, the viability of spermatozoa at 2-4 °C and progressive motility were determined.

The Shapiro-Wilk test was used to verify the hypothesis of normal distribution of other quantitative traits. Hence distribution of the investigated numeric indicators differed from the normal one, the validity of differences was checked by the Mann-Whitney U test. In all procedures for statistical analysis, significance level (P) was calculated, the critical significance level taken was less than or equal to 0.05. The tables show the average values of the indicators (*M*) and their standard errors (±SEM). For data processing, the Statistica 10.0 software was used (StatSoft, Inc., USA).

Resultes. A comparative evaluation of the stallion mane hair composition identified significant differences in an amount of certain chemical elements in animals of different groups (Table 1).

1. Chemical element contents (mg/g) **in the mane hair of purebred Arabian stallions depending on the percentile range** (*M*±SEM, Terskii Stud farm No. 169, Stavropol'skiy Krai, 2016-2017)

Element	Percentile interval		
	< 25	25-75	> 75
	Macroelements		
Ca	500.100±21.2400	839.000±44.9100***	1223.000±61.0800***
K	275.100±16.0000	527.400±28.6000***	1248.000±167.8000***
Mg	160.300±21.1200	372.300±22.2800***	632.500±27.3900***
Na	94.560±4.2200	149.500±4.8800***	286.100±38.5500***
P	249.200±55.3540	496.200±19.6000***	765.300±29.7230***
	Essential microelements		
Co	0.010±0.0007	0.020±0.0010***	0.079±0.0130***
Cr	0.043±0.0040	0.130±0.0140***	0.514±0.1510**
Cu	4.760±0.1100	5.670±0.1250***	7.800±0.5800***
Fe	25.630±1.8100	48.760±3.2900***	168.200±24.7500***
I	0.018±0.0040	0.062±0.0060***	0.232±0.0570**
Mn	0.555±0.0370	1.030±0.0630***	3.830±0.5350***
Se	0.278±0.0130	0.395±0.0130***	0.559±0.0150***
Zn	126.300±3.0800	146.900±2.4900***	181.900±4.3200***
	Conditionally essential microelements		
B	1.540±0.1369	7.050±1.1200**	21.550±1.1400***
Li	0.049±0.0060	0.118±0.0065***	0.249±0.0270***
Ni	0,082±0,0030	0,123±0,0090***	0,441±0,0630***
Si	2,900±0,3110	5,700±0,2850***	8,780±0,6660***
Sr	1,0600±0,1002	2,500±0,1780***	4,190±0,1200***
V	0,028±0,0030	0,072±0,0070***	0,239±0,0270***
	Toxic elements		
Al	7.980±0.7580	24,150±2,2700***	79,800±13,4000**

Continued Table 1

As	0.016±0.0007	0,024±0,0008***	0,058±0,0100***
Cd	0.002±0.0002	0,004±0,0003**	0,010±0,0010***
Pb	0.027±0.0010	0,061±0,0070	0,806±0,2060*
Sn	0.003±0.0001	0,009±0,0010**	0,051±0,0080***

*, **, *** P < 0.05; P < 0.01; P < 0.001, respectively, compared to the group of a percentile interval < 25.

Filtering ejaculates of animals having different hair chemical composition revealed that the ejaculate volume dependes on the amount of zinc, boron, phosphorus, lead, selenium and strontium (Table 2).

2. Ejaculate volume after filtration (ml) **in the groups of purebred Arabian stallions depending on the percentile range of chemical element concentration in mane hair** (*M*±SEM, Terskii Stud farm No. 169, Stavropol'skii Krai, 2016-2017)

Element	Percentile interval		
	< 25	25-75	> 75
As	30.51±6.730	36.84±21.260	43.25±23.240
B	51.40±17.690	35.62±20.250	25.90±9.760**
Cu	35.99±11.830	38.30±7.990	35.57±12.700
Fe	38.72±9.910	34.13±8.060	41.77±13.380
Li	45.56±12.820	37.34±13.810	28.06±11.830
Na	46.00±8.610	30.61±3.010*	42.02±9.950
P	61.97±6.950	25.84±1.970***	36.29±5.880**
Pb	25.77±3.790	32.06±4.070	55.57±6.040*
Se	53.69±6.520	35.33±5.100*	24.24±1.930***
Si	28.19±5.000	40.68±10.600	38.27±13.600
Sn	40.67±12.770	35.24±6.650	37.17±18.070
Sr	58.71±6.120	33.77±4.350**	22.54±1.290***
V	38.73±9.910	32.75±8.370	44.71±11.300
Zn	28.74±3.560	32.76±4.230	54.69±8.880*

*, **, *** P < 0.05; P < 0.01; P < 0.001, respectively, compared to the group of a percentile interval < 25.

The mane hair mineral content reflects exposure of elements from the diet [12] and is linked, as a whole, to the value of their exchange pool in the body [13]. Close linkage between the elemental state of stallion and their reproductive system functioning is identified [14, 15], which in turn, is determines by the effect of elements on mammals' spermatogenesis and sperm quality [16-18].

We found a 66.9-90.3 % increase in the total filtrate volume as the zinc content in mane hair grew, which complied with the earlier findings [19] and can be explained by important role of zinc in the normal functioning of the prostate and the reproductive system as a whole [20, 21]. The physiological importance of zinc, which contains in the prostate secretion, is to implement mechanism of the sperm head and tail separation, as well as the chromatin decondensation. Zinc in seminal plasma stabilizes the cell membrane and nuclear chromatin of spermatozoa [22, 23]. This element also plays an important regulatory role in condensation and acrosome reaction [24)]. However, it is difficult to explain the significant (almost twice) decreasing in ejaculate volume related to elevation of boron level. Negative effects of growing dietary boron on the volume and sperm composition were not detected earlier [25].

An ejaculate volume decreased as the amount of hair strontium, a conditionally essential element, increased. An increase in Sr content from the minimum to the maximum within the 25-75 and > 75 percentile intervals resulted in a decrease in the ejaculate volume by 73.9 % (P < 0.01) and 49.2 % (P < 0.001), respectively. A similar trend was noted for the effect of strontium on the TNS index, which, on the whole, indicates a negative effect of this element on the stallion reproductive ability. However, it is difficult to interpret the obtained data without information about the physiological norm of strontium as a component of the elemental status. Perhaps the population we have formed is characterized by an increased pool of this element. Meanwhile, strontium is capable to demonstrate unique properties for the activation of oocytes, followed by stimula-

tion of their development to the 8-cell or blastocyst stages [26-28]. This can be used to increase the fertilizing capacity of spermatozoa and, as a result, effective reducing amount of single dose.

On the contrary, the filtrate volume increased by 115.6-73.3 % ($P < 0.05$) as hair lead level rising. In whole, a high exchange lead pool is linked to the infertility caused by the induction of a spontaneous premature acrosome reaction, and an increase of this element concentration in the seminal plasma can adversely affect the fertility potential of sperm in vitro [29].

It must be noted that the physiological standards is applicable only to essential and macroelements, but not to toxic elements. The physiological standard lies within the 25th-75th percentiles, and the values above and below this percentile interval for vital elements can be considered as abnormal. Despite the fact that the minimum ejaculate volume (25.84 ml) was recorded in the group with the phosphorus content corresponding to the physiological norm (25th-75th percentiles), the sperm concentration in fresh semen here was maximum (268.2 million/ml) .

3. Quality indicators of diluted ejaculates in the groups of purebred Arabian stallions depending on the percentile range of chemical element concentration in mane hair (*M*±SEM, Terskii Stud farm No. 169, Stavropol'skii Krai, 2016-2017)

Element	Percentile interval					
	< 25		25-75		> 75	
	A	B	A	B	A	B
As	50,57±8,630	128,2±38,14	47,97±7,930	121,8±28,67	47,05±6,290	132,7±16,49
B	47,34±6,250	129,4±14,63	48,71±9,450	127,9±32,52	48,57±4,030	120,1±30,24
Cu	53,38±2,410	131,9±19,47	46,80±2,160	128,8±29,51	46,63±1,740*	115,7±32,62
Fe	52,14±6,120	145,4±6,56	47,78±8,690	115,1±18,33	45,70±5,130	131,5±4,78
Li	50,71±10,530	130,2±25,28	47,18±6,420	120,9±33,24	48,48±6,980	134,3±16,61
Na	49,57±9,620	126,8±15,79	48,20±7,690	124,4±13,85	47,42±5,680	130,0±17,20
P	46,57±5,560	125,1±13,60	49,38±9,140	127,9±22,52	47,91±5,740	124,2±21,50
Pb	50,33±7,850	135,3±14,04	49,95±1,460	126,1±11,35	43,14±2,400	123,4±16,56
Se	45,28±7,400	128,5±20,45	49,69±8,580	125,9±30,29	48,52±4,850	125,1±33,04
Si	54,95±2,950	124,9±35,05	47,89±1,570*	131,5±28,99	42,71±2,060**	116,9±17,23
Sn	48,71±8,130	131,9±13,90	49,00±5,810	130,5±16,70	46,87±10,230	114,3±23,03
Sr	48,00±5,650	133,7±7,56	48,22±8,300	120,5±13,44	48,95±8,500	131,7±13,65
V	52,14±2,310	145,4±6,56	48,38±2,070	118,5±8,45	44,49±2,490*	124,3±6,24*
Zn	51,95±7,340	133,8±15,39	48,16±6,570	125,9±16,22	45,14±5,550	120,0±16,00

N o t e. A — motility, %; B — viability, h.
*, ** $P < 0.05$; $P < 0.01$, respectively, compared to the group of a percentile interval < 25.

Animals with a low content of copper, silicon and vanadium in the hair had the most active spermatozoa (Table 3). Exceeding, when compared to the stallions with large (> 75th percentile) and mean (25th-75th percentiles) quantities of these elements, was 6.8 ($P < 0.05$) and 6.6 % for copper, by 12.3 ($P < 0.01$) and 7.1 % ($P < 0.05$) for silicon, and by 7.3 ($P < 0.05$) and 3.7 % for vanadium, respectively. Several studies have found an inverse relationship between high copper content and sperm quality [30, 31]. The copper pool is also associated closely with the activity of copper-containing enzymes [32].

The viability of diluted semen, when stored at 2-4 °C, depended on the vanadium concertation and was the highest in animals with a low (<25th percentile) level of this element, that is, by 17.0 % ($P < 0.05$) and 22.7 % higher compared to stallions with high and medium hair vanadium level, respectively.

Superficially, unordinary changes in the reproductive ability of stallions depending on the value of the selenium pool were identified. In particular, as the amount of selenium in the hair increased from 0.278±0.013 to 0.395±0.013 and 0.559 0.015 μg/g, the concentration of spermatozoa in the ejaculate decreased by 39.2 % ($P < 0.05$) and 18.1 % ($P < 0.05$). There was a reduction in the sperm motility. It is appreciable that selenium deficiency has a very negative effect on animal fertility [33]. According to it, while increasing dietary Se, an

improvement in stallion sperm quality should be expected. However, the information about the limits of the element content in the mane hair is absent. Perhaps, examined boundaries exceeded the physiological rate. At the same time, there are data which shows the ambiguous effect of selenium additives on reproductive qualities [34, 35]. In some studies, selenium supplementation did not change semen amount and quality [36]. We observed a reduction in the amount of active sperm in the ejaculate with the increasing of hair selenium content. A similar result was obtained for boron, lithium, and strontium (Table 4).

4. Amount of active spermatozoa in the ejaculate (billions) **of the purebred Arabian stallions depending on the percentile range of chemical element concentration in mane hair** (*M*±SEM, Terskii Stud farm No. 169, Stavropol'skii Krai, 2016-2017)

Element	Percentile interval					
	< 25		25-75		> 75	
	TNS	TNS PM	TNS	TNS PM	TNS	TNS PM
As	6.83±2.540	3.60±1.570	7.88±3.550	3.82±1.960	9.14±2.350*	4.26±1.030
B	9.75±2.750	4.66±1.720	8.08±3.210	3.98±1.660	5.97±1.890*	2.93±1.070*
Cu	8.13±2.940	4.36±2.170	8.13±1.810	3.84±1.490	7.48±2.040	3.50±1.410
Fe	8.72±1.970	4.51±0.890	7.02±3.040	3.41±1.620	9.28±2.640	4.30±1.040
Li	9.58±0.240	4.72±0.630	7.41±3.770	3.59±0.520	7.58±1.760	3.69±0.400*
Na	9.81±2.260	4.76±1.080	7.19±2.210	3.58±1.870	7.81±2.990	3.67±1.350
P	9.53±0.920	4.36±0.900	6.78±0.710*	3.44±1.620	8.98±3.570	4.36±2.090
Pb	5.98±2.110	3.33±2.340	7.31±1.620	3.68±1.460	10.63±1.910	4.68±1.750
Se	10.5±0.970	4.78±0.630	7.54±0.830*	3.83±0.450	6.38±0.500**	3.11±0.310*
Si	6.81±1.360	3.78±1.040	8.82±2.410	4.27±1.860	7.33±2.290	3.17±1.470
Sn	7.21±2.650	3.60±1.840	8.25±1.930	4.09±1.740	8.16±2.030	3.77±1.370
Sr	10.79±0.870	5.24±0.590	7.75±0.690*	3.71±0.350*	5.64±0.900**	2.91±0.580*
V	8.72±1.970	4.51±0.900	6.74±1.930	3.34±1.640	9.88±2.300	4.43±1.930
Zn	7.45±1.780	3.83±1.030	7.74±1.520	3.83±1.990	9.01±2.120	4.07±1.410

N o t e. TNS — total number of spermatozoa, TNS PM — total number of progressively motile sperm cells.
*, ** $P < 0.05$; $P < 0.01$, respectively, compared to the group of a percentile interval < 25.

Thorough analysis of cryopreserved sperm quality associated with concertation of chemical elements in mane revealed the dependence of viability and sperm motility only on the lead and tin content. In particular, with a maximum increase in the amount of lead in the hair, there was a decrease in the semen motility after thawing from 27.66 to 20.00 % ($P < 0.05$) (Table 5). A similar decrease in the semen viability after thawing from 78.4 to 48.9 h ($P < 0.05$) with a high tin content was noted when compared to the > 75 and < 25 percentile intervals.

5. Quality of thawed semen of the purebred Arabian stallions depending on the percentile range on the content of chemical elements in the mane hair (*M*±SEM, Terskii Stud farm No. 169, Stavropol'skii Krai, 2016-2017)

Element	Percentile interval					
	< 25		25-75		> 75	
	A	B	A	B	A	B
As	23.57±5.160	67,1±13,00	23,07±5,630	64,6±14,55	23,37±2,720	77,8±17,05
B	22.85±3.020	69,6±14,20	24,09±5,730	71,1±55,99	21,95±7,720	62,9±17,61
Cu	26.91±4.550	78,6±17,69	22,74±5,720	67,8±32,84	20,78±5,400	61,1±14,00
Fe	26.00±5.250	76,5±14,66	22,06±5,290	63,3±16,63	23,17±2,570	72,8±16,35
Li	26.42±5.390	70,3±17,21	22,27±6,750	68,4±15,6	22,28±3,450	68,1±11,66
Na	26.50±6.530	73,3±17,62	21,97±8,580	68,7±11,47	22,85±3,800	64,5±11,95
P	23.92±2.970	67,7±15,67	24,37±3,710	70,7±15,75	20,28±4,150	65,8±10,35
Pb	27.66±3.180	67,5±25,23	23,79±2,040	72,8±33,28	20,00±1,780*	58,3±21,27
Se	21.00±5.710	68,1±15,14	25,16±4,570	71,7±11,41	21,51±5,440	63,1±14,30
Si	26.41±5.980	70,5±12,63	23,20±7,290	74,0±12,04	20,31±5,240	55,9±19,09
Sn	26.14±4.220	78,4±7,18	23,42±6,810	75,3±8,56	20,52±11,560	48,9±9,55*
Sr	23.57±2.570	75,4±14,24	22,51±5,700	64,4±16,05	24,62±4,040	71,5±15,65
V	26.00±5.250	76,1±14,66	23,07±6,760	66,2±14,98	21,00±5,410	66,6±14,79
Zn	26.8±5.630	74,5±18,15	22,76±6,320	68,8±20,68	20,85±6,260	63,0±23,24

N o t e. A — motility, %; B — viability, h.
* $P < 0.05$ compared to the group of a percentile interval < 25.

To date, extensive experimental data of toxic element effects on sperm quality and fertility has been accumulated. The action of toxic elements is multiple and manifests itself in reducing sperm concentration [37] and suppressing sperm motility [38]. Significant negative correlations have been established between lead concentration in sperm, motility ($r = -0.65$, $P < 0.001$) and sperm viability ($r = -0.62$, $P < 0.001$) [39].

Thus, the mane hair elemental analysis can be used for the reduced stallion fertility identification. Increasing content of strontium up to 4.1±0.12, selenium up to 0.559±0.015, boron up to 21.55±1.14 μg/g in the hair is connected with a decrease in ejaculate volume, quantity and spermatozoa motility. The motility and viability of stallion spermatozoa after freezing are inversely related to the lead and tin levels in the body. With an increasing the tin content in the hair up to 0.806±0.206 μg/g, the progressive spermatozoa motility after thawing decreased from 27.66±3.18 to 20.00±1.78 %, or by 7.7 % on average). With an increasing the lead concetration in the hair up to 0.051±0.008 μg/g, the viability of spermatozoa after thawing reduced by 29.5 h, or by 37.6 %. The highest spermatozoa motility was characteristic of animals with low (less than 25th percentile) copper, silicon and vanadium levels in hair. The viability of fresh semen correlated with the vanadium content in the hair and was the largest in animals with a low content of this element. Further work involves the determination of reference and percentile ranges for the content of chemical elements in mane hair in connection with the pool of these elements in the body.

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EFFICIENCY OF BUSULFAN USE FOR ELIMINATING PRIMORDIAL GERM CELLS IN THE GONADS OF CHICKEN EMBRYOS

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Abstract

The use of primordial germ cells to produce genetically modified and chimeric poultry is one of the promising areas of biotechnology. It is also considered as an alternative to traditional methods of selection and genome modification. The production of transgenic and chimeric animals using this technology provides the introduction of donor primordial germ cells into the dorsal aorta of recipient embryos during the migration of this cell type from the blood to the gonads. In the case of the colonization by donor germ cells of the recipient embryos gonads, further differentiation of donor cells to mature germ cells (both male and female) is possible. One of the main factors that determine the effectiveness of conducted manipulations is eliminating own primordial germ cells in a recipient embryos. In this regard, it remains urgent to develop effective methodical techniques for removing this type of cells. The aim of our study was to optimize methodological approaches for eliminating primordial germ cells in the gonads of chicken embryos. Busulfan was used as an alkylating agent in concentrations from 10 to 250 μg/embryo. Dimethyl sulfoxide (DMSO) was used to dilute busulfan, as well as its combination with DMEM medium in various ratios. The busulfan solution was injected into the chicken embryos prior to incubation or after 24 hour incubation. The efficiency of elimination of primordial germ cells in embryonic gonads was assessed on day 7 of incubation based on histological studies using immunohistochemistry on the expression of the SSEA-1 gene (stage-specific embryonic antigen-1). It has been established that the method of preparation and introduction of busulfan into embryos, as well as the dose of this drug determine the effectiveness of elimination primordial germ cells. Reduction of the negative effect on the development of chicken embryos when using DMSO for the dilution of busulfan was shown when the DMEM medium was included in the solution formulation at a concentration of 10 %. The high efficiency of eliminating primordial germ cells was observed when busulfan was used at a concentration of 100 μg per embryo. In this case, the number of germ cells in the gonads declined by 92 % compared to the control. The use of busulfan at a higher concentration while maintaining the effectiveness of this drug was characterized by an increase in embryonic mortality. At the same time, higher effectiveness of eliminating primordial germ cells was achieved with the introduction of busulfan after 24-hour incubation of embryos: the number of germ cells in the gonads of 7-day-old chicken embryos was 12.5 % less than in the case of injection of this drug before incubation.

Keywords: primordial germ cells, chicken, embryos, busulfan

The use of donor primordial germ cells to produce transgenic [1-3] and chimeric [4-6] poultry is an alternative to traditional selection and genome modification. Due to the peculiarities of poultry reproduction and embryology, this technology opens up new opportunities for directed genome modification [7-9] and the reconstruction of valuable breeds and lines using cryopresereved material [10-12].

Primary embryonic cells (PES) are the precursors of the gamete cells. In embryogenesis, they differentiate into male and female germ cells. This greatly

expands the possibilities of realizing their potential while creating transgenic and chimeric individuals with prescribed properties. Primordial germ cells of chicken embryos are formed in the epiblast and migrate through the hypoblast into the gonads via blood [13]. Accordingly, when donor PEC are introduced into the dorsal aorta of recipient embryos during the migration of own PEC from the blood into the gonads, the donor cells may colonize gonads recipients [14, 15].

The transplantation efficiency of donor PEC can be increased through recipients embryos preconditioning focused on the eliminating own primordial germ cells. There are several methods to eliminate endogenous embryonic cells of different vertebrate species, such as γ-irradiation, x-radiation [16-18], chemosterilization [19]. In the latter, busulfan is used. It is an alkylating agent which causes DNA damage in target cells that leads to deactivation of all cellular mechanisms and cell destruction. Tests on laboratory and farm animals showed selective effect of busulfan on the male cells [20-22].

In this research, we studied the efficiency of administering busulfan to remove primordial germ cells in gonads of chicken embryos. This is the first comparative estimates of the primordial germ cell elimination when busulfan solution was prepared and injected to chicken embryos in different ways and at different concentrations.

Within the framework of developing technology to produce transgenic individuals with prescribed properties, this paper suggested optimization of methodology for elimination of primordial germ cells in chicken embryo gonads using busulfan.

Techniques. Pervomaiskaya breed chicken embryos were studied. To eliminate endogenous primordial germ cells, a solution of busulfan in dimethylsulfoxide (DMSO) and nutritional medium DMEM (Dulbecco's modified Eagle's Medium) (Invitrogen, USA) was used. The resulting solutions were sterilized by filtration through filter with a 22 μm pore size. Before busulfan introduction, the eggshells were treated with 70 % alcohole as desinfectant. Further manipulations with embryos were carried out under sterile conditions (in a laminar box). The introduction of 10 μg busulfan per embryo was carried out in two ways: through puncture in the shell at the blunt end of the egg before incubation or by administration of the solution to the embryonic disc through the eggshell hole at the blunt end of an egg after 24 hours of incubation. In the first case the injectate volume was 100 μl, and in the second case it was 50 μl. Eggs were incubated (Rcom Maru 190 Deluxe, Rcom, Korea) at 37.5 °C and 55 % humidity in compliance with the standard for chicken eggs. In determining busulfan dose optimal for the effective removal of primordial germ cells, their presence in gonads was studied when administrated busulfan amounted 40, 70, 100, 150, 200 and 250 μg per embryo.

The busulfan efficiency in embryonic gonads was evaluated histologically. Untreated embryos served as control. The embryos were isolated on day 7 of incubation. Fixation was carried out in Bouin's Fixative Solution (picric acid:acetic acid:formalin, 15:1:5) for 48 hours. Histological specimen were prepared according to a common methods, involving tissue dehydration in increasing concentration alcohols, impregnating in a mixture of xylene paraffin and embedding into paraffin [23]. The preparations were stained with hematoxylin and eosin. Embryo cross-sections in the gonad area were used for the analysis, (the lumbar region, the place of primary kidneys localization). The histological analysis was carried out with a Ni-U microscope (Nikon, Japan), with interpretation and analysis of images using NIS-Elements software (Nikon, Japan). PES on histological sections was identified immunohistochemically with avidin-biotin systems (Vector Laboratories, USA) [21]. Primary antibodies were anti-SSEA-1

(stage-specific embryonic antigen-1). The antigen-antibody complex was identified using horseradish peroxidase detected by 3,3-diaminobenzidine tetrachlorate (DAB) (Vector Laboratories, USA).

Statistical data processing was performed in MS Excel using variation statistics methods. The tables show average values (X) and mean errors ($\pm x$).

Results. Prior to administration of bisulfan, we optimized composition of its diluent. Busulfan is commonly dissolved in dimethylsulfoxide (DMSO) as, when using aqueous solutions, busulfan can partially precipitate. Given DMSO cell toxicity, we studied the effect of the busulfan dessolved in DMSO and DMEM in different ratios on the development of embryos and the efficiency of primordial germ cell elimination (Table 1).

1. Development of Pervomayskaya breed chicken embryos and efficiency of primordial germ cell elimination under different composition of busulfan solution and administration techniques ($X\pm x$)

Composition of injected solution	Embryos treated, *n*	Embryos developed to day 7 of incubation, *n* (%)	Tested gonads, *n*	Gonad diameter, μm,	PEC number in gonads (histological section), *n*
Injection before egg incubation (100 μl per embryo)					
Control (untreated)	15	15 (100)	30	218±10	14±1
Bu + DMSO	20	15 (75)	30	185±11	8±1
Bu + 90 % DMSO + 10 % DMEM	20	16 (80)	32	191±9	8±1
Bu + 70 % DMSO + 30 % DMEM	20	17 (85)	34	190±10	10±1
Bu + 50 % DMSO+ 50 % DMEM	20	17 (85)	34	201±12	10±2
Injection in 24 hours of incubation (50 μl per embryo)					
Control (untreated)	15	14 (93)	28	211±9	12±1
Bu + ДМСО	20	12 (60)	24	172±12	7±1
Bu + 90 % DMSO + 10 % DMEM	20	14 (70)	28	178±8	7±1
Bu + 70 % DMSO + 30 % DMEM	20	15 (75)	30	188±10	9±1
Bu + 50 % DMSO + 50 % DMEM	20	16 (80)	32	190±14	10±1

Note. Bu — bysylfan (10 μg per embryo), PEC— primordial embryonic cells.

Embryonic mortality for DMSO solution of busulfan was 18-23 % higher compared to the control. Introduction of DMEM to resultant solution of busulfan reduced the negative effect of DMSO on embryo development up to 20 %. However, as the DMEM content in the injectable solution increased to 30 % or more, the effectiveness of busulfan decreased. Optimal efficiency of embryonic development and primordial germ cell elimination resulted from the administration of 10 μg busulfan in 90 % DMSO and 10 % DMEM. In this case, the administration of busulfan after 24-hour incubation was more effective than when bisulfane was used prior to incubation. The number of primordial germ cells in the gonads was 12.5 % less than in the embryos treated with busulfan at an earlier incubation time. In further studies, we used a busulfan solution contained 90 % DMSO and 10 % DMEM which were injected after 24-hour incubation of embryos.

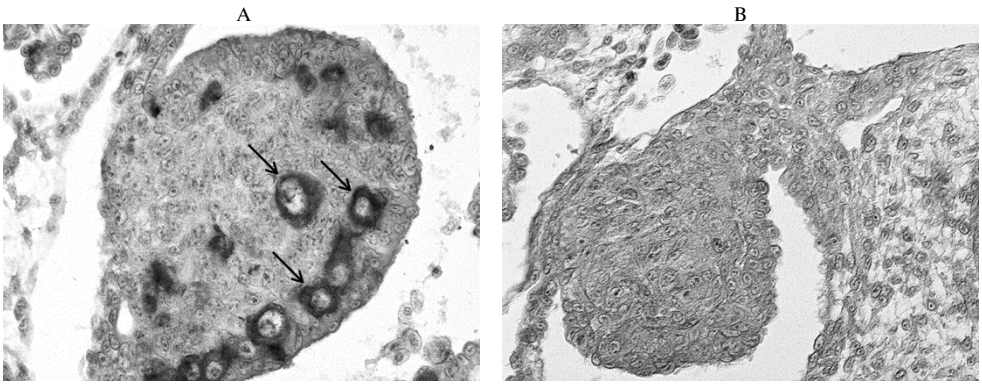
2. Efficiency of primordial germ cells (PEC) **elimination in gonads of Pervomayskaya breed chicken embryos depending on a dose of busulfan** ($X\pm x$)

Parameter	Dose of busulfan, μg per embryo						
	control	40	70	100	150	200	250
Embryos treated, *n*	15	20	20	20	20	20	20
Embryos developed to day 7 of incubation, *n* (%)	14 (93)	10 (50)	8 (40)	5 (25)	2 (10)	1 (5)	0 (0)
Tested gonads, *n*	28	20	16	10	4	2	0
Gonad diameter, μm	211±9	176±6	154±4	111±7	99±3	96±1	
PEC number in gonads (histologic section), *n*							
min	4	1	0	0	0	0	
max	22	13	11	2	1	1	
avg	12±1	6±1	4±1	1±1	1±1	1±1	

Histological study of 7-day-old chick embryos revealed a change in the

size of gonads and the presence of primordial germ cells which depended on the busulfan concentration (Table 2). So, at a dose of 40 μg busulfan, the average gonad diameter was 17 % less than that in the control. With increasing busulfan amount, the diameter was decreasing. The difference with the control reached 27, 47, 53 and 55 % for 70, 100, 150 and 200 μg per embryo, respectively. At 250 μg of busulfan, there was no further development of all 20 embryos treated.

Reducing gonad diameter was due to a significant decrease in the number of primordial germ cells (Fig.). At 40 and 70 μg of busulfan, this index was 2- and 3-fold lower, respectively, compared to the control (see Table 2). At a dose of more than 100 μg per embryo, there were single primordial germ cells in the gonads. It was also reported that busulfan decreased the size of the testicles and testicle tubules in laboratory animals and cocks [24, 25].



Immunohistochemical study of gonads in 7-day-old embryos of Pervomayskaya breed chicken: A — control (untreated), stained primordial cells are indicated by the arrows; B — administration of busulfan at 100 μg per embryo, no primordial germ cells found. Magnification ×400, light microscopy (Nikon, Japan).

Thus, the data obtained indicate the efficacy of busulfan to remove primordial germ cells from gonads of chick embryos. The optimal dose of the agent is 100 μg per embryo. This amount of busulfan led to a 53 % reduce in the diameter of gonads and a 92 % decrease in the number of primordial germ cells. Higher busulfan amount, up to 250 μg, caused abnormalities in embryo development resulted in high embryonic mortality.

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HATCHABILITY IMPROVEMENT IN MULARDS BASED ON SELECTION OF VIABLE PRONUCLEI CARRIERS AT ARTIFICIAL INSEMINATION

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Abstract

Present state and possible solutions are discussed for the problem of hatchability improvement in mulards, interspecies hybrids of Muscovy and domestic ducks. Different techniques of semen collection from Muscovy males were studied: manual massage and temporal placement of female in a cage with a male with subsequent semen collection into an artificial vagina. It was found that the latter technique improves ejaculate volume and sperm cell motility resulting in 5 % improvement of mulard egg fertility. Recommended sperm concentration is ca. 150 million per dose; semen should be introduced into the oviduct to the depth of 4 cm. Thus, effective way of collecting sperm in musk drakes was shown for the first time. Also, we studied an influence of the breed and the line on the hatchability of mulards and the expediency of selecting carriers of genetically compatible pronuclei to increase the output of hybrid young animals when crossing musk and domestic ducks. In the crosses, the parents were musk drakes, cross Jubilee, lines Y1, Y2 and Y3 characterized by good meat qualities, and house ducks of the Bashkir color breed (high egg-laying line BC2) and the Peking breed (high fertile line A4). Trials aimed at the investigation of the effects of genetic breeds and lines on the efficiency of hybridization showed the absence of significant differences in the hatch of mulards between different genetic combinations. However, mulards from Muscovy males (line Y1, cross Yubileynyi) and Pekin females (line A4, cross Agidel 34) had 4.5-7.2 % ($P \leq 0.05$-0.01) higher live bodyweight at slaughter as compared to other studied combinations. Here, a new method for selection of carriers of genetically compatible pronuclei without any violation of their genomic integrity was designed and experimentally tested. The method involves artificial insemination with a mixture of highly active semen of alien species and low-active semen of the native species (2:1). Carriers of livable pronuclei identified by this method improved the hatch of mulards by 20.5 % ($P \leq 0.001$) compared to randomly taken individuals from the breeding flock. Heritability coefficient (from mothers to daughters) of mulard egg hatchability of 27 % evidences that hatchability in mulards can be improved via the selection of compatible families together with the evaluation of parents based on the productive performance in mulards.

Keywords: ducks, Muscovy ducks, Pekin ducks, mulards, selection, hatchability, hatch, livability

As is known, interspecific hybridization is common in developing new plant varieties and animal breeds, however, in poultry farming it is not yet properly widespread due to low hatching of hybrid offspring [1-3]. In the available literature, there is disparate information on hybrids of chicken or waterfowl species but mostly these are a few isolated cases having no economic value [4-6]. Poultry farming knows only one example of use of such hybrids called mulards derived from cross between female common Muscovy duck (*Cairina moshata*) and domestic duck (*Anas platyrhynchos* L.). Mulards are characterized by high viability and excellent taste and nutritive parameters of meat [7-9]. However, this hybrid had not been yet widely spread due to low hatchability of eggs. Mu-

lard hatchability is usually 30-35 % which prevents its wide used in commercial poultry [10-12].

Egg hatchability in poultry depends on genetic specificities and farming methods [13-15], breeding [16-18], housing [19-21] and egg incubation technology [22-24].

The study we are here presenting is the first to determine optimized technique of semen collection from Muscovy males, to assess effects of breeds and lines on hatchability of mulards. Also, we estimated practical effect of selecting parents with genetically compatible pronuclei to increase the output of hybrid birds when crossing musk and domestic ducks.

Our purpose was to improve interspecies hybridization of musk and domestic ducks by providing better sperm quality and selection of parental lines and carriers of genetically compatible pronuclei to be involved in crossings.

Techniques. Experiments on interspecies hybridization were carried out on musk ducks (*Cairina moshata*) and domestic ducks (*Anas platyrhynchos* L.) (Russian Research and Technological Institute of Poultry Farming, Moscow Province; Blagovarskii Poultry Breeding Farm, Republic of Bashkortostan). Muscovy males (cross Jubilee, lines Y1, Y2, and Y3), domestic ducks of the Bashkir color breed (BC2 as maternal line of cross BC12), and Pekin females (A4 as maternal line of cross Agidel 34) were involved in crossing. The duck crosses mentioned have been created in Russia and are characterized by high reproductive and productive properties [8, 25[. Poultry feeding and keeping were in line with the accepted recommendations (Methodological guidelines for breeding of the agricultural poultry. Sergiyev Posad, 2015) and with technological design standards (Methodological Guidelines for Technological Designing of Poultry Farming Enterprises RD-APK 1.10.0504-13. Moscow, 2013).

Common technique of semen collection from Muscovy males (two groups of $n = 30$) applied included 5-fold manual massage of the abdominal part of body or temporal placement of female in a cage with a male with subsequent semen collection into an artificial vagina. Ejaculate volume, counts and activity of sperm cells (motility in points) in ejaculate were determined by commonly accepted methodology. Microscope MBR-1 (Russia) with ×400 zooming was used, and sperm cell activity was assessed by 10-point scale [26]. Pre-developed biotechnical environment which reduces the destabilizing effect of secrets of ovarian tube mucosa on alien sperm was used upon artificial insemination. Dosage per one insemination was nearly 150 million sperm cells; sperm was introduced into the ovarian tube at 4 cm depth.

One hundred eggs from each group were incubated (a Stimul IV-8 incubator, Stimul-Inc Company, Russia). For estimation of true fertilized eggs and the age of embryonic death, all eggs after incubation were opened. Incubation protocol was as generally accepted [27].

Individual carriers of viable pronuclei under interspecific crossing were identified by artificial insemination with highly-active alien semen of 9-10-point motility in mixture with low-active native semen of 4-5-point motility in 2:1 ratio. Carriers of viable pronucleus were those resulted in at least 60 % hatchability of viable chicks.

Selected Pekin female carriers of viable pronuclei were reproduced upon intraspecific mating. The obtained offspring were grown and divided into 6 breeding pens of 4 ducks each, with individually evaluated productivity. Individual caps and standard wing band set were used to control the origin of offspring obtained by incubation.

Body weight, meat quality, meat chemical composition, nutritional value, and heritability of the traits were estimated as per commonly accepted meth-

odologies.

Statistical analysis was performed using software Statistica 10.0 (StatSoft, Inc., USA) and Microsoft Excel tools. Results were presented as mean (*M*) and standard error of mean (±SEM). Student's *t*-test was used to confirm validity of differences between the means. The values were statistically significant at $P \leq 0.05$.

1. Comparative analysis of semen fertility in Muscovy males (*Cairina moshata*) of Jubilee cross line Y1 depending on the technique of semen collection (*M*±SEM)

Parameter	Manual massage	"Decoy duck"
Assessed number of Muscovy males	30	30
Males who secreted semen	18	24
Ejaculate volume, cm^3	0.28±0.03	0.33±0.04
Number of sperm cells, bill/cm^3	2.38±0.07	2.42±0.06
Sperm cell activity, points	9.00±0.06	9.50±0.40
Number of inseminated Pekin ducks	50	50
Eggs incubated	100	100
Egg fertility, %	80.0±3.7	85.0±4.2
Egg hatchability, %	40.0±5.1	42.4±5.8
Mulards hatched, %	32.0±2.7	36.0±3.1

Results. As is clear from the data (Table 1), after 5-fold manual massage only 60 % of males had secreted sperm, while use of "decoy duck" increased their number up to 80 %. At that, obtained ejaculate was characterized by 8.5 % higher volume ($P \leq 0.0001$) and a 5.5 % increase in sperm cell activity ($P \leq 0.001$). The increase of volume and higher activity of sperm cells, apparently, are due to additional amount of reproductive gland secretion in ejaculate. Clearly, this promoted higher fertility of sperm cells introduced into ovarian tube of ducks line A4, which were used in hatchability tests to assess quality of the Muscovy sperm obtained by different methods.

Mulards were produced by artificial insemination without affecting the integrity of the bird's genome. Male parents were Muscovy males (cross Yubileynyi, lines Y1, Y2, and Y3) characterized by good meat properties. Female parents were domestic Bashkir color breed ducks (BC2, the maternal line of cross BC12) with high egg hatchability, and also Pekin ducks (A4, the maternal line of cross Agidel 34) with high fertility. We have compared outcomes of interspecific mating depending on the bird's genotype, i.e. breed and line (Table 2).

2. Incubation of eggs from crossing Muscovy (*Cairina moshata*) males of Jubilee cross line with domestic ducks (*Anas platyrhynchos* L.) of various breeds and lines (*M*±SEM)

Parameter	Line of Muscovy ducks (♂)		
	Y1	Y2	Y3
Bashkir color breed (line BC2) (♀)			
Eggs incubated	100	100	100
Eggs fertilized, %	82,0±3,4	80,0±4,2	81,0±3,4
Egg hatchability, %	43,9±2,6	42,5±3,1	43,2±3,7
Hatchability of hybrid offspring, %	36,0±1,9	34,0±1,8	35,0±2,1
Pekin breed (line A4) (♀)			
Eggs incubated	100	100	100
Eggs fertilized, %	81,0±3,5	82,0±4,1	80,0±3,6
Egg hatchability, %	43,2±4,2	43,9±4,2	42,5±3,8
Hatchability of hybrid offspring, %	35,0±3,6	36,0±3,4	34,0±3,0

As followed from the obtained data, no valid differences were found in incubation outcomes for eggs from various crossing combinations. Hybrid offspring differed only in plumage color and meat body type at slaughter age. Mulards reproduced from combination ♂line Y1 (Muscovy ducks) × ♀line A4 (Pekin duck, cross Agidel 34) were superior to other combinations in body weight at slaughter age by 4.5-7.2 % ($P \leq 0.05$-0.01). Accordingly, it is feasible to use Muscovy males of line Y1 (cross Yubileyniy) and Pekin females of line A4 (cross Agidel 34) for marketable products in commercial breeding of mulards.

Indicidual carriers of viable pronuclei at interspecific crossing were identified based on methodology developed within the scope of the conducted re-

search. That is, high-active alien semen mixed with low-active own semen in specific proportion (2:1) was used for artificial insemination. Finally, 30 Pekin ducks of line A4 (cross Agidel 34) have been selected. Insemination of these birds by sperm of Muscovy males of line Y1 (cross Jubilee) led to mulard hatchability of at least 60 %, and for several laying birds this indicator reached 70-75 %. Estimation of incubation properties of the individuals, which were selected as carriers of viable pronuclei, in offspring (F_1) from interspecific crossings line ♂Y1 (Muscovy ducks) × ♀line A4 (Pekin duck, cross Agidel 34) showed that hatchability of hybrid birds significantly (by 20.5 %) exceeded the control, where randomly selected ducks from the pedigree stock were inseminated. Herewith, no valid differences between the birds groups by fertility and survivability have been observed. Thus, in view of the number of incubated eggs $n = 200$ in each group of selected birds, fertility comprised 80.5±4.4 vs. 81.5±4.3 % in the control, hatchability comprised 69.6±4.2 vs. 42.9±3.8 % in the control, and mulard hatchability comprised 56.0±3.8 vs. 35.5±3.1 % in the control.

Coefficient of mother-daughter heritability of hatchability for hybrids in line A4 was 27 %. The comparatively low heritability gives evidence of feasibility to select combining families of domestic ducks with assessment of the offspring quality.

It should also be noted that produced interspecific hybrids ♂line Y1 (Muscovy males) × ♀line A4 (Pekin female, cross Agidel 34) was characterized by high viability. Survivability from 1 day age to slaughter age reached 95.5-99.0 %. Body weight, meat properties (yield of chest and leg muscles), chemical composition of meat (water, protein, fat, ash, calcium, and phosphorus) and its caloric value at slaughter age in interspecific hybrids were in line with interim indicators as compared to original forms. Herewith, organoleptic indicators of meat in mulards were characterized by specific and high taste properties.

Therefore, semen sampling from Muscovy males requires temporary placement of Muscovy female in cage with male with further collection of ejaculate in artificial vagina. For production of mulards, it is feasible to use males of line Y1 (Jubilee cross) as father line, and line A4 (cross Agidel 34) as maternal line. It was proposed to increase mulard hatchability by selection of genetically compatible pronuclei. Presented data indicate that reproductive properties of Muscovy and domestic ducks could be improved by creation of the specialized breeding forms to increase mulard hatchability by up to 70 %.

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PREPARATION OF SELENIUM NANOPARTICLES BY USING SILYMARIN AND STUDY OF THEIR CYTOTOXICITY TO TUMOR CELLS

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Abstract

The past few years have seen substantial progress in veterinary oncology: new methods have been developed for the diagnosis and treatment of oncological diseases in animals, and the range of possible therapeutic interventions has been broadened. Of interest, in particular, are the prospects for the creation of veterinary pharmaceuticals with the use of various nanoparticles, including colloidal selenium, on the surface of which are immobilized biologically active substances that have antitumor action. Selenium nanoparticles are cytotoxic to tumor cells and have also been considered as effective carriers for the in vivo targeted delivery of drugs, genetic materials, proteins, and so on. The well-tunable polyvalent structures of the selenium nanoparticle surface provide a convenient platform for integrating several therapeutic agents or biomacromolecules with covalent or noncovalent surface conjugation. We synthesized selenium nanoparticles in complex with silymarin, a flavonoid hepatoprotector extracted from the fruit of milk thistle [*Silybum marianum* (L.) Gaertn.], and we evaluated the cytotoxicity of the resultant preparation to normal and tumorous cells. By using electron microscopy and dynamic light scattering, it was found that the developed procedure ensured the preparation of stable suspensions of silymarin-conjugated selenium nanoparticles with sizes ranging from 20 to 40 nm. The obtained conjugate was shown to be markedly cytotoxic to the Hep-2 tumor cell line, suppressing cell respiration approximately 6.5-fold as compared to the control, whereas the respiration of SPEV-2 normal cells was inhibited approximately 2.3-fold. Initial colloidal selenium had much weaker effects on both cell types, and pure silymarin had no statistically significant influence on SPEV-2 cells (in contrast to Hep-2 cells). The results of this study could be used in developing next-generation anticancer agents and are of interest in the implementation of green chemistry-based approaches.

Keywords: *Silybum marianum*, flavonolignans, silymarin, selenium nanoparticles, conjugation, cytotoxic effects

Despite the fact that progress achieved in modern veterinary medicine enabled to reduce animal mortality caused by oncological diseases, cancer is often continues to be treated as one of the most sever diseases of our time. According to available data, over 50 % of all dogs and cats aged over 10 years die from cancer, while oncological diseases remain among the most encountered reasons for asking for the veterinary assistance [1].

The past few years have seen substantial progress in veterinary oncology: new methods have been developed for the diagnosis and treatment of oncological diseases in animals, and the range of possible therapeutic interventions has been broadened [2]. However, it should be noted that arsenal of the veterinary office practically lacks specific medication designated for treatment of the oncological diseases in animals. In particular, important perspectives are associated with novel veterinary pharmaceuticals of various nanoparticles, including colloidal selenium, on the surface of which biologically active substances having antitumor action are immobilized.

Selenium (Se) is microelement of great importance for health of humans and animals [3, 4]. Physiological role of selenium is mainly associated with its presence in selenomethionine and selenocysteine amino acids included in relatively small set (nearly 25) of selenoproteins possessing, in particular, oxidation-reduction and immunomodulating properties [5].

Of great interest is ability of selenium compounds to have cytotoxic effect on tumor cells due to potential of selenolate and hydrogen selenide to effectively react with oxygen and thiols resulting in non-stoichiometric absorption of thiols and NADPH, oxidative stress and, finally, in cell death due to apoptosis, necrosis or necroptosis [6, 7]. Oncologic inhibitory effect of selenium nanoparticles was illustrated, in particular, in a number of publications [8, 9].

Selenium nanoparticles are also considered to be effective carriers for the in vitro targeted delivery of drugs, genetic materials, proteins, and so on. The well-tunable polyvalent structures of the selenium nanoparticle surface provide a convenient platform for integrating of several therapeutic agents or biomacromolecules with covalent or non-covalent surface conjugation [10]. Amino acids [11, 12], fungal polysaccharides [13], vegetable extract from the leaves of *Terminalia arjuna* [14], folic acid [15], cell cultures of *Saccharomyces cerevisiae* [16], and so on are used at biosynthetic (“green”) production of selenium nanoparticles. In the study of the mechanisms of action of the modified selenium nanoparticles, some researchers indicate their trend towards activation of mitochondrial apoptosis in cell line MCF-7, thus causing oxidation stress and in furtherance dysfunction of mitochondrion and, thus, stunting growth of the oncological cells [15]. Moreover, they indicate reduction of the membrane potential of mitochondrion and over-production of the active oxygen forms in Hep-2 cells under the effect of selenium nanoparticles [17].

In the past years, nearly 30 of chemical matters with cancer-prevention effect, which could be useful in reduction of the oncological diseases in humans, have been described [18]. Among them, in particular, much attention was drawn to naturally-occurring polyphenolic antioxidants [19]. Flavonolignans extracted from the medical plant *Silybum marianum* (L.) Gaerth., having strong antioxidant properties, may inactivate both free radicals, as well as reactive oxygen species in a cell. Moreover, they block receptors and transport systems in the cell membrane, which ensure transfer of toxic substances into a cell, reduce activity of macrophage cells participating in antigen presentation, reduce production of γ-globulins, and block lipoxygenase and cyclooxygenase, thus, having anti-inflammatory, immunomodulating, and anticancerogenic effects [20].

In particular, it was shown that silibinin, one of the flavonolignans of *Silybum marianum,* which currently account for 70 % of the total number of flavonolignans [21], increase in vitro and in vivo H3 and H4 histone acetylation in heteroplastid cell line Huh-7 in nude mice [22, 23]. Silibinin inhibits HDAC activity in cells of non-small-cell lung cancer and reduction of their intra-cell composition [24]. Silymarin is a silibinin analogue also falling under the category of vegetable-based flavonolignan type hepatoprotectors extracted from seeds of

Silybum marianum.

We were the first to suggest the technique for production of the stabilized conjugate of selenium nanoparticles from the initially unstable suspensions with the use of silymarin and to study cytotoxicity of such conjugate for immortalized kidney cells of pig embryo (line SPEV-2) and cells of hepatocellular carcinoma of humans (line Hep-2).

The purpose of this study was to develop the method for stabilization of the selenium nanoparticles suspension upon formation of the complex with silymarin and to assess cytotoxic properties of the obtained product with regards to tumor cells.

Techniques. Selenium nanoparticles suspension was obtained by adding 40 ml L-cysteine suspension to 67 ml of selenous acid suspension. For production of the former suspension, 100 ml of distilled water was added to 0.128 g of selenous acid (Ural Plant of Industrial Chemistry — UZPH AO, Russia), for production of the later 100 ml of distilled water was added to 0.726 g of dry L-cysteine (Neolab OOO, Russia); pH of suspensions were brought up to 8.5 with 0.1 M NaOH. Obtained suspended matter (initial nanoselenium preparation) became red-brown.

Diameter (d) of synthesized nanoparticles was measured with the use of a transmission electronic microscope Libra 120 (Carl Zeiss, Germany) and by dynamic light scattering method (DLSM) at analyzer Zetasizer Nano-ZS (Malvern, Great Britain), as described [25].

Commercial silymarin (TEVA Czech Industries s.r.o., Czech Republic) was used for obtainment of the selenium nanoparticle conjugate. Silymarin concentration in final preparation was defined by high-efficiency liquid chromatography (HELC) methodology. Test was conducted at liquid chromatograph Stayer (Akvilon ZAO, Russia) with spectrophotometric detector A_{288} subject to the instructions attached. Column Onix Monolithic C 18 (made by Akvilon ZAO under license of Merck KGaA, Germany) was used for separation of the components.

Cytotoxic studies were conducted in cell lines SPEV-2 (immortalized kidney cells of pig embryo) and Hep-2 (tumoral cells of human hepatocellular carcinoma). Cells were grown in plastic flasks with Dulbecco modified Eagle's medium (DMEM) (Biological Industries, Israel) containing L-glutamin, 15 mm HEPES, 10 % fetal bovine serum and antibiotics (20 μg penicillin of 1000000 U and 25 μg gentamycin of 1000 U per 100 μg of medium). Culturing was conducted in a CO_2-temperature-regulated chamber at 37 °C in atmosphere containing 5 % of CO_2. Cells were detached from the plastic substrate by trypsin-versene solution at 37 °C within 10-15 minutes followed by precipitation in centrifuging at 900 g for 20 minutes. Precipitate was re-suspended in phosphate saline buffer at pH 7.2-7.4 with repeated centrifuging in the above mode. Following re-suspending of cells in full DMEM, they were placed in wells of cell culture plate (1×10^5 cells per well).

To study the cytological effect of the synthesized nanoparticles, cells were pre-cultured for 18-24 hours until formation of a layer occupying 80 % of the well surface. Selenium nanoparticles preparation conjugated with silymarine was lyophilically dried, re-dissolved in incubation medium (pH 7.2-7.4), and placed in wells of the plate with coontinous cell cultures (8 repeats at a dose of 8 μm of silymarin per well). For comparison, silymarin solution of the same concentration was used in the growth medium. In control wells no preparations were added. Viability of the cultured cells was assessed by their ability to reduction of Nitrotetrazolium blue (MTT) to formazan (MTT-test, MTT tetrazolium assay technology) [26] with determination of formazan concentration.

Statistical processing of obtained results was performed by standard

methods with the use of Student's *t*-test for assessment of the significance of differences between samples in trial and control tests. Based on calculation results of the arithmetic mean (*M*) and standard deviation (±SD), standard error mean (±SEM) and limits of a confident interval were identified for sample, accounting for the Student's *t*-test coefficient at 95 % confidence level (p = 0.05) and number of measurements *n* = 8.

Results. Based on data obtained by dynamic light scattering (DLS) method (Fig. 1), size of particles in the initial selenium preparation comprised d ≈ 43-110 nm. However, colloidal solution of non-conjugated nanoparticles has quite low stability with brick-red precipitation (allegedly amorphous selenium) occurred within 10-15 minutes. For the purpose identified, we have created the following original method of obtaining silymarin conjugate with selenium nanoparticles. Silymarin, 1.38 g, was dissolved in 100 ml of 0.1 M NaOH. Further, 67 ml of selenous acid solution was added to 100 ml of silymarin solution following with addition of 40 ml of L-cystein solution. By adding 0.1 M HCl to neutralize alkaline excess, pH was adjusted to 8.5. Based on DLS method (see Fig. 1), size of 97 % of all nanoparticles in the selenium-silymarin preparation was within the range of d ≈ 16-44 nm.

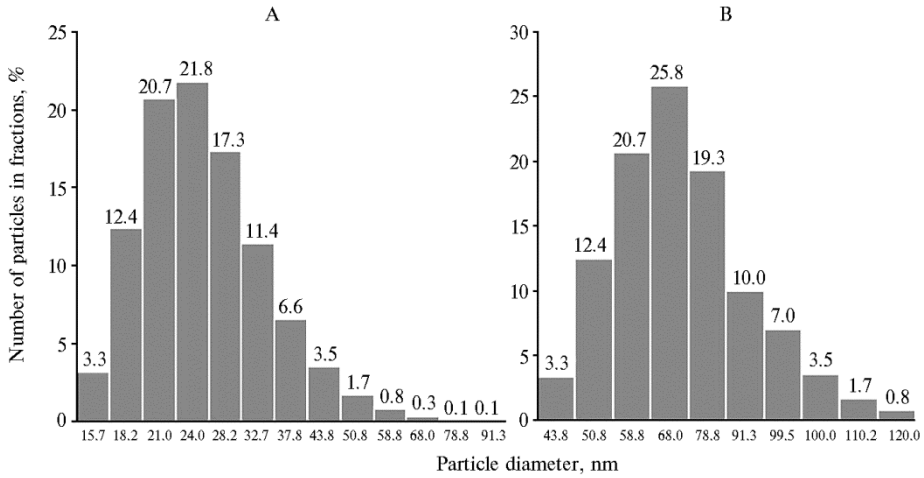

Fig. 1. Distribution of nanoparticles by size in the selenium preparation conjugated with silymarin (A)**, and without silymarin** (B) **based on dynamic light scattering methodology** (an analyzer Zetasizer Nano-ZS, Malvern, Great Britain).

Unlike the initial nanoselenium preparation, the obtained suspended matter of the conjugated selenium nanoparticles with silymarin became red-brown, transparent, visually not opalescent, and remaining stable for a long time. Thus, the achieved stability of the colloidal suspension of selenium-silymarin was accompanied by an increase in its dispersion (decrease of d values) typical for colloidal systems of such type, as was further confirmed by electron microscopic study (Fig. 2).

It was found that adding dried selenium conjugated with silymarin in the medium for cell incubation did not result in any changes in pH.

Cytotoxicity test of two produced preparations of selenium and native silymarin conducted on cell cultures SPEV-2 and Hep-2 found that pure silymarin did not cause statistically significant decrease in viability of SPEV-2 cells (Fig. 3, A). However, when this preparatioon was added to Hep-2 cell cultures, it resulted in decrease of formazan concentration by 10.3 % (see Fig. 3, B), as evidenced by the established inequalities $t_{actl.} = 2.45 > t_{estm.} = 2.14$; $p_{actl.} = 0.028 < 0.05$. Addition of the suspension of the initial nanoselenium resulted in reliable suppression

of SPEV-2 culture viability in MMT-test (by 7.3 %), whereas selenium conjugated with silymarin had resulted in a 55.6 % decrease of formazan concentration (see Fig. 3, A).

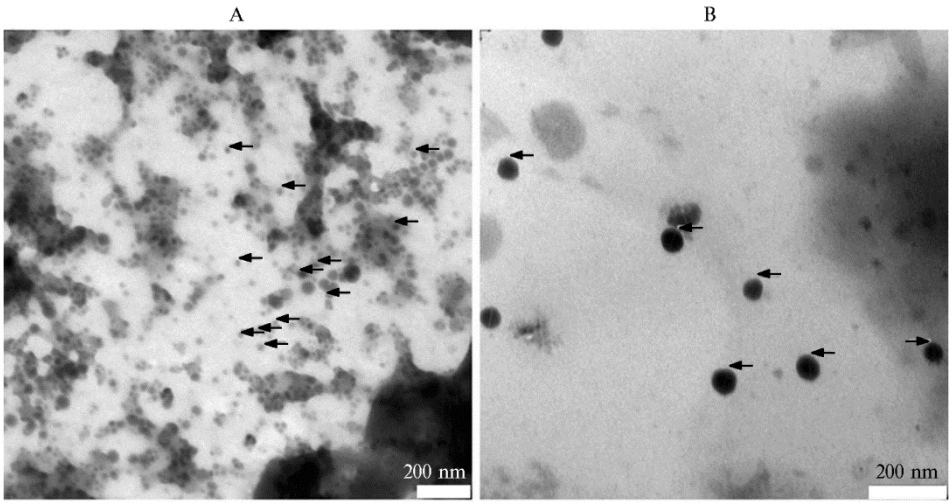

Fig. 2. Electron microscopy of nanoparticles (marked by arrows) **in selenium-silymarin conjugate** (A) **and of initial selenium nanoparticles** (B) (nanoparticles are marked by arrows).

More apparent response to added conjugated preparation had been observed in tumor cell line Hep-2 (see Fig. 3, B): selenium conjugated with silymarin caused a decrease in the number of viable cells, as per concentration of formazan, by 84.6 % (initial colloidal selenium solution decreased this value for the same line only by 34.6 %).

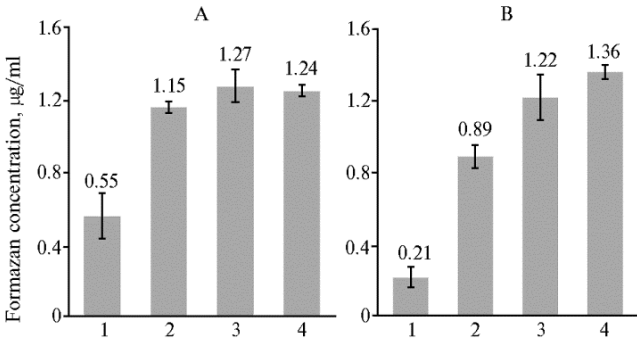

Fig. 3. Viability of cell cultures SPEV-2 (A) **and Hep-2** (B)**, expressed via formazan concentration, when influenced by selenium nanoparticles conjugated with silymarin** (1)**, initial selenium nanoparticles** (2)**, and pure silymarin** (3) **as compared to control** (no preparations added) (4). Vertical bars indicate confidence intervals for mean values at 95 % significance level.

Accounting for the results of this test and the properties of the initial substances described in publications we considered above, the enhanced cytological effect of selenium nanoparticles with silymarin could be caused by the synergy due to conjugation of the components. It is assumed that observed effect of the produced conjugate could be used for development of approaches to creation of next-generation anticancer agents. It appears that the advanced biosynthesis of selenium nanoparticles with the use of silymarin is of interest in terms of implementation of the principles of "green" chemistry [27].

Therefore, addition of silymarin in reaction mixture upon reduction of the solenoid acid with L-cystein results in formation of the stable suspension with organoleptic properties (color and transparency) typical for colloidal suspended matters of selenium at average more probable diameter of particles of 25 nm, which is triple less than in the initial less stable colloidal selenium solution. The produced conjugate of selenium nanoparticles with solymarin has clear cytologi-

cal effect on the line of tumor cells Hep-2 with approximately 6.5 times decrease in the number of viable cells as compared to the control, and approximately 2.3-times decrease of such value in cell lines SPEV-2.

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IMPROVEMENT OF FERRET FUR PELT QUALITY BY USING PRODUCTS OF RECYCLING RAW MATERIALS OF ANIMAL ORIGIN

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Abstract

The waste materials of manufacture of basic animal products are adverse anthropogenic factors affecting environment and, at the same time, they are a source of valuable biologically active substances which can be used. We developed a protocol for complex application of biologically active recycling products, collagen and keratin, obtained by original patented technologies from recycled waste materials of leather and textile industries in combination with melatonin (original modified preparation) for using in ferret breeding. The preparation Melacoll is a stabilized complex of melatonin and collagen. Melacoll has a prolonged effect (up to 4 months) and the toxicity class 4. The keratin-containing substance quality (more than 95 % keratin) makes it possible to use this additive as a protein supplement to fodder. In combined application of Melacoll and keratin, we observed the uniform and statistically reliable weight gain of animals during 4 months exceeding the indices obtained for both small and large doses of keratin (from about 0.59 kg to 2.63±0.13 kg, $t_{obs} > t_{table}$). By month 5 this index decreased which showed completion of animal development and achieving the slaughter weight one month earlier than the standard slaughter terms. Higher concentrations of implanted Melacoll promoted the increase in pelt size. In farm trials, application of Melacoll and keratine led to a reliable 12 % increase in pelt size as compared to the control. The pelt lifetime defects (e.g. broken guard hair) in Melacoll-implanted ferrets was the greatest (37.5 %) whereas in the animals fed with keratin as the fodder additive these defects were the smallest (12.5 %) due to the standard diet enriched with sulphur-containing amino-acids which are responsible for hair strength. Complex use of keratin and Melacoll reliably increased hair density while Melacoll itself did not exert any marked effect on this trait. Optimal concentration of the proposed keratin-containing supplement to the ration in raising slaughter ferret youngsters is 0.6 %, and that of Melacoll is 1 ml of 6 mg/ml substance.

Keywords: fur animal breeding, raising slaughter ferret youngsters, animal raw materials, wastes, recycling, keratin, collagen, melatonin, pelt quality

Ggreen economy aims at protecting the environment, developing an environmentally friendly industry and increasing resources through innovative non-waste technologies for processing raw materials with the maximum balance of valuable components in the products [1]. Secondary raw materials of animal origin are, on the one hand, rather powerful factor of negative anthropogenic environmental impact, on the other — a reserve of valuable biologically active substances that can be used to create various biolgicals for different purposes [2-5]. Keratin, a sulfur containing protein unique in its composition and properties can be extracted from wool and feather recycling waste [6, 7], untanned leather waste products may be a source of collagen [8, 9], and wastes of meat processing industry may serve as raw material for melatonin. a hypophysis hormone. These products are used in the pharmaceutical and cosmetic industries [10-13]. Keratin is a hair-treating agent for certain diseases of human hair [14], is used as a kera-

tin-based wound dressing with curative effect [15], and it is also studied in connection with the application in other areas of biology and medicine [16-19]. In Russia, the use of enzymatic hydrolysis of keratin as a fodder additive for fur animals has been suggested [20]. Foreign studies of melatonin, especially its pharmaceutical forms having a prolonged effect, are mainly associated with medical uses (effects on biorhythms, treatment of sleep disorders) [21, 22]. In Russia, melatonin preparations with prolonged effect for acceleration of fur animal maturation have been developed (RF patents Nos. 2040897, 2096044, 2122787) and are widely used.

Application of products obtained by processing secondary protein-containing raw material can be expanded. For example, in fur farming, it remains relevant to search for preparation that can positively affect the quality of down-and-fur raw materials and semi-finished products. The most attractive formulations are based on natural substrates, combining biological activity and relatively low cost.

We proposed a scheme for the complex usage of biologically active products, collagen and keratin, obtained by innovative original patented technologies based on recycling leather and textile waste, in combination with melatonin (the original modified preparation) and showed that their using in the fur-farming improved both general animal condition and pelt quality.

Our subjective was to estimate effects of products of processing secondary protein-containing waste on fur and pelt quality in ferret youngsters.

Techniques. Collagen was extracted from hide split (the recyclable waste basically not used in lather industry) according to the description (RF Patent No. 2129805). Solubilized keratin was extracted from sheep wool, the recyclable waste of wool processing in the textile industry, by alternating alkaline and acid treatments to purify fibrillary keratin from the accompanying substances followed by its dissolving in a slightly alkalescent medium (RF patent No. 2092072). Collagen served as a fodder additive.

Melatonin (a pituitary hormone, the product of the processed meat waste, code 931684 according to the All-Russian Classifier of Products, a group of intermediates products of synthetic medicines) with prolonged effect was obtained by the original method based on the commercial preparation Melatonin Powder (N-Acetyl-5-Methoxytryptamine) (Shanxi Sangherb BioTech Inc., China). The obtained collagen was a stabilizing agent. Melatonin (6 mg/ml) was immobilized on collagen via incorporation into collagen matrix hydrogel by physico-chemical adsorption [23, 24]. Castor oil (N.A. Semashko Moschempharm, Russia), polyvinyl acetate (PVA) (TEX, Russia), polyvinyl alcohol (PVA) BC-05 (Liwei Chemical Co. Ltd, China), Kuraray Poval® 18-88 (Kuraray Co. Ltd, Japan), as well as a mixture of PVA BC-05 and Kuraray Poval 18-88 in the ratio 1:1 were used as organic copolymers that promote the gradual substance dissolving in the animal's body with a dosed release of the active component. Collagen and melatonin without binding agent was a control. Stability of melatonin-collagen complex, named conventionally as Melakoll, was estimated by the melatonin amount which diffused through the semipermeable membrane for dialysis against the blood-substituting solution (BSS, Russian Research Institute of Hematology and Transfusiology, St. Petersburg). Optical density of the dialysate, which contain diffused melatonin, was measured by a photocolorimeter UFC-2 (Zagorsk Optical and Mechanical Plant, Russia) in the range of λ = 315-980 nm (optical path length 5 mm), in which the working wave length was determined for better monitoring of the melatonin output from complex. Melatonin levels in the dialysate were measured by the calibration graphs. Acute toxicity of the Melakoll complex was assessed on white mongrel mice (males, n = 5,

in control group m = 16.7±0.8 g, in test group m = 16.9±0.9 g) in accordance with State Standard 12.1.007-76 "Harmful substances. Classification and general safety requirements" and the requirements set out in State Standard 32296-2013 "Methods of chemical products testing on the human body. The basic requirements of the assessment acute toxicity test, while intragastric intake by the fixed dose method". The experiments were carried out in accordance with the of the Geneva Convention protocols and the principles of proper laboratory practice (National Standard of the Russian Federation State Standard R 53434-2009), and also according to the recommendations of The Guide for the Care and Use of Laboratory Animals (National Academy Press Washington, D.C. 1996)

For farm test (carried out in Russian Sable Enterprise, Moscow region), 104 males of black ferret (*Mustela putorius*) youngster of cage housing, age 2.5 months at the beginning of the experiment, were divided into groups (8 animals each). At the beginning of the experiment, 1 ml of Melakoll, as an accelerator of vital biorhythms, were subcutaneously injected to ferrets from groups I-V into the **nape** of the neck. In groups I, II, III, IV and V, the drug concentration was 3; 6; 9; 12 and 15 mg/ml, respectively. Group VI was a control where animals were injected with PVA in combination with collagen, that is, with an immobilization matrix and a linking agent. Throughout the experiment, groups I-VI, as well as group VII (control animals that did not receive either Melakoll or keratin) were fed according to the norms adopted in the farm. Animals of groups VIII-XIII, in addition to basic diet, received keratin for 5 days with 5-day intervals during the whole experiment to exclude overfeeding because of high (95 %) keratin content in the feed additive [25-28]. Keratin supplement dosages in groups VIII, IX, X, XI, XII and XIII were 0.2; 0.4; 0.6; 0.6 (average for the remaining experienced groups); 0.8 and 1 % of feed per animal, respectively. Additionally, at the beginning of the experiment, the ferrets of group XI were injected with 1 ml of Melacoll, the melatonin concentration of 6 mg/ml selected based on the RF patents Nos. 2040897, 2096044 and 2219910.

During the monitoring period, the animals were weighed individually with an accuracy of 0.01 kg. At the end of the experiment, the ferrets were slaughtered. Pelts have been subjected to the primary processing (skinning, fleshing, correction and conserving). The pelts were removed using a cut along the rump, with the head fur, paws and tail remained. The pelts were cleaned of the meat prunes, of bones from paws and tail, of cartilages from the ears, and of tendons, then degreased without damaging the hair roots, corrected the hair outward, with longitudinal stretching (without overstretching) (State Standard 11146-65 "Undressed leather of the white and black ferret. Technical specifications"), and air-dried.

The pelts were sorted in accordance with the requirements of State Standard 11146-65 "Unprocessed leather of white and black ferret. Technical specifications", with regard to grade, size, defects, color. The grade was determined organoleptically by totality hair commercial features (puffiness, gloss, density, length and softness, fur maturity) and skin appearance. For determining pelt size, the length from the interpupillary line to the tailhead and the width in the pelt middle were measured, the obtained values were multiplied and the size was assigned as large, medium or small (State Standard 11146-65). Defect-free pelts were either without or with defects which did not significantly change the quality. If the defects exceeded the area of tolerance, the pelts were referred to a group with a small, medium or large defect.

Rump hair density was determined by direct counting hair roots at horizontal sections of 1 cm^2 pelt area or per microscope field of view (mm^2). In direct calculation, a piece (1 cm^2) was cut out, then, fixing pelage with a thread to

form a beam. Guard, awn and then downy hairs were selected from the beam with tweezers; guard and awn hairs were counted apiece, downy hairs were counted by hundreds. An average for 3 samples was calculated. Microscopic histologic section were prepared according to the standard method (S.A. Kasparians et al., Methodological Recommendations for Determining Quality Indicators for Tanning and Fur-and-Meat Raw Materials. Moscow, 1986, in Russ.), stained with hematoxylin and eosin, fixed with Canadian balsam under covered glass and viewed at a 10-fold magnification (a microscope Micromed Eureka 40x-1280x with a digital ocular (OOO Observational devices, St. Petersburg). Micrometric ruler scale division was 8.4 μm, the field of view was 0.7 mm^2. The arithmetical mean was determined for the number of hair follicles in 10 fields of view of the microscope with recalculation for 1 cm^2.

During statistical processing, the arithmetical mean (*M*) and standard error of the mean (±SEM) were calculated. The statistical significance of the mean differences was assessed by the Student's *t*-test [29-31].

Results. The quality of products in fur farming depends on the balanced and biologically proper value of feeds and biostimulators. The collagen-stabilized form of melatonin with a prolonged effect was supposed to be used as biorhythms regulator to accelerate animal growth while solubilized keratin should serve as a protein fodder additive to stimulate dense pelage formation.

The collagen hydrogel, used as the basis for the melatonin preparation, had neutral pH 6.5-7.0 and averaged 1.3 % of mass fraction of solid substances. The counts of microorganisms in the substance did not exceed the values allowed by Sanitary Regulations and Standards 2.3.2.1293-03 "Hygienic requirements for the use of food additives" (approved by the Chief State Sanitary Physician of the Russian Federation, April 18, 2003, http://docs.cntd.ru/document/901862338). Concentration of immobilized melatonin (6 mg/ml) corresponded to that of preparations which accelerate pelage maturation (RF patents Nos. 204897, 2096044, 2122787).

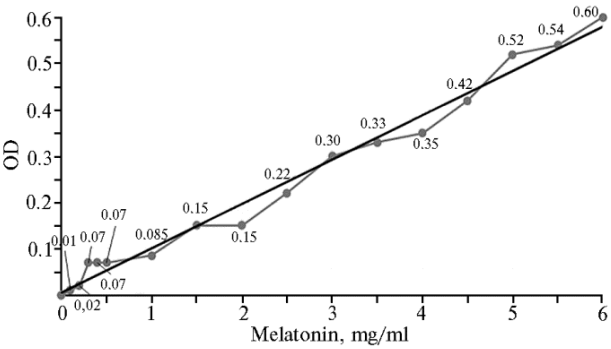

Fig. 1. Calibration graphs of optical density (OD) **depending on melatonin concentration** (λ = 315 nm).

The comparison of calibration graphs for melatonin solutions in BSS (data not given) showed that it is possible to reliably estimate melatonin concentration at 315 nm (Fig. 1).

The greatest stability showed sample No. 6, conventionally named Melacoll, from which 13.3 % of the initial amount of melatonin was released during the observation. A decisive role in this was played by copolymers, a mixture of two polyvinyl alcohols (Table 1).

1. Dynamics of melatonin releasing from the stabilized collagen complexes depending on copolymers

Composition	Days					
	10		20		30	
	melatonin concentration in dialysate					
	mg *M*±SEM)	%	mg (*M*±SEM)	%	mg (*M*±SEM)	%
1. Collagen + melatonin (CM)	0.40±0.01	6.7	1.00±0.01	16	1.50±0.01	25.0
2. CM + castor oil	0.25±0.01	4.2	1.00±0.01	16	1.20±0.01	20.0
3. CM + PVA	0.20±0.01	3.3	0.50±0.01	8.3	1.00±0.01	16.0

Continued Table 1

4. CM + PVOH VA-05	0.30±0.01	5.0	0.50±0.01	8.3	0.90±0.01	15.0
5. CM + PVOH Kuraray Poval® 18-88	0.40±0.01	6.7	0.60±0.01	10.0	0.90±0.01	15.0
6. CM +PVA mixture	0.20±0.01	3.3	0.50±0.01	8.3	0.80±0.01	13.3
7. Collagen + PVOH mixture	0.00	0	0	0	0	0

N o t e. PVA — polyvinyl acetate, PVOH — polyvinyl alcohol.

2. Average weigh (g) **of white mice organs in testing acute toxicity of Melacoll by a fixed dose method** (M±SEM, n = 5)

Organ	Control	Test
Heart	0.178±0.004	0.179±0.005
Lungs	0.276±0.013	0.274±0.012
Liver	1.899±0.060	1.910±0.061
Kidney	0.502±0.020	0.509±0.022
Spleen	0.240±0.013	0.243±0.014

Four hours after the laboratory mice received intragastric Metacoll, their general condition (i.e. a decrease in activity, lack of appetite, a need for water) indicated stress but pathologies did not develop. In 24 hours, the activity of the mice became normal, the appetite returned completely. On days 7 and 14 and hereafter, the animals were active, ate feed completely, no deviations in their condition were noted. No one mouse died during the experiment. At 2.5 months after the experiment beginning, the animals were slaughtered for pathological examination (Table 2). However, the difference in organ weight in the control without Melacoll and in the test with Melacoll was insignificant: t_{obs} = 0.01; t_{table} = 2.3 (i.e., $t_{obs} < t_{table}$, P > 0.05). These results indicate that Metacoll has no pronounced toxic effect on animals and can be attributed to substances of the toxicity class 4 in accordance with State Standard 12.1.007-76 "Harmful substances. Classification and general safety requirements".

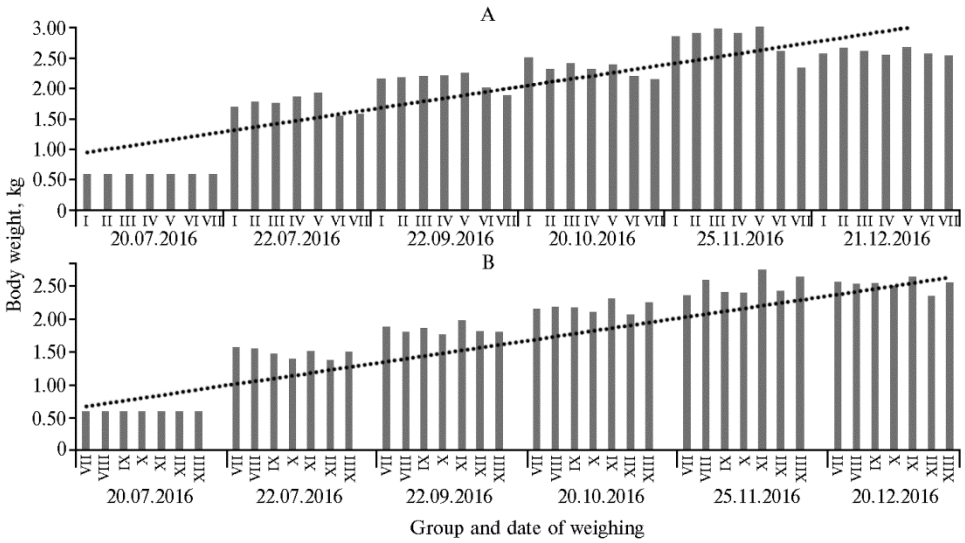

Fig. 2. Dynamics of weight gain of black ferret (*Mustela putorius*) received Mellacol injection (A) **and dietary keratin additive** (B) (a farm trial, Russian Sable Enterprise, Moscow Province, 2016). For description of groups and preparations used, see the section «Techniques». Controls: group VI (injection of mixture of collagen and PVA) and group VII (animals which did not receive either Melacoll or keratin). Dotted lines reflect an increment trend.

A characteristic feature of keratin composition is a high proportion of sulfur-containing amino acids cystine and cysteine which are extremely important for pelage formation in fur farm animals, especially in growing youngsters. The denser fur, the higher the animal's need for dietary sulfur-containing amino acids [28, 32, 33]. So, intact keratin should favorably affect fur formation. However, it should be noted that the level of digestible protein in the diet of fur animals is 8-11 g per 100 kcal [27, 32]. This amount avoids the toxic effects of ni-

trogen-containing products of protein decomposition [32]. At the same time, the lack of digestible protein decreases the hair tolerance to damaging, as a rule, mechanical, effects [32].

The resulting product was a homogenous liquid, gray beige in color (from light to dark tones), pH 6.5-7.5; dry matter ratio was 3.0-5.0 % and keratin content, as per total nitrogen, reached 95-98 % of dry matter. Microbiological purity of the final product was high, with microbial contamination less than 10^1 CFU/cm^3, yeasts, moldy and yeast-like fungi were absent. In farm trial, we used this substance in small doses which are not significantly modifying the protein ratio of 0.23-1.13 g of keratin per 156 g feed portion, but raising amount of sulphur-containing amino acids.

At different Melacoll dosage applied at the beginning of the trial, there was a direct correlation for the first four months between the used dose and the monthly increase in the body weight of ferret youngsters (Fig. 2, A). During this time, the body weight, as compared to the initial values, increased approximately 5-fold in the experimental groups, whereas only 3.6-fold in the control. At month 5 of life, the body weigh of the experimental animals decreased by an average of 0.5 kg and was almost equal to that in the control group. It should be noted that during each month there was no statistically significant difference between the weight of animals received minimum and maximum dose of Melacoll. Thus, on weighing dates (July 20, 2013, August 22, 2016, September 22, 2013, October 20, 2016, October 25, 2013, December 20, 2016), the t_{obs} values in groups I and V were 0.01, 0.30, 2.08, 1.62, 1.20 and 0.90, respectively, at t_{table} 2.14. At the same time, the difference in the monthly weight gain was statistically significant (Table 3).

The obtained data indicates that Melacoll accelerated animal development for almost a month, since continuation of the experiment negatively affected ferret weight in the experimental groups by the end of the 5th month.

3. Statistical significance of monthly differences in body weight gain of black ferrets (*Mustela putorius*) received injection (min/max) **of Melacoll** (a farm trial, ferret cages, Russian Sable Enterprise, Moscow Province, 2016)

Weighing date	Student's *t*-criterion	
	group I (min), *tobs.*	group V (max), *tobs.*
20.07-22.08	16.39	11.80
22.08-22.09	4.25	2.17
22.09-20.10	5.08	2.67
20.10-25.11	2.35	3.88
25.11-20.12	1.88	0.86

$T_{tab.}$ 2.14

N o t e. Design of the experiment and application of tested preparations are described in section «Techniques».

When using keratin as a feed additive (see Fig. 2, B), the growth acceleration for the first 2 months was more pronounced at low doses than for larger doses. Over time, the difference in the body weight of animals which got small and large doses of keratin (groups VIII and XIII) became statistically unreliable. The values of t_{obs} 0.39 < t_{table} 2.14 (P > 0.05) in October and t_{obs} 2.0 < t_{table} 2.14 (P > 0.05) in December indicated the effect of small doses.

Cumulative effect of Melacoll and keratin (group XI) is a matter of special interest. The weight gain in the test animals was statistically significant for 4 months at *tobs.* of 16.59, 5.16, 2.29 and 2.58, respectively, vs. $t_{tab.}$ 2.1. In this, the increment exceeded the values which were obtained for keratin only, regardless of the dose used, and the maximum effect, like for Melakoll, was recorded for month 4.On month 5, the body weight in this group decreased by 3 %, but the differences were statistically insignificant (t_{obs} 1.58 vs. t_{table} 2.14). It indicates that the animals have grown to slaughter weight in November, that is, 1 month before the expected date. A similar trend in weight loss was also observed in the groups where pure keratin was used as a protein additive.

Our findings are in line with the available publications on feasibility of pro-

longed-release melatonin and keratin supplements for feeding young animals in fur farming [2, 4, 20, 28]. At the same time, according to our data, the combined use of these two biologicals has more pronounced effect.

Melatonin, by stimulating animal maturation, positively affects the size and weight of pelts [28, 32]. Melacall had similar effect (Table 4). An increase in the concentration of implanted Melacoll resulted in a larger fur pelt size, with the largest found in group V where the concentration of melatonin was the maximum. For implantation of collagen and PVA mixture (control group VI), the pelt size was practically the same as in group VII (untreated control, t_{obs} 1.11 < t_{table} 2.14, P > 0.05), which confirms the existing opinion on the functional role of melatonin in hair biology of fur animals [27, 28, 32]. While keratin feeding, the largest pelt size was found in groups VIII and XIII (912 and 918 cm^2, respectively), and in group XI where keratin was used together with Melacoll (998 cm^2). Differences between this indexes in groups VIII and XIII were not statistically significant (t_{obs} 1.21 < t_{table} 2.14, P > 0.05), which confirms our hypothesis about the advisability of using small doses of keratin.

In group XI, the differences in pelt size as compared to control (12 %) were significant (t_{obs} 2.89 > t_{table} 2.14, P > 0.05), unlike groups VIII and XIII in which only keratin was administered and the increase in pelt size compared to the control was 7-9 % (t_{obs} 2.21 < t_{table} 2.14, P > 0.05). In this, the pelt size in the group XI was significantly larger than in group VIII (t_{obs} 2.99 > t_{table} 2.14, P > 0.05) and in group XIII (t_{obs} 2.96 > t_{table} 2.14, P > 0.05). In evaluation according to the State Standards, all pelts were assigned to the grade 1 (full-haired with high, frequent, shiny hair and thick fluff, with a fluffy tail). All pelts exceeded 600 cm^2 in size (see Table 4), that is, they were characterized as large.

4. Pelt grade and size in black ferrets (*Mustela putorius*) which received Melacoll and keratin separately and jointly (a farm trial, ferret cages, Russian Sable Enterprise, Moscow Province, 2016)

Group	Grade	Size, cm^2 (M±SEM)	Size category	Defects			Imperfect pelts, %
				small	medium	large	
Melacoll injected							
I	1	930±39	Large	5.6	No	No	5.6
II	1	947±40	Large	16.7	No	No	16.7
III	1	951±32	Large	5.6	No	No	5.6
IV	1	961±48	Large	12.0	No	No	12.0
V	1	1023±30	Large	0	16.7	No	16.7
Controls							
VI	1	869±42	Large	16.7	No	No	16.7
VII	1	861±30	Large	5.7	16.7	No	22.4
Dietary keratin supplement							
VIII	1	912±38	Large	16.6	No	No	16.6
IX	1	889±35	Large	No	11.1	No	11.1
X	1	908±36	Large	No	5.5	No	5.5
XI (+ Melacoll injection)	1	998±32	Large	No	No	No	0.0
XII	1	872±47	Large	9.0	No	No	9.0
XIII	1	918±34	Large	7.0	No	No	7.0

Note. For description of groups and preparations used, see the section «Techniques». Controls: group VI (injection of mixture of collagen and PVA) and group VII (animals which did not receive either Melacoll or keratin).

One of the important indicators of the pelt quality is the kind of defect [20, 28]. Imperfections which reduce pelt quality may occur during animal's lifetime depending on nutrition and keeping conditions, effects of environment and physiological state of animal, or these may result from unprofessional actions or negligence of personnel at slaughtering and during primary processing of raw materials [20, 25]. Particular interest for us was the studying of lifetime defects, which include broken guard hair, bald patches, scrapes, poor hair, bites, and tangled hair [25, 26]. Of the above-mentioned, the guard hair damage, bald patch

and scrapes were found (Table 5). The lifetime defects of pelts were the most frequent (37.5 %) in animals of groups II, IV and V which received implanted melatonin. In groups VIII-XIII fed with keratin as a protein supplement to the basic diet, the lifetime defects reached a 25.0 % level. At Melacoll implantation, guard hair damage occurred. In control groups, 25 % pelts were characterized by guard hair damage, whereas in the pelts from animals that received keratin this defect was found only in two groups, one pelt per each (see Table 5). In our opinion, this may be due to the better availability of sulfur-containing amino acids responsible for hair strength [20, 27].

5. Frequency of lifetime defects of pelts in black ferret (*Mustela putorius*) which received Melacoll and keratin separately and jointly (a farm trial, ferret cages, Russian Sable Enterprise, Moscow Province, 2016)

Group	Pelts with lifetime imperfections, psc/%		
	broken guard hair	bald patches	scrapes
Melacoll injected			
I	1/12.5	0	0
II	2/25.0	0	0
III	1/12.5	1/12.5	1/12.5
IV	3/37.5	0	0
V	3/37.5	0	0
Controls			
VI	2/25.0	0	0
VII	2/25.0	1/12.5	0
Dietary keratin supplement			
VIII	1/12.5	0	1/12.5
IX	0	1/12.5	0
X	0	1/12.5	0
XI (+ Melacoll injection)	0	0	0
XII	1/12.5	1/12.5	0
XIII	0	0	1/12.5

N o t e. For description of groups and preparations used, see the section «Techniques». Controls: group VI (injection of mixture of collagen and PVA) and group VII (animals which did not receive either Melacoll or keratin). In each group, 8 pelts were examined.

6. Fur density in black ferret (*Mustela putorius*) which received Melacoll and keratin separately and jointly (a farm trial, ferret cages, Russian Sable Enterprise, Moscow Province, 2016)

Group	Direct count on rump, psc/cm^2 (M±SEM, n = 3)	Histological estimates of hair follicle number	
		per field of view (M±SEM, n = 10)	average, pcs/cm^2
Melacoll injected			
I	6304±530	44.1±1.6	6300
II	6340±543	44.3±1.7	6328
III	6410±541	44.9±1.7	6414
V	6533±653	45.7±1.4	6529
Controls			
VI	6069±509	42.5±0.9	6071
VII	6111±511	42.8±1.8	6114
Dietary keratin supplement			
VIII	6840±548	47.8±1.6	6828
X	6832±583	47.8±1.4	6829
XI (+ Melacoll injection)	7141±614	49.9±1.8	7129
XIII	6870±587	48.1±2.3	6871

N o t e. For description of groups and preparations used, see the section «Techniques». Controls: group VI (injection of mixture of collagen and PVA) and group VII (animals which did not receive either Melacoll or keratin). For the differences between groups I and VII, II and VII, III и VII, V и VII, and VI and VII t_{obs} are 0.04, 0.05; 0.07; 0.09; 0.01 at t_{table} 2.77, respectively.

The commercial value of fur pelts is mainly due to the fur density which is crucial for fur puffiness and thermal conductivity, as well as for the suitability of pelts for manufacturing valuable finished fur products. We estimated this index in the groups with the maximum Melacoll and keratin effects, as it followed from body weight dynamics, pelt size, and the kinds of pelt defects (Table 6). Melakoll did not exert a marked influence on the fur density, which was confirmed by an assessment of the statistical significance of differences (see Table

6). It should be noted that some scientists opine against the use of melatonin as a stimulant, because according to their data, this leads to a decrease in fur quality deterioration because of unnatural growth acceleration [32, 33].

Keratin increased the fur density by 12 % compared to the control (t_{obs} 4.1 > t_{table} 2.77, P > 0.05). In the combined using keratin and melakoll, the hair density increased significantly by 16 % compared to the control (t_{obs} 3.1 > t_{table} 2.77, P > 0.05). The results of direct fur counting correlated well with the histological estimates of hair follicle number. The main factors of fur density are the hair number per bundle, the number of grouped bundles and their distribution. Histological comparison of pelt horizontal sections in experimental group XI and control group VII (Fig. 3) revealed a greater number of bundles in group XI and confirmed the fact that the number of bundles and hairs depend both on the hereditary features and on the external factors, such as feeding and keeping conditions, which affect realization of the animal genetic potential.

Thus, the combined use of keratin and melatonin-containing Melacoll mutually enhanced the effect of each biostimulant. Keratin enriches the diet with sulfur-containing amino acids that activate hair growth, while melatonin, due to accelerated biorhythms, contributes to a better eating and digestion. These factors ultimately ensure the acceleration of animal growth and positively affect the pelt quality.

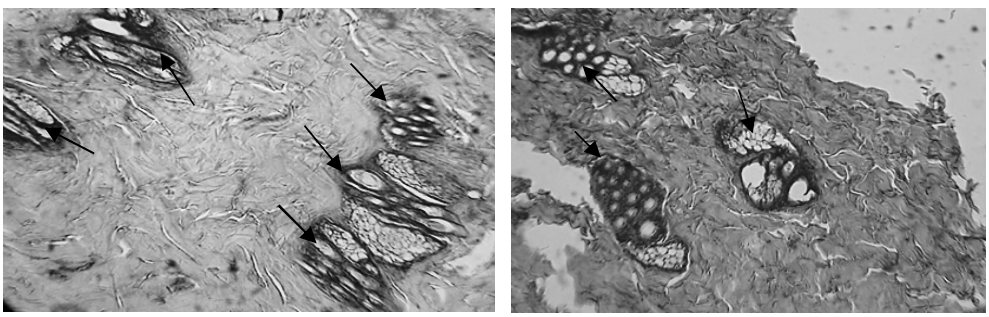

Fig. 3. Horizontal section of a pelt in black ferret (*Mustela putorius*): a combined use of injected Melacoll and dietary keratin (group XI, on the left) and animals which did not receive these preparations (group VII, on the right) (a farm trial, ferret cages, Russian Sable Enterprise, Moscow Province, 2016). Hematoxylin and eosin staining, a magnification of ×10; arrows note hair bundles.

So, the obtained results lead to the following conclusions. An original melatonin-collagen complex with prolonged effect, conditionally named Melacoll, was produced from unusable wastes and used to stimulate the growth of ferret youngsters. Also, the effect of keratin-containing substance was tested, the quality of which allows us to use it as a protein fodder additive. An increase in concentration of the implanted Melacoll increases the pelt size, whereas keratin shows positive effect at low doses. In combination of Melacoll and keratin, the resultant effect exceeds that of each preparation separately. There is an even, statistically significant gain in body weight for 4 months. During this period, the animal growth is completed and they reach a pre-slaughter stage. In the farm trials, the combined use of Melacoll and keratin significantly increased pelt size and quality, as followed from estimates of fur density and defects. The largest number of pelt lifetime defects (37.5 %), in particular, guard hair damage, was noted in administering Melacoll without keratin additive. Direct counting and histological study confirmed that the combined use of keratin and Melacoll significantly increases the fur density, although Melacoll itself does not noticeably affect expression of this trait. Dietary keratin (0.6 % of feed weight) and 1 ml injection of Melacoll (6 mg/ml) are optimal to accelerate growth of the ferret youngsters.

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ACTIVITY OF DIGESTIVE ENZYMES IN DUODENAL CHYMUS AND BLOOD IN BROILERS OF PARENTAL LINES AND THE MEAT CROSS DEPENDING ON DIETARY BIOACTIVE ADDITIVES

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Abstract

The achievement of maximal productivity in poultry requires knowledge of the genetic productivity potential and adjustment of nutrition according to bird's physiological peculiarities. It is well established that early growth and development in poultry depend on the functional formation of the digestive system. Exocrine function of the pancreas plays one of the most important roles in this process. Earlier studies showed that the weight of the pancreas in broiler hybrid chicks is substantially higher compared to the parental lines till 35 days of age. Detailed comparative investigation of the corresponding distinctions in the digestion, however, requires physiological studies on fistulated chicks. The aim of the study presented was to determine and compare the activities of digestive enzymes in duodenal digesta and blood in hybrid broiler chicks (cross Smena 8) and in chicks of parental lines (Cornish and Plymouth Rock) when using bioadditives of several types, i.e. a spore forming probiotic, the mixture of low molecular weight organic acids, phytobiotic complex Intebio, feed additive with living bacteria (OOO Biotrof, Russia). It was found that amylase activity in the duodenal digesta was 22.0 % higher in the hybrid and 35.8 % higher in Cornish parental line as compared to Plymouth Rock line; lipase activity was higher 5.8- and 2.3-fold, respectively, due to the high dietary level of crude fat. The activity of proteases, however, was significantly lower in hybrids (by 9.0 %, $p < 0.05$) as compared to Plymouth Rock line and 32.3 % higher when compared to Cornish chicks ($p < 0.001$). The activity of blood amylolytic enzymes followed the reverse trend compared to the duodenal digesta; activity of other blood enzyme bore no differences between the genotypes. A diet formulation, as a proportion of protein, fat, and carbohydrates, can substantially influence the activity of the digestive enzymes in the intestinal lumen and in blood. Low molecular weight organic acids and the enzyme-based preparation in the diets for meat-type chicken stimulate activity of digestive enzymes in the duodenal digesta and blood with respect to the genotypic physiological peculiarities of the birds.

Keywords: intestinal digestion, digestive enzymes, blood pancreatic enzymes, broiler chicks, parental lines, meat cross, enzymes, probiotics, phytobiotics, organic acids

The achievement of maximal productivity in poultry requires knowledge of the genetic productivity potential and adjustment of nutrition according to bird's physiological peculiarities [1]. Study results [2, 3] show that growth and development in poultry depend on functional formation of the digestive system ensuring the initial metabolism, on which digestibility and digestion of food nutrients very much depends. Basic research of digestive physiology promotes development of the perspective systems and nutrition technologies for growing modern and newly created highly-productive poultry breeds and crosses.

Our previous findings showed [4, 5] that growth of chickens depends on weight of pancreas which performs not only the exocrine function, but also participates in metabolic regulation via production of hormones and bioactive substances. Obtained information evidences for significant superiority of hybrids over the chickens of parental lines by weight of pancreas at 35 days of age.

Presently, publications on digestive physiology studied using fistulated hens are sparse [6-9], and we completely failed to find similar comparative data for the youngsters of hybrids and their parental lines. It was found [10-13] that digestive ferments (amylase, lipase, and trypsin) enter the blood and constantly circulate in the blood stream. However physiological role of blood pancreatic ferments has not been studied enough and there are different views on entero-pancreatic circulation of the digestive enzymes [14].

It is evident that enhanced efficiency of feed conversion into animal products required digestive glands to effectively function of and their ability to adapt to varied nutritive conditions. Nowadays, this problem is more relevant due to the unprecedented extension of the list of regulated parameters of rations, and also due to necessity for replacement of the expensive highly nutritious components by low calorie alternatives with slow hydrolyzed ingredients, e.g. non-starch polysaccharides, trypsin inhibitors, erucic acid, glucosilonates, etc.). In recent years, a lot of biologically active additives (ferments, taste boosters, acidulants, phyto- and probiotics, etc.) are suggested which are marketed as the means for recovering from gastrointestinal problems. However, their impact on animal and human metabolism and health is poorly studied.

Adaptation of the digestive system to feed additives is crucial for poultry homeostasis and health, which determines relevance of sych studies. During physiological studies in fistulated hens, activity of amylase, protease, and lipase in gastrointestinal contents and blood plasma was studied for the first time in parental lines and their cross upon use of various bioactive additives in feeding.

Purpose of this research is to compare activity of digestive ferments in duodenal chymus and blood of hens at use of biologically active additives.

Techniques. Activity of the digestive ferments was studied in chronic experiment on 20-42-day old broiler chicken of cross Smena 8 and on young 49-70-day old birds of the parental lines of this cross (Selection and Genetic Center Smena, Moscow Province; B5 as the paternal line, B9 as the maternal line). Implantation of fistules in duodenum was performed with the use of sedative and anesthetic agents according to principles of human treatment of animals. T-shape fistula was installed in the ascending limb of duodenum close to the point of confluence of three pancreatic and two biliary ducts. In 3-5 days following surgical interference, birds recovered after surgery and could be involved in the study.

Physiological test was performed by group method, 5 heads per each group, where different types of the analyzed biologically active additives were included in main diet of poultry. Feed was prepared according to zootechnical regulations. Poultry was fed proportionally: 30 g per head in the morning on the empty stomach, and the rest daily feed was supplied during the day. Duodenal chyme (5 ml) was collected 1 hour after feeding, promptly centrifuged for 5 minutes at 5000 rpm, and diluted with cooled Ringer solution (1:10).

Chymus amylase activity was determined on a photoelectric photometer KFK-3 (Zagorskiy Optical Mechanics Plant, Russia) at $\lambda = 670$ nm by starch hydrolyze [15] and expressed in milligrams of the digested starch per 1 ml of chymus over 1 minute. Lipolytic activity was measured on a semi-automatic biochemistry analyzer BS-3000P with flow cell (Sinnowa Medical Science & Technology Co., Ltd, China) with the set of reagents for lipase (DIAKON-VET OOO, Russia). Protease activity was determined by decomposition of Hammerstein Grade

Casein (EMD Millipore Corp., Billerica, USA) under colorimetric measurement on a KFK-3 at $\lambda = 450$ nm [16].

Blood samples were collected from the axillary vein of birds, on the empty stomach, supplemented with sodium citrate and centrifuged at 5000 rpm for 3 minutes. Blood plasma had been studied for activity of amylase and lipase at a Chem well 2900 (T) device (Awareness Technology, USA) with relevant reagent kits (Human GmbH, Germany). Trypsin activity [17] was assessed on a BS-3000P analyzer.

Statistical processing of the results had involved calculation of the mean (*M*) and standard mean errors (±SEM). Reliability of differences was assessed by Student's *t*-test. Resulting differences were deemed statistically significant at $P < 0.05$.

Results. During the experiment, we tested effects of few types of feed additives, namely a probiotic supplement based on *Bacillus subtilis*, a mixture of low-molecule organic acids, a phytobiotic based on *Bacillus subtilis* with addition of volatile oils, and an enzymatic probiotic Cellobacterin-T (Biotrof OOO, Russia) (Table 1).

1. Diets to study the effect of various feed additives on digestive function in meat broiler chickens of cross Smena 8 and young birds of their parental lines

Group	Diet composition
I	Basic vegetable diet (BD) balanced by all principal nutritional components
II	BD + sporous probiotic based on *Bacillus subtilis* (500 g/t)
III	BD + mixture of low molecule organic acids (1000 g/t)
IV	BD + phytobiotic based on *Bacillus subtilis* with addition of volatile oils (500 g /t)
V	BD + Cellobacterin-T (1000 g/t)

Basic diets the parental lines and their hybrid were different accounting for feeding standards (Table 2).

2. Recipes and nutritional value of combined feed in the basic diet (BD) **used to study to study the effect of various feed additives on digestive function in meat broiler chickens of cross Smena 8 and young birds of their parental lines**

Ingredient	Content in combined feed, %	
	BD of hybrids ("finish")	BD of parental lines
Wheat	40.35	49,48
Soybean meal	2.95	0
Sunflower cake	25.00	18,58
Wheat bran	0	17,31
Corn	17.00	10,00
Maize gluten	4.06	0
Soybean oil	6.98	0
Limestone (36 %)	1.47	2,50
Monocalcium phosphate	0.62	1,04
Culinary salt	0	0,25
Sodium sulphate	0	0,18
Feeding methionine	0	0,10
Mineral blend (0.08 %)	0	0,08
Choline chloride	0	0,08
Lysin 98	0.35	0,35
Vitamin blend (0.02 %)	0	0,02
Premix	1	0
Nutritional value per 100 g of combined feed:		
energy value, ccal	320	255
fat, g	9.02	5,35
fiber, g	5.21	6,73
protein, g	19.79	15,59

Note. "Finish" means the third diet for growing broilers, from day 21to day 42 of age.

Our research found that duodenal chymus of meat chicken is characterized by high activity of digestive ferments (Table 3) that is in line with data of Chinese scientists [18].

Obtained results (see Table 3) showed that amylase activity in duodenal

chymus of hybrids is higher compared to the chicken of maternal line B9 by 22.0 % and of parental line B5 by 35.8 % ($P < 0.01$). It is mainly due to poultry feeding [19, 20], since in diet of broiler chickens as compared to that of the both parental lines the fiber content per 100 g feed was less by 1.52 %, whereas easier digested carbohydrates were higher. As to metabolic energy, diet of hybrid birds significantly (by 20.30 %) exceeded the combined feed of young birds of parental lines. Since broiler diet contained 9.00 % of raw fat while its content in the diet of parental lines was approximately 2-fold lower (5.35 %), lipase activity in duodenal chime of hybrids increased compared to the parents, namely being as much as 5.8 times higher in birds of the maternal line and 2.3 times higher in birds of the paternal line. Other pattern was noted in the stomach for activity of proteolytic enzymes. At high content of raw protein in diet of broiler chickens, their protease activity was 9.0 % lower ($P < 0.05$) than that of the maternal line, but 32.3 % higher ($P < 0.001$) as compared to the paternal line. This may be due to the fact that the hydrolysis of various components of the feed along the digestive tract occurs unevenly. Decomposition of fats is the first to occur be broken down, mainly in duodenum, whereas amylase and protease are mainly present in jejunum [21]. Hence, digestive system in meat chickens is developed adequately to the nutritional value of the provided feed depending on the genetic properties which define intensity of metabolic processes.

3. Activity of digestive ferments in duodenal chymus and blood of meat broiler chickens of cross Smena 8 and youngsters of their parental lines depending on the type of dietary additives used (M±SEM, $n = 25$, physiological test)

Ferment	Group				
	I	II	III	IV	V
Broiler chickens of cross Smena 8					
Chymus enzyme activity					
Amylase, $mg \cdot ml^{-1} \cdot min^{-1}$	341±27.4	354±33.3	270±34.5	309±37.3	462±28.2*
Lipase, IU/l	1734±215.4	1455±161.8	1069±211.0*	1462±212.7	1749±227.3
Protease, $mg \cdot ml^{-1} \cdot min^{-1}$	33±1.0	31±0.9	32±1.4	33±1.3	36±0.6*
Blood enzyme activity					
Amylase, IU/l	244±37.2	455±56.1*	386±38.5*	311±50.5	454±43.2*
Lipase, IU/l	20±4.1	31±5.0	27±4.5	27±2.9	42±5.5*
Trypsin, IU/l	29±2.0	21±2.9*	53±5.3	41±3.8*	35±2.3
Chickens of parental line B5					
Chymus enzyme activity					
Amylase, $mg \cdot ml^{-1} \cdot min^{-1}$	219±21.1	154±15.6*	231±25.5	155±8.6*	199±25.7
Lipase, IU/l	750±54.7	912±92.2	982±76.5*	632±41.7	896±70.5
Protease, $mg \cdot ml^{-1} \cdot min^{-1}$	22±1.8	27±2.0	30±1.7**	22±1.7	28±0.9*
Blood enzyme activity					
Amylase, IU/l	395±43.5	350±59.5	322±20.5	245±21.5*	436±35.2
Lipase, IU/l	29±2.2	32±2.4	28±2.1	38±4.5	42±8.5
Trypsin, IU/l	35±5.4	41±1.0	34±3.5	46±6.5	43±9.1
Цыплята материнской линии В9					
Chymus enzyme activity					
Amylase, $mg \cdot ml^{-1} \cdot min^{-1}$	266±31.0	407±40.5*	305±41.0	348±36.5	215±31.4
Lipase, IU/l	301±37.5	212±16.4*	597±50.3**	226±59.7	504±65.0*
Protease, $mg \cdot ml^{-1} \cdot min^{-1}$	36±0.8	37±1.1	36±1.0	34±1.5	35±1.0
Blood enzyme activity					
Amylase, IU/l	290±25.1	382±33.3*	263±6.5	336±26.2	311±27.6
Lipase, IU/l	15±0.9	15±0.1	19±0.6*	16±1.8	25±3.5*
Trypsin, IU/l	29±0.5	27±0.5	30±0.9	27±3.2	25±1.7

N o t e. See description of groups in Table 1.
*, ** Differences from the control are statistically significant at $P < 0.05$; $P<0.001$, respectively.

Activity of blood amylolytic enzymes of meat chicken displayed the regressive trend as compared to such activity in stomach (see Table 3). In blood of broiler chickens this values decreased, as compared to these of young chickens, by 18.8 % for the maternal line and by 61.9 % for the parental line ($P < 0.01$). Lipase activity in blood varied in all studied groups, but insignificantly. We also did not observe any valid differences between the groups in activity of proteolytic ferments. These results are coherent with hypothesis of enteropancreatic circula-

tion of digestive ferments. Availability of pancreatic ferments in blood confirms the opinion that they may enter into the blood stream, flow with blood into the pancreas and be repeatedly secreted into the intestine without decaying in the intestine to amino acids [11, 23, 24].

In broiler chickens, dietary probiotic (see Table 3) had not led to significant changes in fermentative activity of duodenal contents. Herewith, blood amylase activity increased by 86.5 % ($P < 0.05$), and trypsin activity decreased by 37.6 % ($P < 0.05$). It appears that slowdown of proteolytic activity was due to competitive effect of sporous probiotic on digestive ferments, in particular proteolytic [24-27]. Probiotic had no significant effect on activity of the digestive ferments in small intestine and blood of paternal line B5 youngsters, whereas promoted a 53.0 % increase in intestine amylase activity ($P < 0.05$) of maternal line B9 youngsters. Similar changes were observed in blood, i.e. amylase activity increased by 31.7 % ($P < 0.05$). During tests, lipase activity in stomach decreased by 29.6 % as compared to the control.

Accordingly, the dietary probiotic had selective effect on activity of the digestive ferments in meat chicken. Activity of blood enzymes in broilers changed, and amilolythic activity in blood and stomach increased in maternal line, whereas activity of the stomach lipase decreased.

Low-molecule organic acid is one of the most effective antibiotic surrogates. These compounds, including those in form of salts, are characterized by high antibacterial activity. In our test, they had significantly changed the lipase activity in duodenal contents: this activity rate was high in control broiler chickens, but it had been decreased by 38.4 % upon addition of organic acids in the diet. In blood, such values went up: upon addition of organic acids amylase activity increases by 58.2 %, protease activity increases by 82.7 % ($P < 0.05$). In chickens of parental lines, organic acids had promoted higher activity of lipase (by 30.9 % for line B5, by 98.3 % for line B9) and protease (by 36.4 % for line B5) in duodenal contents as compared to control chicken. Accordingly, one may assume that organic acids promote secretion of the intestinal juice which stimulates activity of the pancreatic ferments, mainly lipase, in gut, increases activity of blood amylase and trypsin in broilers, and activates blood lipase in youngsters of maternal line.

Results obtained upon addition of dietary phytobiotic confirm its stimulating effect on the digestive process in broiler chickens and young birds of paternal line, which had been manifested by the increase of blood trypsin activity by 41.4 % ($P < 0.05$) and 31.4 %, respectively. In such cases, better appetite and protein metabolism could be expected, taking into account the role of trypsin in regulation of the metabolism and increase of blood vessel diameter.

Fermentative preparations as feed additives are widely used in chicken farming, but the mechanism of their effect on digestive processes had not been studied in full. Our tests with dietary fermentative preparation Cellobacterin-T had shown (see Table 3) that Cellobacterin-T increased gut amylase activity in broiler by 35.5 % and protease activity by 9.1 % ($P < 0.05$) as compared to the control. At the same time, blood activity of pancreatic ferments increased: by 86.1 % for amylase, by 110.0 % for lipase, and by 20.7 % for trypsin ($P < 0.05$) as compared to the control. Lipolythic activity (in line B9) and proteolythic activity (in line B5) in duodenal contents increased due to the fermentative preparation supplement (see Table 3), with similar changes observed in blood.

It should be noted that used additives affect activity of the digestive ferments in poultry gastrointestinal tract interacting with a large number of physical and chemical factors, as well as with rapidly changing microbiota. Thus, the design of formulations and schemes of their application in poultry must be focused

on various properties and mechanisms of action of probiotics, phitobiotics, acidifiers, ferments, etc., to use them as the improvers of sustainability of gut microflora ecosystem and gut health, and also as the additional energy sources [28].

Therefore, hybrid broiler chickens overcame young birds of parental lines by activity of digestive ferments in duodenal chymus, except protease in maternal line. By blood amylase activity, hybrids were inferior to chickens of parental lines due to type of poultry nutrition. Use of probiotic did not have significant effect on duodenal enzymatic activity in healthy poultry, except for chickens of maternal line in which amylase activity increased and lipase activity decreased. Herewith, blood amylase activity increased in broiler chickens and chickens of maternal lines. Organic acids affect digestion by increasing gut lipase activity in chickens of parental lines, and also gut proteolytic activity in paternal line, whereas blood activity of digestive ferments increased in broilers and chickens of maternal line. Phytobiotics did not have significant effect on gut digestive ferments, but after entering blood, they increased trypsin activity in broilers and chickens of paternal line. Used fermentative preparations promoted higher activity of gut and blood digestive ferments in hybrids, except for gut lipase, higher duodenal protease in chickens of paternal line, and higher gut and blood lipase in chickens of maternal line.

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HAIR MACRO- AND MICROELEMENT LEVELS AS ESTIMATES OF MINERAL STATUS IN HORSES OF STUD AND LOCAL BREEDS FROM DIFFERENT RUSSIAN REGIONS

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Abstract

The optimal balance of chemical elements plays a vital role in the functioning of animal body. In horses, such balance is especially important because of specific hard work, in some cases on the verge of physiological limits. Besides, sometimes under the traditional technology of horse herd the rearing conditions may be extremal, for example, when horses are outdoors around the clock in severe winters of the northern Yakutia at extreme subzero Arctic temperatures. Estimate of mineral profile of a horse is the most important marker of physiological well-being, viability, productivity and adaptability to stresses. The purpose of this study was to identify levels of micro- and macroelements in hair of horses (*Equus ferus caballus*) of various genetic groups that are bred and used in various technological conditions in different regions of the Russian Federation. Totally, we used 198 hair samples of horses of stud breeds (Russian Trotter, Thoughbred, Purebred Arabian, Purebred Akhal-Teke) and local (indigenous) Bashkir, Kabardin, Mezenskaya, Vyatskaya breeds, Tuva and Yakut horses. The stud horses were kept at stables and the other in herds on pastures in 11 regions of the Russian Federation. In hair samples, 5 macroelements (Ca, K, Mg, Na, P) and 8 essential microelements (Co, Cr, Cu, Fe, I, Mn, Se, Z) were determined. Genetic determination of the content of macro- and microelements in the hair were evaluated in the Thoroughbred horse, Purebred Arabian and Purebred Akhal-Teke breeds (Stavropol Krai) kept under identical climatic conditions and common technology. Genetic profile was assessed for 16 autosomal DNA microsatellite loci, and the relationship between the level of homozygosity and distribution of chemical elements was evaluated in each of these breeds. All the studied groups significantly differed in hair chemical elements depending on genetic group (breeds), areal and rearing. Animals from the Republic of Sakha (Yakutia) had a deficit for 11 out of 13 chemical elements analyzed when compared to the average indices for all surveyed regions The Republic of Bashkortostan and the Republic of Kabardino-Balkaria shared the second place for the deficit of macro- and microelements, the Republic of Kalmykia and Stavropol Krai were the third and followed by Arhangelsk, Briansk and Lipetsk regions. The most favorable situation was in the Republic of Tyva and Krasnodar Krai where the surplus was registered for 12 and 11 elements respectively and some of them amounted up to 220 % compared to the average for the country. The advantage of stables keeping of horses, which provides the best balance of chemical elements in the body by regulation of nutrients in the diet, was observed. Hair mineral levels in horses of different origin correlated to their genetically homogeneous groups. Raising the level of hair

macro- and microelements and an increase in heterozygosity of the three purebred breeds, as detected by 16 DNA microsatellite loci polymorphism, was found out to correspond. The Akhal-Teke breed that is distinguished by a greater heterozygosity was the most well-balanced for macro- and microelements. The micronutrient level database for horses of various breeds and breed groups form different Russian regions is of interest for the studying factors determining mineral metabolism in animals that should facilitate the innovative development of horse breeding.

Keywords: *Equus ferus caballus*, horses, trotting and race breeds, herd breeds, indigenous breeds, microelements, hair level of microelement, area, rearing, microsatellite DNA, genetic studies

Macroelements and essential microelements are of vital importance in the metabolism. Stable pool of chemical elements is one of the key factors for normal functioning of every living system [1, 2]. Deviations in the chemical elements content, due to the action of exogenous and endogenous factors, lead to disturbances up to developing pathologies [3]. At elementosis, a shortage or excess of minerals causes significant harm to livestock by reducing productivity and fertility, slowing growth down, lowering the immune status, and increasing mortality of animals [4].

Radical intensification of technologies at robotized megafarms and big commercial farm complexes raises the relevance of the research focuses on the elemental status of farm animals and poultry [5]. Obviously, objective tests are required to control providing an optimal pool of nutrients. Recently, in this regard, the attention of researchers is attracted to the so-called accumulating biomaterials, i.e. skin epithelium and horny layer, hair, and bone tissue [6]. Unlike buffer systems (blood, urine, lymph), they reflect the long-term intake (within a month or more) of microelements in the body. Animal hair, meanwhile, serves as the most informative biomaterial, which recreates the elemental status of organism, as a whole [7, 8]. The constancy of hair chemical composition is provided by the keratin coat which prevents penetration of external contaminants and the loss of internal components [9]. The advantages of using hair to assess the elemental status of farm animals are safe and easy sample collection, including mass screening, which are simply to transport and store for a long time without the use of special equipment [10].

The importance of these data is due to a diversified uses of horses by people, from meat and dairy breeding to olympic sports, and it is especially relevant in Russia because of wide geographic spreading of hours breeds in contrasting natural and climatic zones [11].

This paper is the first to characterize the pool of basic macro- and microelements in Russian landrace and thoroughbred horse breeds under various breeding technologies in contrasting climatic conditions It was revealed that in the Republic of Sakha (Yakutia), animals suffer from deficit in 11 out of the 13 studied chemical elements, while the most prosperous regions are the Republic of Tyva and the Krasnodar Territory. The advantage of stable keeping was shown. Our findings showed rise of mineral concentrations in hairs of three purebred breeds as their heterozygosity increases.

Our aim was to reveal, based on the analysis of mane hair, the peculiarities of elemental state of horses (*Equus ferus caballus*) from heterogeneous genetic groups, with view to regional conditions of animal keeping and breeding.

Techniques. The survey (2017) involved adult horses from 11 Russian regions (the Republic of Yakutia, Tyva, Bashkiria, Kalmykia, Dagestan, Kabardino-Balkaria, Stavropol and Krasnodar, Arkhangelsk, Lipetsk, Bryansk regions). The stud breeds were Russian Trotter, Thoughbred, purebred Arabian, purebred Akhal-Teke, also local (indigenous) Bashkir, Kabardin, Mezenskaya, Vyatskaya breeds and Tuva and Yakut horses were involved. Horses were grouped according to the breed, the region of their origin, rearing and use, and also given the technology of their keeping. In each group, the average concentration of microele-

ments in the main hair was determined [12]. The group indicators were compared with each other and with the sample average [13].

Mane hairs were collected as per the instructions and recommendations of the Order of the Ministry of Health of the USSR of July 27, 1978 No. 701 "Additions to the Order of the Ministry of Health of the USSR of 12.08.77 № 755" and "The Guide for Care and Use of Laboratory Animals" (National Academy Press, Washington, DC, 1996). In the samples (n = 198), after digestion using a microwave SW4 (Berghof, Germany), the macro- and microelements were measured by atomic emission (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS) (a mass spectrometer Elan 9000 and an atomic emission spectrometer Optima 2000 V, Perkin Elmer, USA).

The amount of macro- and microelements in the mane hair, as depended on a genotype, was studied in Thoroughbred horse, purebred Arabian horse and purebred Akhal-Teke, with stabling in identical climatic conditions [14] (Tersky horse-breeding farm No. 169 and Stavropol stud farm No. 170, Stavropol Krai). Genotyping was performed with 16 autosomal microsatellite loci: AHT4, AHT5, ASB17, ASB2, ASB23, CA425, HMS1, HMS2, HMS3, HMS6, HMS7, HTG10, HTG4, HTG6, HTG7, and VHL20. DNA was extracted from hair follicles using the commercial ExtraGene™ DNA Prep 200 kit (Isogen Laboratory, Moscow). Separation and detection of amplification products was performed on a genetic analyzer AB 3130 (Applied Biosystems, USA). The degree of homozygosity was determined for each breed (the experimental samples included 319 purebred Arabian horse, 359 Thoroughbred horses and 141 Akhal-Teke horses).

Statistics was analysed using Microsoft Excel 2010 and Statistica 8 software (StatSoft, Inc., USA). While mathematical processing, the mean values (M), their standard errors (±SEM) and coefficients of variation (Cv) were calculated. Statistical significance of differences was assessed by Student's t-test at $p \leq 0.05$.

Results. In this report, we have analized the data on the five macroelements (calcium, potassium, magnesium, sodium, and phosphorus) and eight essential microelements (cobalt, chromium, copper, iron, iodine, manganese, selenium, and zinc). Also we have identified differences in this indicator between the investigated groups from different regions (Table 1). The highest calcium content was found in the horses from the Krasnodar Krai. Calcium participates in the formation of the bone tissue, it is important for the functioning of muscle tissue, heart, nervous system, skin. Hypercalcemia often leads to zinc and phosphorus deficiency in the body. The main causes of calcium deficiency are its inadequate intake with a diet, excess of phosphor, zinc, cobalt, magnesium, iron, potassium and sodium, as well as soft drinking water and shortage of vitamin D [15]. The minimum calcium amount was found in native horses from Yakutia and Bashkiria.

A high phosphorus level was observed in purebred riding horses from Dagestan. Phosphorus plays an important role in the brain function, cardiac and skeletal muscles. It also participates in transmembrane transport of the substances necessary for enzyme activity [16]. The lowest phosphorus content was pointed out for Vyatskaya breed horses from Lipetsk region and also for Yakut and Kabardino-Balkarian native horses.

The maximum concentration of potassium was found in horses of the Krasnodar Krai, whereas its minimum content was characteristic of native horses of Yakutia, Kabardino-Balkaria and Bashkiria. The highest magnesium level was in Mezen horses from Arkhangelsk region, the least among was detected in Yakutskaya breed. Herd horses from Tyva were characterized by significantly higher manganese and sodium contents compared to horses from other regions (see Table 1).

1. Chemical elements in mane hair (μg/g) of native and purebred horses from different Russian regions (*M*±*m*, 2017)

Region	Macroelements					Essential microelements							
	Ca	K	Mg	Na	P	Co	Cr	Cu	Fe	I	Mn	Se	Zn
1	883.16±48.21	385.73±62.55	259.88±13.90	185.68±25.88	361.87±9.89	0.04±0.01	0.49±0.05	5.39±0.16	82.28±15.34	0.42±0.06	5.10±1.18	0.08±0.01	125.37±3.51
2	1827.21±103.75	1518.95±360.78	471.84±51.96	1210.47±388.76	512.58±26.40	0.16±0.02	0.66±0.06	5.88±0.24	321.94±41.26	0.37±0.09	19.02±2.05	0.12±0.01	140.94±4.59
3	1064.05±66.84	560.25±84.61	314.05±22.09	261.89±63.43	430.40±14.65	0.09±0.01	0.80±0.14	5.83±0.22	161.28±20.93	0.29±0.06	5.55±0.57	0.22±0.02	142.80±4.33
4	1271.5±247.81	952.00±147.36	369.85±78.12	324.83±51.98	456.47±86.35	0.10±0.02	0.42±0.09	4.08±0.69	232.13±49.88	0.35±0.07	7.32±1.67	0.46±0.09	124.99±21.99
5	1479.75±215.77	918.50±70.44	474.25±43.80	282.50±48.42	700.00±43.64	0.09±0.04	0.27±0.10	5.49±0.20	180.74±78.28	1.38±0.81	5.34±2.25	0.53±0.023	124.50±7.66
6	1467.35±41.59	429.35±53.96	407.35±20.60	142.16±25.78	370.60±10.77	0.08±0.01	0.20±0.02	5.99±0.18	115.08±15.66	0.20±0.02	8.18±1.71	0.29±0.02	150.35±3.24
7	1243.89±86.65	878.92±105.48	534.37±48.25	311.81±45.48	557.63±20.86	0.07±0.29	0.29±0.04	5.94±0.19	164.18±23.07	0.34±0.06	4.86±0.95	0.51±0.02	136.88±3.16
8	2388.33±424.15	2953.17±1063.06	526.50±88.18	551.98±316.94	519.00±69.23	0.16±0.05	0.31±0.09	6.54±1.35	189.57±62.06	0.87±0.19	14.68±4.00	0.32±0.07	127.17±6.46
9	1420.05±108.09	1338.40±152.02	566.25±49.00	399.85±64.34	436.70±23.06	0.04±0.01	0.25±0.01	5.00±0.16	47.21±5.85	0.32±0.06	12.68±3.17	0.15±0.01	140.05±5.36
10	1369.60±93.74	1199.90±738.29	373.05±56.59	581.68±367.28	344.80±8.88	0.14±0.02	0.42±0.05	4.97±0.12	239.36±31.26	0.27±0.03	8.24±1.46	0.29±0.01	106.19±2.67
11	1291.46±57.24	709.53±99.95	376.87±20.00	170.08±27.83	575.40±27.93	0.12±0.01	0.34±0.04	6.26±0.13	442.53±61.40	0.40±0.13	12.95±1.65	0.11±0.01	130.53±3.86

N o t e. Republic of Yakutia (*n* = 30), 2 — Republic of Tuva (*n* = 19), 3 — Republic of Bashkiria (*n* = 20), 4 — Republic of Kalmykia (*n* = 6), 5 — Republic of Dagestan (*n* = 4), 6 — Republic of Kabardino-Balkaria (*n* = 20), 7 — Stavropol Krai (*n* = 38), 8 — Krasnodar Krai (*n* = 6), 9 — Arkhangelsk Province (*n* = 20), 10 — Lipetsk Province (*n* = 20), 11 — Bryansk Province (*n* = 15).

Among the groups, there were significant differences in the hair cobalt content: the highest amount was in horses from Tyva and the Krasnodar Krai, the least amount was in Mezen horses from Arkhangelsk region and native horses from Yakutia. Herd horses of native breeds from Bashkiria, Tyga and Yakutia were leaders in chromium content, while the least amount was characteristic of the animals from Kabardino-Balkaria. The Russian Trotter horses from Bryansk region were superior in the iron content, and the minimum amount was found in animals of local Mezen breed. The increased quantity of iodine has been detected in stud horses from Dagestan and the Krasnodar Krai, Kabardian horses showed lower iodine level. Excess selenium was found in Thoroughbred, Arabs and Akhal-Teke horses from Dagestan, the Stavropol Krai and Kalmykia, the selenium content was low in herd horses of native breeds from Yakutia and Tyva, as well as horses of Russian trotting breed from the Bryansk region.

To estimate total deficit (surplus) of all studied macro- and microelements in horses from different regions, a percentage of the regional average was calculated for each element (Table 2). This indicator makes it possible to compare the influence of various external factors on the hair elemental composition [13]. However, adding positive or negative deviations from the average even more apparently differentiates these regions in terms of the content of the most important chemical elements in the horse mane hair.

2. Deficit (–) and surplus of chemical elements in the mane hair of native and purebred horses from different Russian regions vs. the average per the country (%) (2017)

Регион	Macroelements					Essential microelements							
	Ca	K	Mg	Na	P	Co	Cr	Cu	Fe	I	Mn	Se	Zn
1	–32.16	–57.92	–38.2	–51.12	–21.92	–54.98	20.94	–5.02	–53.77	17.31	–40.06	–69.7	–7.37
2	40.36	65.7	12.21	218.63	10.59	80.07	62.89	3.62	80.9	3.34	123.53	-54.55	4.13
3	–18.26	–38.89	–25.31	–31.06	–7.14	1.29	97.45	2.74	–9.38	–19	–34.77	–16.67	5.51
4	–2.32	3.85	–12.04	–14.5	–1.51	12.55	3.66	–28.1	30.43	–2.24	–13.97	74.23	–7.65
5	13.67	0.19	12.78	–25.64	51.03	1.29	–33.36	–3.26	1.56	285.44	–37.24	100.75	–8.01
6	12.72	–53.16	–3.13	–62.58	–20.04	–9.96	–50.64	5.56	–35.34	–44.14	–3.87	9.84	11.08
7	–4.45	–4.12	27.08	–17.92	20.31	–21.22	–28.43	4.67	–7.75	–5.04	–42.88	93.17	1.13
8	83.47	222.15	25.21	45.3	11.98	80.07	–23.49	15.25	6.52	142.99	72.53	21.21	–6.04
9	9.09	46.00	34.66	5.25	–5.78	–52.73	–38.3	–11.89	–73.47	–10.62	49.02	–43.18	3.47
10	5.21	30.89	–11.28	53.11	–25.61	57.56	3.66	–12.42	34.5	–24.59	–3.16	9.84	–21.54
11	–0.79	–22.60	–10.38	–55.23	24.15	35.05	–16.08	10.31	148.66	11.72	52.19	–58.34	–3.56

N o t e. Republic of Yakutia (n = 30), 2 — Republic of Tuva (n = 19), 3 — Republic of Bashkiria (n = 20), 4 — Republic of Kalmykia (n = 6), 5 — Republic of Dagestan (n = 4), 6 — Republic of Kabardino-Balkaria (n = 20), 7 — Stavropol Krai (n = 38), 8 — Krasnodar Krai (n = 6), 9 — Arkhangelsk Province (n = 20), 10 — Lipetsk Province (n = 20), 11 — Bryansk Province (n = 15).

In the Republic of Sakha (Yakutia), horses had shortage of 11 out of the 13 studied chemical elements, comparing to the average for all the regions surveyed. The republics of Bashkiria and Kabardino-Balkaria shared the second place in terms of macro- and microelements shortage, the third was the Republic of Kalmykia and the Stavropol Krai, then Arkhangelsk, Bryansk and Lipetsk regions. The most prosperous were the Republic of Tuva and the Krasnodar Krai where there was a surplus for 12 and 11 elements, respectively, with some elements up to 220 % to the national average. Perhaps, the identified regional shortage of many chemical elements was associated with the biogeochemical peculiarities of a particular territory.

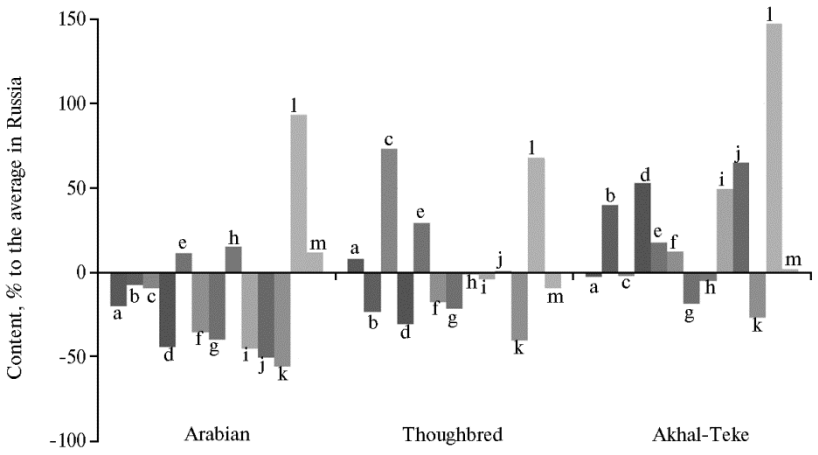
An explanation for the clear regional differentiation in the content of elements may be the fact that the horse hair composition is related to the technologies of keeping and feeding, which are mainly used in a particular region. So, in the Republic of Sakha (Yakutia), horses were kept outdoors all year round and used forage from under the snow in wintertime. Elemental composition of hair under herd technology was different from that of horses kept in stables on bal-

anced diets (Table 3). In herding technology, horses consumed pasture fodder and natural water throughout the year with minimal additional coarse fodder in the winter months, so the accumulation of elements in the hair corresponded to their quantity in the environment. Animals in the stables, as a rule, received the necessary elements with feed, feed additives and water in abundance.

3. Chemical elements (μg/g) in the mane hair of native and purebred horses from different Russian regions depending on breeding technology (2017).

Element	In total (n = 198)	Технология содержания			
		herd (n = 69)		stable (n = 129)	
	average in Russia	average	deviation from average in Russia	average	deviation from average in Russia
Ca	1301.76	1195.55	–106.21	1354.87	+53.11
K	916.71	748.36	–168.35	1000.89	+84.18
Mg	420.50	333.95	–86.55	463.77	+43.28
Na	379.90	489.96	+110.05	324.87	–55.03
P	463.49	423.23	–40.26	483.62	+20.13
Co	0.09	0.09	0.00	0.09	0.00
Cr	0.41	0.63	+0.22	0.29	–0.11
Cu	5.67	5.65	–0.02	5.69	+0.01
Fe	177.97	171.17	–6.80	181.37	+3.40
I	0.36	0.37	+0.01	0.35	–0.01
Mn	8.51	9.06	+0.55	8.23	–0.28
Se	0.26	0.13	–0.13	0.33	+0.07
Zn	135.35	134.71	–0.64	135.67	+0.32

N o t e. Herd technology was used in Republic of Yakutia, Republic of Tuva, Republic of Bashkiria, stable technology was used in Republic of Kalmykia, Republic of Dagestan, Republic of Kabardino-Balkaria, Stavropol Krai, Krasnodar Krai, Arkhangelsk Province, and Lipetsk Province.



Chemical elements in the mane hair of purebred Arabian (n = 20), Thoughbred (n = 20) and Akhal-Teke (n = 20) horses: a — Ca, b — K, c — Mg, d — Na, e — P, f — Co, g — Cr, h — Cu, i — Fe, j — I, k — Mn, l — Se, m — Zn (Stavropol Krai, 2017).

To assess the dependency of metabolic processes on the genetic heterogeneity, we compared hair samples from tribal horses of three purebred breeds, i.e. purebred Arabian, Thoroughbred and Akhal-Teke, bred without crossing for several centuries (Fig.). This has resulted in a specific genetic profile of each of the breeds. The influence of other factors (geochemical province and keeping technology) on the elemental state of main hair [14] was offset by the fact that the experiment was carried out in the same territory (the Stavropol Krai) under stabling technology. A trend toward increasing in heterozygosity at 16 autosomal microsatellite DNA loci corresponded to the vector of increasing content of chemical elements in mane hair of horses in three purebred breeds compared to the average value for all regions of Russia.

The Arabian breed, the most homozygous of these breeds (0.383), had

the greatest backlog in the content of the elements compared to the average value in Russia. Then there were purebred Thoroughbred horses with homozygosity of 0.339 and the most prosperous Akhal-Teke horses with homozygosity of 0.318. Probably, genetic polymorphism of a breed can be one of the factors which determine inherited intensity of the chemical element metabolism.

Data similar to ours and indicating the dependence of hair mineral composition (metabolic pool of chemical elements) of farm animals on the conditions of the biogeochemical province were obtained in other works [17-21]. There reports on a relationship between diet composition and the accumulation of chemical elements in body depots [22-25]. Data on the expressed link of the chemical elements metabolism with the horse's belonging to a genetically homogeneous group is in line with the results in laboratory animals [26].

Thus, we have found out distinctions in the mane hair levels of macro- and microelements which are related to the specific biogeochemical profiles of the territories, keeping conditions and the primary purpose of the breed use. Stabling was shown to have superiority because of ensuring the best balance of chemical elements in animal nutrition due to normalized composition of the rations, whereas at herding, animals receive chemical elements in the ratio characteristic of a particular biogeochemical province. For three purebred breeds studied, the vector of increasing amounts of hair macro- and microelements was in conformity with the vector of heterozygosity at DNA microsatellite loci. Akhal-Teke breed which is the safest in terms of element balance is also distinguished from the other two breeds, Thoroughbred and Arabian horses, by greater heterozygosity. Our research has shown the urgency of creating database on the micronutrient status of horses. This will bring to better understanding factors that determine metabolic processes in these animals, and improve horse breeding, which is original and important livestock branch.

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INVASIVE AND NONINVASIVE DETECTION OF ADAPTIVE RESPONSE IN MEAT POULTRY AFTER PREVENTIVE APPLICATION OF A STRESS-PROTECTIVE ANTIOXIDANT COMPOSITION

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Abstract

Prevention of technological stress among chickens at commercial faring is an important task but the methods for evaluating effectiveness of these measures in using pharmacological agents have not yet been developed. A method has been developed to diagnose the stressful conditions in commercial stock herds that makes it possible to control physiological state of chickens via corticosterone detection in poultry manure. The method is used to assess development of nonspecific adaptive response in chickens under the influence of various technological factors without direct impact on the body. Here, in farm trails of pharmacological prevention of stresses under intensive poultry production we first proved that the non-invasive assay of corticosterone level in manure extracts may estimate an adaptive response in chickens after technological stress. The SPAO complex (Stress-Protector Antioxidant, a pharmacological composition developed at the Department of Physiology and Pharmacology, South Ural State University) was used as an anti-stress agent. SPAO active complex contains lithium citrate, vitamins, vitamin-like substances etc. that affect metabolism. Blood and poultry manure samples were collected from the Hubbard F15 crossbred chickens during the transfer from the section of rearing flocks to the adult herd at 120 day-old poultry (group 1 of 12371 females and 1246 males, and control group of 12865 females and 1255 males). SPAO complex increased viability in the herd up to 4.6 %, egg production up to 2.53 %, average hatching up to 2.28 % and decrease feed costs per broiler chick on average by 16.0 % (presumably due to the direct effect of the components of the complex on the hypothalamic-pituitary-adrenal system). Pharmacological prevention of stresses allowed to extend economic use of hens in the experimental group for 2 weeks, to increase the gross yield of eggs up to 12.4 pcs whereas an earlier depletion of the productive performance occurred in the control group. In use of SPAO complex blood corticosterone level decreased 2.7 times and corticosterone in extracts of poultry manure was 22.3 % lower. Corticosterone levels in hen blood and the extracts from manure showed statistically significant correlations both before stressing ($r = 0.89$, $p < 0.05$) and after stressing ($r = 0.68$, $p < 0.05$) which indicates a high reliability of the proposed noninvasive method of stress diagnostics to estimate the effectiveness of pharmacological prevention of stress in industrial poultry.

Keywords: stress in chickens, pharmacological prophylaxis of stress, industrial poultry production, SPAO-complex, diagnostics of stress, non-specific adaptation reactions

Recognition and prevention of stresses in chickens remain an actual challenge of modern poultry farming [1, 2] in consideration of commercial efficiency coupled with requirements of humane technologies of animal rearing, welfare of animas raised for food and their protection [3]. In practice, laboratory methods for diagnosing stresses are practically not used [4, 5], and approaches which are based on monitoring of productivity, mortality and external signs of poultry adaptation to stress are not effective enough to diagnose stress reactions prior to the onset of pathological processes [6, 7].

Blood leukocyte indices [8] and level of hormones, mainly those of hypothalamic-pituitary-adrenal system [9], are characteristic indicators of the ac-

tivity of adaptation systems. However, they vary widely under the influence of various factors, including individual's features [10], are associated with circadian rhythms [11] and therefore are limitedly applicable in industrial conditions [5]. Blood sampling causes additional activation of bird's hypothalamic-pituitary-adrenal system [12] similarly to that under technological stresses. This diminishes practical effectiveness of stress monitoring though in laboratory studies these methods are acknowledged as the most accurate and reliable [13]. Indicators of bird's immune system activity, particularly, expression of the proinflammatory cytokines IL-1β, IL-6, heat shock proteins HSP-70 and lysozyme [14] has not been sufficiently studied and are not applicable in commercial poultry [15].

Non-invasive recognition of stresses in chicken based on estimates of hypothalamic-pituitary-adrenal-adrenal hormones in biomaterials collected without direct effects on animals, e.g. eggs [16], feathers [17] and litter [18] is a perspective direction in stress control. A.V. Miftakhutdinov [19] developed method based on specific markers of poultry physiological state that makes it possible to identify stress in meat chickens under commercial farming without direct exposure to a bird. This method is used in the analysis of nonspecific adaptation reactions under the influence of various technological factors.

In the present study, we at first time showed that the accuracy of the non-invasive determination of the amount of corticosterone in the litter extracts after the stress is sufficient to use this test when assessing the effectiveness of anti-stress therapy in commercial poultry.

The aim of the work was to estimate the reliability of the developed non-invasive method in detection of chickens' adaptive reactions in order to control the effectiveness of pharmacological prevention of stresses.

Techniques. Blood and litter samples collected from the chickens of the Hubbard F15 cross were studied at transfer of the replacement stock youngsters from the workshop for growing to the adult flock. The transfer was accompanied by intramuscular injection of Nobilis vaccines (MSD Animal Health, USA) against infectious rhinotracheitis, chicken infectious bronchitis, infectious bursal disease, Newcastle disease, chicken rotovirus tenosynovitis as per recommendations of the manufacturers.

Test group of 12,371 females and 1,246 males and control group of 12,865 chickens and 1,255 males were selected during growing of replacement stock. Poultry aged 120 days was transferred to adult flock. Observation was carried out until the end of the productive period, i.e. for 55 weeks in the test group and for 53 weeks in the control group. Survival rate, egg production, egg fertilization, hatchability, egg weight, and feed consumption were assessed. In the test group, the birds received a pharmacological complex for animals, a stress-protective antioxidant SPAO (white water soluble powder; developed in South Ural State Agrarian University), which includes lemon-acid lithium salt, vitamins, vitamin-like and other substances that affect metabolism [20]. Chickens drank the drug with water through water medicators at a dose of 185 mg/kg of body weight two days before the transfer and vaccination, and a day and for 2 days after transfer and vaccination. The control group did not receive SPAO at transfer and vaccination.

Blood was collected within 30-45 minutes after the stressing impact (transfer to another workshop and vaccination). Litter was collected 3 hours after manipulations. To avoid effect of circadian rhythm, samples were collected in the test and control groups at the same time.

Corticosterone was extracted according as per the protocol [19]. Blood and litter corticosterone level was assessed by a competitive enzyme immunoassay (ELISA) in a Tecan Sunrise ELISA analyzer (Tecan, Austria) at $\lambda = 450$ nm,

with DRG kits Corticosterone ELISA KIT (DRG, Germany) and corticosterone-conjugated peroxidase competing for binding to polyclonal antiserum to corticosterone which was used to cover wells of microplate. The smallest measurable concentration of corticosterone in the test is 1.63 nmol/l. The cross reactions for corticosterone reached 100 %, for progesterone were 7.4 %, for deoxycorticosterone were 3.4 %, for 11-deoxycorticosterone were 1.6 %, for cortisol were 0.3 %, and for other steroids were less than 0.1 % (according to the instructions attached to the kit). In accordance with the procedure [19], the corticosterone concentration in litter extracts more than 50 nmol/l indicates activation of the hypothalamic-pituitary-adrenal system and stress in meat chickens.

The table shows the arithmetic mean (X) and the squared deviations from the means ($\pm S_x$). Differences between the groups were estimated using the non-parametric Mann-Whitney U test (MW-U). The figure shows an average with a standard error (squares) and the minimum and maximum values. The Kruskal-Wallis test (KW-H) was used for intergroup comparisons. Non-parametric Wilcoxon test (W) was applied to compare the dependent variables within the group before and after exposure to factors presumably causing stress. Analysis of the statistical relationship between the experience and control before and after the stress was performed by the Kendall method of rank correlation.

Results. Chicken placement is preceded and accompanied by catching, estimating habit, transporting, intramuscular vaccination and preparing the body for egg production, breaking and forming new hierarchies of subordination in the herd, changing the lighting regime and diet. Due to a combined impact of several technological factors, this period is, in our opinion, one of the critical ones for the future productivity and reproductive qualities of the parent herd [21, 22], so we chose period to evaluate the antistress activity of the drug.

In total, the hens of the parent flock from the test group received the SPAO complex 5 times. As a result, in the test group (Table), as compared to control, survival for the whole period was 4.6 % higher, egg production was 2.53 % higher, egg yield per initial layer increased by 12.4 pcs., the average hatchability was 2.28 % higher, while the cost of feed per reproduction of one broiler chicken decreased by 16.0 % on average. The control hens were withdrawn from the production cycle 2 weeks earlier than the test birds because of an earlier depletion of the productive potential, which is manifested in a decrease in egg production and fertility. This may serve as one of the proofs of the expediency of pharmacological prevention of stress in commercial poultry farming.

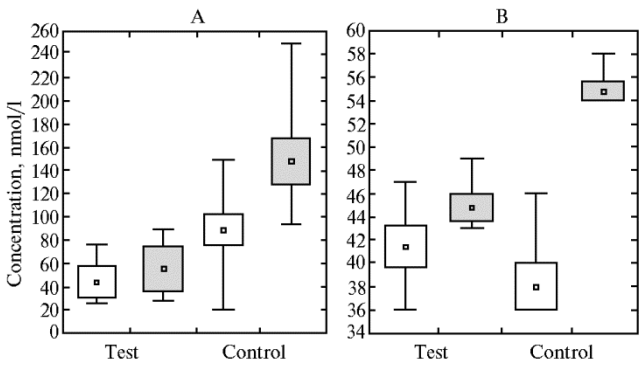
Productivity and reproductive parameters in Hubbard F15 cross in farm test ($X \pm S_x$)

Indicator	Test (n = 13617)	Control (n = 14120)	P
Finial survival in total, %	89.0	84.4	
Average survival of parent flock, %	93.92±3.64	91.69±4.65	P = 0.0439
Average egg-laying, %	70.34±10.35	67.81±9.59	P = 0.2219
Total egg output per laying hen, psc.	139.0	123.6	
Average number of eggs fertilized, %	80.56±7.60	77.65±4.64	P = 0.0174
Average hatching, %	77.30±4.14	75.02±3.93	P = 0.0178
Average egg weight, g	59.58±5.46	61.69±5.46	P = 0.1120
Average feed consumption per reproduction of one broiler chicken g	833.51±743.78	993.39±795.72	P = 0.0016

Glucocorticoids are commonly used to assess the extent of impacts on and adaptive responses of the body [15]. Prior to transfer and vaccination, the concentration of corticosterone in chickens from both groups was statistically equivalent (44.00±8.68 and 89.0 ±17.06 nmol/l, respectively).

After using the antistress agent in the test group, an increase in the concentration of blood corticosterone (up to 55.50±7.41 nmol/l) was not statistically

significant (P = 0.3105). However, the chickens of the control group experienced a sharp increase of corticosterone synthesis in response to stress (up to 148.14±26.52 nmol/l, P = 0.0425).



Corticosterone concentration in blood (A) **and litter extracts** (B) **of Hubbard F15 chickens prior** (left) **and post vaccination** (right) **with the use of a stress protector antioxidant preparation** (test) **and without stress protector** (control) (farm trials). A: prior to vaccination, Kruskal-Wallis test KW-H(1; 14) = 3.4554, p = 0.0630; post vaccination KW-H(1; 14) = 9.8216, p = 0.0017. B: prior to vaccination, Kruskal-Wallis test KW-H(1; 10) = 2.0979, p = 0.1475; post vaccination KW-H(1; 10) = 7.4503, p = 0.0063.

When recognizing nonspecific adaptation response by concentration of stress hormones, a significant problem is the activation of stress mechanisms at blood sampling, which affects the outcomes, leads to a distortion of the results and often does not allow obtaining reliable data [5]. Given this, we performed a noninvasive assessment of corticosterone in the litter extracts in the test and control groups (Fig.) to confirm the data obtained.

Before the transfer and vaccination, the amount of corticosterone excreted with litter (see Fig.) in test was the same as in control (P = 0.1475). After the vaccination, statistically unreliable (P = 0.0800) elevation of corticosterone level, from 41.40±4.04 to 44.80±2.68 nmol/l, occurred in the test birds. In the control, on the contrary, there was a statistically significant (P = 0.0431) increase in corticosterone excretion, from 38.00±4.47 to 54.80±1.79 nmol/l. Comparing the indices (see Fig.) indicates on a statistically significant increase in the amount of corticosterone in litter (by 18.2 %, P = 0.0063) of the chickens from the control group after a technological impact, as compared to the test birds.

In comparing corticosterone in blood and litter extracts in the test and in control birds, the Kendall rank correlation coefficient prior to stressing was statistically significant and high (r = 0.89, p < 0.0500), and post stressing value was statistically significant and medium (r = 0.68, p < 0.0500). The revealed decrease in the correlation coefficient after the manipulations can indirectly indicate that blood collection as an irritant has less effect on birds in a state of relative rest than on poultry exposed to this technological stress factor. The observed regularity agrees with the theory of nonspecific adaptive response according to L.Kh. Garkavi et al. (23) and other findings pointing to the potential effects of stressors of different strengths and duration [24]. At the same time, the observed high and medium coefficient of statistically significant correlations indicates the practicality of the method of noninvasive stress recognition in chickens by the corticosterone in litter extracts. Simple manipulations, clear results and the wide availability of ELISA make it possible to use the method we suggested in commercial poultry farming to recognize stress impact and to evaluate the effectiveness of the anti-stress therapy. When using the diagnostic method under consideration, it must be taken into account that the older the chickens, the less pronounced changes in blood glucocorticoids [6]. These differences, apparently, can be considered as a result of gradually progressing physiological and biochemical changes in the hypothalamic-hypophyseal-adrenal system, which facilitate a more rapid restoration of glucocorticoid level [25].

Thus, the pharmacological prevention of stresses during the growing of replacement stock youngsters and keeping adult parental flock makes it possible to increase the survival and fertility of the poultry and extend the period of its economic use. The use of a stress-protective antioxidant complex at vaccination and transfer of chickens slows down the stress development, as followed from a 2.7 times decrease in blood corticosterone level and a 22.3 % lower corticosterone in litter extracts. Decreased induction of corticosterone under the action of stressors is an important indicative parameter of the effectiveness of a pharmacological antistress agent. The corticosterone concentrations in blood and litter extracts statistically significantly correlate, which indicates the expediency of using the developed noninvasive method to determine the effectiveness of pharmacological prevention of stress in commercial poultry farming.

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OBTAINING A STABLE CELL LINE EXPRESSING RECOMBINANT I329L PROTEIN OF AFRICAN SWINE FEVER VIRUS

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Abstract

The African swine fever virus (ASFV), a large DNA virus with icosahedral morphology, is the only representative of the family *Asfarviridae*. ASFV has a wide list of mechanisms for evading the host's immune system. This fact hinders the development the vaccines against ASF. One of the approaches used by the virus in immune evasion is the mimicry of Toll-like receptors (TLR) by immunomodulating proteins. ASFV immunomodulatory proteins are the most valuable tools for the understanding of the pathogenesis of the disease and to create a means of combating the disease. One such ASFV protein is pI329L, the antagonist and TLR3 signaling inhibitor, which reduces the interferon response of the body. Protein pI329L inhibits TLR3-mediated activation of NF-κB and induction of INF-b through the activation of TLR3 with its ligand — viral DNA, RNA and poly (I:C). Removing this protein from ASFV particles is a rational approach to developing a weakened virus vaccine. Therefore, I329L is characterized as a viral TLR3 antagonist, which negatively affects interferon antiviral response of the host. Purpose of the work was to obtain a CHO cell line stably expressing a TLR3 antagonist, the recombinant I329L protein of ASFV. Here, we designed the plasmid pBMN-I329-his, carrying the full-length I329L gene with 6xHis-tag at the C-terminus. By electroporation with plasmid pBMN-I329-his of the CHO cell line and further stabilization on a selective antibiotic (5 μg/ml puromycin), a stable CHO-I329L-His cell line was derived. The insertion of the I329L gene into the genome of the cell was confirmed by PCR using primers of the specific gene, followed by nucleotide sequencing, using as the template DNA isolated from the cells CHO-I329L-His. Western Blot confirmed the presence of I329L protein in the cell lysates of CHO-I329L-His. As a result of the analysis it was established that the size of the recombinant protein was 55 kDa compared to calculated 35 kDa. The sequential deglycosylation of endoglycosidases PNGase and EndoH of the target protein resulted in an increase in its electrophoretic mobility and detection of specific bands of ~ 37 and ~ 35 kDa. This fact confirms the high degree of glycosylation of the target molecule, which leads to a lower electrophoretic mobility. Additionally, the recombinant I329L protein was recognized by hyperimmune sera against the ASFV, which indicated its authenticity. The obtained stable cell line CHO-I329L-His is deposited in the cell culture museum of Federal Research Center for Virology and Microbiology and can be used to study the mechanisms of action of immunomodulating proteins, such as pI329L of the ASFV, and, therefore, to get deeper insight of the African swine fever virus biology.

Keywords: African swine fever, ASFV, TLR3 signalling, protein expression, recombinant pI329L, stable cell line

African swine fever (ASF; the causative agent is African swine fever virus, ASFV, *Asfivirus*, *Asfarviridae*) is infectious disease of domestic and wild pigs, characterized by high mortality and contagiousness, which occurs in hyper-

acute, acute, subacute and chronic forms and can be transmitted from sick and infected animals through contacts and by alimentary ways. In the Russian Federation, the ASFV has registered since 2007. Over recent years, the country has considered as an area of disease risk [1].

The virus genome consists of linear double-stranded DNA (dsDNA) and is between approximately 170 and 190 kbp in size, depending on the isolate. All members of the pig family *Suidae* are susceptible to ASFV infection. The main virus reservoir is ticks *Ornithodoros* [2]. The genome nucleotide sequence of reference ASFV strain Ba71V contains 150 open reading frames (ORFs). In ASFV infected cells, 95 polypeptides with a molecular weight from 10 to 220 kDa were detected [1]. ASFV has various mechanisms for evading the host's immune system. It becomes the main hindrance for development the vaccines against disease [3]. One way of immune evasion is through mimicry of Toll-like receptors (TLR). These receptors are responsible for viral double-stranded RNA (DNA) recognition and activate innate immunity [4]. Nowadays, 13 mammalian Toll-like receptors are known. They are activated by various ligands, basically by bacterial and viral components. The receptors differ in adaptor peptides with which their cytosolic fragments are linked [5].

One of the ASFV proteins involved in the modulation by the mediated Toll-like receptor of host defense, is pI329L, the TLR3 antagonist and signaling inhibitor [6, 7]. The functional analysis indicates that pI329L inhibits TLR3-mediated activation of NF-κB and IFN- b through the TLR3 activation with ligand, i.e. the viral DNA, RNA or poly (I:C) [8, 9]. So, the I329L protein serves as a viral TLR3 antogonist that negatively impacts on the interferon antiviral host response [10]. Nowadays, there is no information about the I329L protein of ASFV which is circulating in Russia. There is no data on its genetic stability [11, 12], and also no tools (e.g. stable cell line) for studying mechanism of immunomodulating activity of pI329L protein of Russian ASFV isolates.

In this paper, we for the first time reported data on the recombinant I329L protein authentic to viral ASFV protein, which was produced in Chinese hamster ovary (CHO) cell lines.

The aim of the research is obtaining a stable cell line to express recombinant I329L protein of the African swine fever virus.

Technique. The nucleotide and amino acid sequences of I329L protein was taken from the UniProt database (reference number E0WM90). pBMN-I329L-his plasmid was designed in Clone Manager 7.0 software (Sci-Ed Software, USA). DNA was isolated from the reference strain Stavropol 01/08 ASFV (State Collection of FRCV&M) [20] using the QIAamp DNA Blood Kit and Mini Kit (Qiagen N.V., Germany).

PCR was performed using Phusion polymerase (NEB, USA) according to the manufacturer's instructions. The reaction mixture consisted of 10 μl of 5× Phusion HF buffer, 1 μl of 10 mM dNTPs, 2 μl of 10 μM F329his, 2 μl of 10 μm R329his, 1.5 μl of DMSO, 0.5 μl of Phusion DNA Polymerase, 1 μl of viral DNA and 31 μl of sterile water. A pair of primers used was F329his (forward) 5′-TATATAAAGCTTGCCACCATGCTAAGGGTTTTCATA-3′, R329his (reversed) 5′-TATATATCTAGATTATCAATGGTGGTGGTGATGGTGCTTTCTTCTTA-CACGAGA-3′. R329his containes a His-tag peptide at the C-terminus. Amplification of the target gene was carried out according to the protocol: pre-denaturation for 5 min at 98 °C; denaturation for 30 sec at 98 °C, primer annealing for 30 sec at 52 °C, elongation for 60 sec at 72 °C (25 cycles). An amplified product of the expected size was identified in 1.3 % TBE-agarose gel with ethidium bromide.

DNA was purified from the agarose gel using the Gel Extraction Kit

(Qiagen N.V., Germany) according to the manufacturer's instructions. The PCR product of *I329L* was cloned at HindIII and XbaI restriction sites (NEB, USA) into the pBMN plasmid vector (Addgene, USA), having ampicillin and puromycin resistance genes. Ligation and insertion were performed at a room temperature for 1 hour using T4 DNA ligase (NEB, USA) in a volume of 5 μl (0.5 μl of 10× ligase buffer, 1 μl of the linearized pBMN vector, 0.5 μl of *I329L* PCR product, 0.5 μl of T4 DNA ligase and 2.5 μl of sterile water).

Transforming of *Escherichia coli* XL-10 Gold strain, genotype endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tetR F′(proAB lacIqZΔM15 Tn10(TetR Amy CmR), was made using ligated mixture by standard heat shock method. Plasmid DNA was isolated with Plasmid Mini Kit (Qiagen NV, Germany) The isolated plasmid DNA of clones were tested by analytical restriction with the use of flanking endonucleases HindIII and XbaI. The reproduction accuracy of the cloned fragment was confirmed by sequencing on a genetic analyzer Applied Biosystems 3130xl (Applied Biosystems, USA).

Transfection of the CHO cell line (Chinese hamster ovary cells) was performed by electroporation on a Gene Pulse Xcell device (Bio-Rad, USA). The continuous cell line CHO was incubated in a shaker at 37 °C and 5 % CO_2 in BalanCD medium (Irvine, USA) with 500,000 units of penicillin, 100 μg/ml of streptomycin, and 4 mM L-glutamine. Viability and the amount of living cells were evaluated by staining with 1 % trypan blue solution (Gibco, USA). For this, to a vial containing 20 μl of the cell suspension, 80 μl of 1 % trypan blue solution which stains dead cells was added and shaken vigorously. The results were integrated on an automatic cell counter NucleoCounter NC100 (ChemoMetec, Denmark).

Protein I329L of ASFV was isolated using magnetic particles DynaBeads His-tag (Thermo Scientific, USA). Peptide N-Glycosidase F (PNGase F) (NEB, USA) and endoglycosidase H (EndoH) (NEB, USA) were used for I329L protein deglycosylation. Detection of the target protein was performed by western blotting in a 12.5 % polyacrylamide gel, followed by transfer to a Trans-Blot Turbo system (Bio-Rad, USA) on a nitrocellulose membrane (Bio-Rad, USA). The membrane was incubated with rabbit polyclonal antibodies to 6× His-tag (HRP) (Abcam, USA). The chemiluminescent reagents were used to visualize the reaction (Advansta, USA). The specificity of the recombinant I329L protein was recognized in reaction with hyperimmune swine antisera against the Stavropol 01/08 ASFV (State Collection of FRCV&M) from infected animals, and with commercial species-specific goat antibodies against pig IgG, conjugated with horsradish peroxidase (HRP) (Santa Cruz Biotechnology, USA).

Results. In this research, we used DNA isolated from culture of pig bone marrow cells infected with Stavropol strain 01/08 ASFV. The resulting matrix was used in PCR with gene specific primers (F329his and R329his) to amplifyfragment with restriction sites for subsequent cloning into the pBMN vector. A resultant PCR product of 1023 bp was obtained. In ligation, the ratio of the vector and the insert was 1:3.

Analytical restriction at flanking endonuclease sites confirmed the presence of a specific insert of *I329L* gene (Fig. 1, B) in four clones. They were verified by sequencing for the insert accuracy and the presence of substitutions or deletions. A comparative alignment of nucleotide and amino acid sequences selected the pBMN-I329L-his clone, identical in its sequence to the reference gene *I329L* of strain Georgia/wb/2007 ASFV (FR682468).

To obtain stable CHO-I329L-His cell line, a culture of CHO cells in the phase of logarithmic growth was used. The cells viability after transfection with

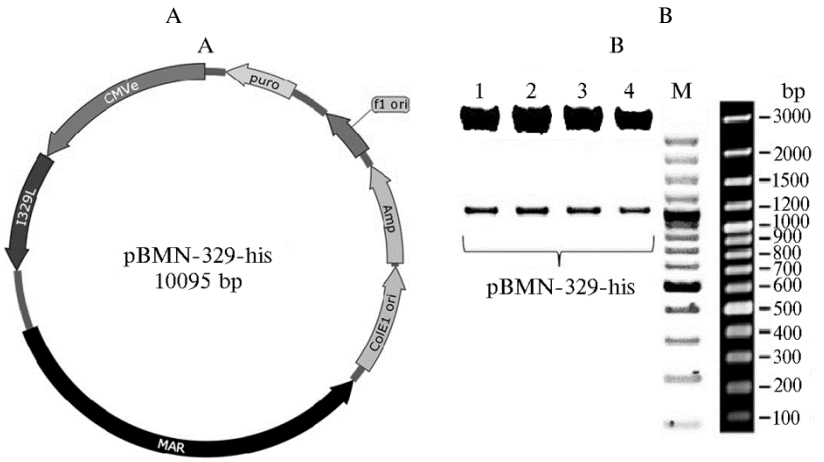

Fig. 1. Map of pBMN-I329L-his plasmid caring the target *I329L* gene of African swine fever virus (ASFV) (A) **and screening the plasmid pBMN-I329L-his clones by analytical restriction using HindIII and XbaI** (NEB, USA) (B)**:** 1-4 — clones Nos. 1-4 pBMN-I329L-his, M - molecular weight marker 100 bp Plus (Fermentas, USA).

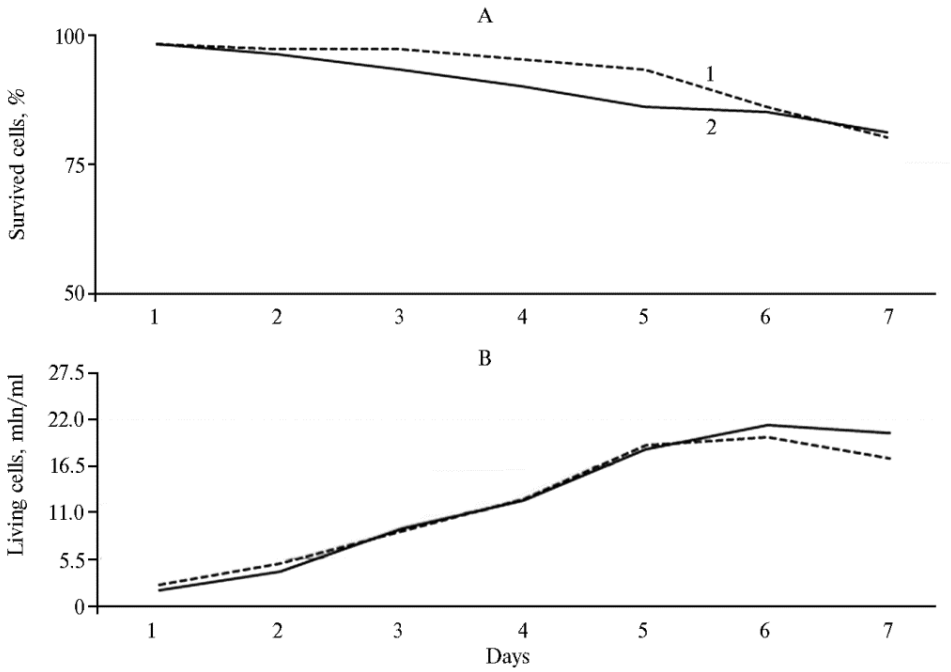

Fig. 2. Vitality (A) **and growth rate (B) of the CHO-I329L-His cell line** (1) **and the original CHO cell line** (2) **during 7-day culture in BalanCD medium supplemented with 5 μg/ml puromycin** (Appli-Chem, Germany).

pBMN-I329L-his plasmid was tested in 24 and in 48 hours with a trypan blue. Twenty-four hours after CHO cells transfection with pBMN-I329L-his plasmid, cells viability was 70 %, the counts of the living cell suspension was 1.0 million/ml; in the control transfection without plasmid, these values were 92 % and 1.0 million/ml, respectively. Analysis performed repeatedly 48 hour after transfection showed that the proportion of living cells increased up to 83 %, and the amount of living and transfected plasmid cells were up to 2.2 million/ml; in control, the figures increased to 94 % and 4.0 million/ml, respectively. The obtained data confirmed the efficiency of CHO cell transfection and the possibility of further selection of transfected cells in a puromycin containing medium (Ap-

pliChem, Germany). Forty-eight hours after transfection, the overall cells pool was transferred to a BalanCD growth medium with puromycin at 5 μg/ml final concentration.

We evaluated the dynamics of viability and the counts of viable transfected cells in comparison to the parent CHO cell line (Fig. 2) in model 7-day culture of suspension. The results showed that the 80 % threshold of viability was reached only on day 7 of culture. Over 7 days, in the stable CHO-I329L-His cell line, there were more than 80 % of viable cells.

ASFV *I329L* gene integration into CHO cell genome was confirmed by PCR with the reference primers for b-actin (NM_001244575.1) and cytochrome b (AB033693) of CHO cell line as an internal positive control. As a result, the *I329L* gene sequences were detected in total genomic DNA isolated from the stable CHO-I329L-His cell line (Fig. 3, A).

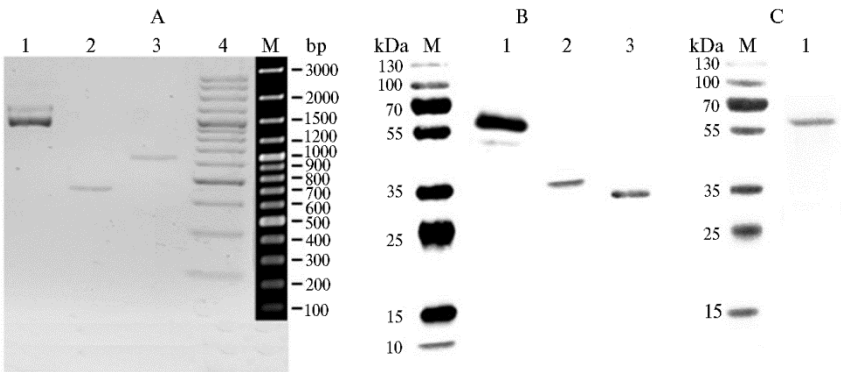

Fig. 3. Analysis of ASFV gene *I329L* integration and CHO-I329L-His cell line by PCR and immunobloting.

A: Detection of the *I329L* gene in CHO-I329L-His cells by electrophoretic separation of PCR products: 1 — amplified *I329L* gene from stable cell line CHO-I329L-His; 2 — amplified b-actin gene from stable cell line CHO-I329L-His; 3 — amplified cytochrome b gene from stable cell line CHO-I329L-His; M is molecular weight marker 100 bp Plus (Fermentas, USA).

B: Immonoblotting of ASFV recombinant protein I329L with His-taq antibodies after deglycosylation by endoglycosidases EndoH and PNGaseF: 1 — protein I329L isolated on magnetic particles DynaBeads His-tag (Thermo Scientific, USA); 2 — protein I329L cleaved with endoglycosidase EndoH (NEB, USA); 3 — protein I329L cleaved with endoglycosidase PNGase F (NEB, USA); M is the molecular weight marker Page Presteined Rule (Fermentas, USA).

C: Immonoblotting of recombinant protein I329L bound to hyperimmune serum antibodies against ASFV: 1 — I329L protein isolated on DynaBeads His-tag magnetic particles (Thermo Scientific, USA); M is molecular weight marker Page Presteined Ruler (Fermentas, USA).

Analysis of the target ASFV I329L protein expression was performed by western blotting with specific antibodies to His-tag located at C-terminus of the molecule. To do so, cells (5×10) selected from the continuous cell line were lysed, followed by concentration with DynaBeads His-Tag magnetic particles. Western blot confirmed the presence of protein I329L in the stable CHO-I329L-His cell line. However, the expressed recombinant I329L protein of 55-60 kDa in size (see Fig. 3, B) did not match to expected molecular weight of 35 kDa. This fact can be explained by the higher glycosylation of I329L protein, which reduces electrophoretic mobility of the molecule. These results are also confirmed by bioinformatic analysis which showed 9 N-glycosylation sites [15, 17[. An additional weak band of 35 kDa (see Fig. 3, B) could be explained by the protein translation from one of the three start codons (ATG) in its extracellular domain [17, 18]. It should be noted that the electrophoregram also showed traces of the target protein proteolysis.

As a result of treatment of the recombinant protein I329L with endogly-

cosidases, its molecular weight decreased to ~37 kDa for EndoH and ~35 kDa for PNGaseF, so originally, the expressed protein I329L was highly glycosylated (see Fig. 3, B). Besides, the molecular weight of I329L treated with PNGaseF was lower than that after EndoH treatment, indicating the presence of a small amount of complex glycans.

In immunoblotting test of binding I329L with hyperimmune serum of animals infected with strain Stavropol 01/08, the target protein I329L formed immune complexes with polyclonal antibodies to ASFV (see Fig. 3, C).

Thus, we developed stable CHO-I329L-His cell line which express the recombinant full-length transmembrane I329L protein of the African swine fever virus ASFV. The CHO-I329L-His cell line has similar phenotypic and growth properties with the parent CHO line. The ASFV recombinant protein I329L is non-toxic for CHO cells. The insertion of the *I329L* gene into genome of CHO cells was confirmed by PCR and by analysis of the target protein expression. The presence of glycosylated forms of protein I329L was revealed. Binding I329L with hyperimmune serum antibodies against ASFV is indicative of its antigen specificity. The CHO-I329L-His cell line is a unique model to study the ASFV interaction with a cell and to develop candidate vaccines based on defective recombinant ASFV. Stable cell line CHO-I329L-His was deposited in the Cell Culture Museum of the Federal Research Center for Virology and Microbiology. The cell culture can be used in studying mechanisms of immunomodulating protein activity and will provide new data on ASFV biology.

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IDENTIFICATION OF THE BOVINE ATYPICAL PESTIVIRUS IN BIOLOGICAL SAMPLES

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Abstract

Atypical cattle pestivirus (BVDV3; HoBi-like) is an unclassified candidate for the genus *Pestivirus* of the *Flaviviridae* family. The agent was isolated for the first time in 2004 from fetal bovine serum from Brazil, and shows a high degree of similarity with the BVDV1 and BVDV2. Its presence in the cattle populations can potentially reduce the effectiveness of control programs of bovine viral diarrhea. The article presents the results of developing a method for identifying of BVDV3 in the biological samples based on polymerase chain reaction. Synthetic oligonucleotide primers complementary to positions 9202-9218 and 9501-9521 of the reference strain D32/00_"HoBi" genome were selected. The basic parameters of the reaction have been worked out. The sensitivity of PCR was 7.4×10^{-1} copies/μl. It has high specificity and does not reveal RNA of the BVDV1, BVDV2 and classical swine fever virus. With the help of the developed PCR 18 samples of fetal bovine serum (FBS) from various sources, 11 types of continuous cell culture lines used for virus cultivation in several Russian research institutes, 10 attenuated live vaccines, 189 internal organs, 1383 blood sera from cattle, 168 blood sera from reindeer, and 63 blood samples from red deer were investigated. The virus was revealed only in seven lots of FBS obtained from two manufacturers and produced in South America. Phylogenetic analysis of amplicons showed all positive lots grouped with BVDV-3 strain D32/00 _"HoBi" (Brazilian group). Given the potential risk of using contaminated fetal serum, further research of the spread of BVDV3 in Russia is needed. The virus was not found in continuous cell culture lines, vaccines used for human, cattle and small domestic animals. Additionally, no evidence has been found out of virus circulation among cattle of various breeds, including those imported from another countries, reindeers and red deer in the Krasnodar territory, Siberia and the Republic of Kazakhstan. The presence of the virus in the FBS used in the production of vaccines does not exclude its spread in Russia. The findings confirm the need for continuous updating and improvement of methods for diagnosing pestiviruses and tightening the rules for the international FBS trading.

Keywords: atypical pestivirus, BVDV3, HoBi-like virus, primers, polymerase chain reaction, cell cultures, fetal bovine serum, vaccines, phylogenetic analysis, cattle, reindeers, red deer

Pestivirus infections cause significant economic losses in dairy and beef cattle farming worldwide. Nowadays, the greatest concern is Bovine viral diarrhea virus (BVDV) which affects mucous membrane, especially two prototype BVDV species of the genus, BVDV types 1 and 2 (BVDV1 and BVDV2). Infection of non-immune animals leads to subclinical pathologies, immunosuppression, diarrhea, respiratory diseases, reproductive pathology and mucosal disease of persistently infected calves [1-4].

Atypical pestiviruses are new, officially not classified group of *Pestivirus*, *Flaviviridae*, tentatively called Bovine viral diarrhea virus type 3 (BDVV3) or atypical pestiviruses (HoBi-like) [5]. The agent was first isolated in Europe from fetal bovine serum imported from Brazil [6]. Later, the pathogen was identified in fetal serums, which were isolated in Australia, Mexico, the USA and pack-

aged in Europe [7-10]. Cattle and buffalo natural infection caused by BVDV3 was reported in Southeast Asia [11-12], in Italy [13-15], and in India [16]. BVDV3 infection is closely related to bovine viral diarrhea — mucosal disease (BVD-MD) and may discredit its control causing false-positives results in diagnostics and impaired prophylactic efficiency of vaccination [5, 17]. Wide use of fetal serum and intensive international trade in purebred breeding animals may lead to transmission of infection worldwide [5, 18], and therefore it is necessary to develop modern highly sensitive and specific diagnostics to detect the viruses of this group, and studying their circulation in different areas, including Russia.

In accessible domestic literature, we could not find any report about PCR detection of atypical bovine pestivirus. Here, this is the first such report on rapid and sensitive method we suggested and used to reveal and sequence atypical bovine pestivirus genome in seven imported batches of fetal bovine serum.

The aim of the research was development of polymerase chain reaction protocol for detection of atypical pestivirus in biological products and studying its circulation among domestic and wild ruminants.

Techniques. To select oligonucleotide primers, we aligned available genomic sequences of BVDV1, BVDV2 and BVDV3 strains from the GenBank (http://www.ncbi.nih.gov) with the use of ClustalW software [19]. BVDV1 strains were NADL (AJ133738.1), Singer (DQ088995.1), Osloss (M96687.1), and PT-810 (AY078406.1); BVDV2 stains were US890 (Z79772.1) and Giessen-6 AY379547.1; and four atypical pestivirus strains were D32/00_"HoBi" (AY489116.1), Th/04_KhonKaen (NC_012812), SVA/cont-08 (FJ232692.1) and IZSPLV_To (HM151361.1). Primers were chemically synthesized by amidophosphite method (an automatic synthesizer ASM-102U, Bioset, Russia). Concentration of primers in the stock solutions was determined spectrometrically.

Viral RNA was isolated using commercial RIBO-prep kit reagents (Central Institute of Epidemiology of The Federal Service for Supervision of Consumer Rights Protection and Human Well-Being, Russia) according to the manufacturer's instruction. The resultant cDNA was diluted 2-fold with 1× TE buffer to 40 μl volume and used for PCR. PCR products were analyzed by horizontal electrophoresis in 2 % agarose gel in Tris-borate buffer with 0.4-0.5 μg/ml ethidium bromide followed by visualization in short-wave UV at $\lambda = 254$ nm (UVT-1 Transiluminator, Biokom, Russia). PCR outcomes were considered positive when a 320 bp fragment was obtained.

Positive control samples (PCS) were obtained by molecular transformation of *Escherichia coli* with pDrive plasmid which contains specific DNA insertion. Plasmid DNA concentration was determined using Quant-iTdsDNA, HS assay kit (Invitrogen, USA) on a QUBIT fluorometer (Invitrogen, USA); the final concertation was 0.333 μg/μl (7.4×10^{10} copies/μl). To assess PCR sensitivity, 10-fold dilutions of PCS were used. The analytical sensitivity was the last PCS dilution resulted in positive PCR outcomes. The specificity was assessed with strains Oregon C24VBVDV1, BL BVDV2 and Shimen isolate of classical swine fever virus (collection of Siberian Federal AgroBioTech Center RAS).

RNA fragment specificity was confirmed by nucleotide sequencing and purification on Sephadex G-50 superfine (GE Healthcare, USA). PCR fragment sequencing was performed for both DNA strands. Primary data of sequencing was analyzed by Sequencher v.4.0.5 software (Gene Codes Corporation, USA). Sequencing was performed with BigDye 3.1 kit (Applied Biosystems, USA) according to the manufacturer's protocol. The reaction mixture (5 μl volume) contained 2 μl solution from the sequencing assay kit, 5 pM oligonucleotide primer and 0.5 μg DNA template. PCR was carried out in a programmed thermostat GeneAmp PCR-system 6700 (Applied Biosystems Inc., USA) and included 30

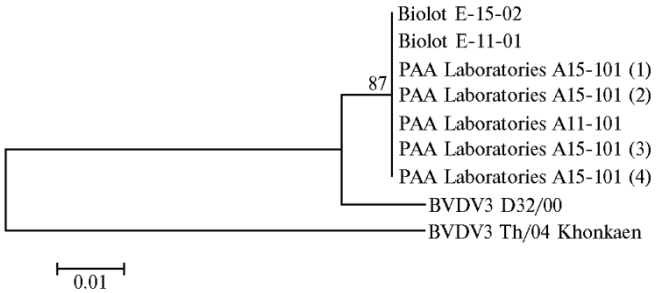
cycles as follows: 10 s at 96 °C, 15 s at 50 °C, and 4 min at 50 °C. After amplification, unbound labeled nucleotides were removed from the reaction mixture on a G-50 Sephadex superfine column. Both DNA strands were sequenced. Primary data of sequencing (chromatograms) was analyzed by Sequencher v.4.0.5 software. A highly conserved 5′-UTR region of pestivirus genomes was used for sequencing. The synthesized fragments were analyzed by a comparison with known sequences of other BVDV3 strains (particularly, BVDV3_D32/00 and BVDV3_Th/04_Khonkaen) using ClustalW multiple sequence alignment program [19].

Eighteen batches of fetal bovine serum from South America, the USA, and New Zealand (twelve, five and one batch, respectively) were tested. In tests, we used continuous cell cultures of bovine coronary artery (BCA), bovine kidneys (MDBK and Taurus), embryonic bovine testis (EBT), rabbit kidneys (RK-13), African green monkey kidney (VERO), mouse fibroblasts L929 and MF, baby Syrian hamster kidney (BHK-21), fetal bovine trachea (FBT), fetal lamb kidney (FLK), feline kidneys (FK-81) (collections of research institutes). Additionally, ten live vaccines for medical and veterinary uses were tested. Also, the biomaterials for PCR assay of viral genome were organs, blood and blood serum of animals of different age and sex groups from Novosibirsk, Tyumen, Omsk, Kemerovo and Kurgan regions, Altai, Krasnoyarsk and Krasnodar territories, the Republic of Kazakhstan, and also of Taimyr and Yamal reindeer and marals from Altai Krai.

Results. We found several species-specific regions of BVDV3 genome. Within each, using Oligo Analyser v.6.31 program, we chose oligonucleotide primers which provide specific amplification of BVDV3 RNA. In pre-tests, the most successful primers, in terms of the resulting PCR product quality, were those complementary to positions 9202-9218 and 9501-9521 of a prototype strain D32/00_“HoBi” (AB871953.1) genome. These primers, i.e. SEQIDNO:1 5′-TTTGCAGCCGAGCGTAG-3′ and, SEQIDNO:2 5′-CCTCCTGCATAC-TGTCACCTT-3′, were used in PCR tests. The sensitivity of the developed PCR was 7.4×10^{-1} copies/μl, with no amplification of genome fragments of other viruses. Most researchers use the 5′-UTR for sequencing. It provides the most accurate results, especially in attributing isolates to species or types (genotypes). 5′-UTR sequences are most often chosen as primers [8, 10, 11, 18]. We also used this region as a target. The primers we developed had sensitivity and specificity similar to those reported by other researchers, and, when used in sequencing, revealed Brazilian group virus in tested fetal sera.

HoBi-like virus was first isolated and characterized by H.G. Schirrmeier et al. [6] in Germany in a fetal serum batch derived from Brazil and packed in Europe. The isolate designated as D32/00_“HoBi” was considered a Brazilian group virus prototype. Then, the genetically varying subtypes having regional distribution, in particular a Thai group, were identified [11, 12]. N. Mishra et al. [16] supposed the existence of a third, Indian group of strains. There may also be a fourth virus group outside the Indian region, particularly in Italy [13-15]. Hence to date, four genetic virus groups have been identified. In available domestic literature, there is a report on detecting atypical pestivirus in a commercial vaccine against plague of small ruminants in the Republic of Tajikistan [22]. HoBi-like virus was identified in seven samples of fetal sera from two producers of South America (Fig.). Phylogenetic analysis revealed clustering of all tested samples with D32/00_“HoBi” strain of Brazilian group.

It should be noted that we did not find BVDV3 either in tested 10 live vaccines, intended for immunization of human, farm and small domestic animals, or in 11 tested continuous cell lines. Also, the BVDV3 genome was not found in



Unrooted phylogenetic tree constructed by a Neighbor joining method (NJ) in MEGA v.4 using Kimura model. Bootstrap support values are shown in the nodes. Samples PAA Laboratories A15-101 (1-4) came in from different sources.

189 samples of organs and in 1383 blood serum samples of various cattle breeds, including imported ones, and in 168 blood sera of reindeer and 63 blood samples of marals. Although we have not ascertained atypical pestivirus circulation in the surveyed areas, its presence in Russia is quite possible, because the pestivirus was detected in seven batches of fetal sera that have been used for several years in four Russian institutes not only in researches but also for vaccine production.

Thus, based on PCR with reverse transcription, we developed a highly sensitive specific method for atypical pestivirus detection and identification in biological material. The use of synthetic oligonucleotide primers, complementary to positions 9202-9218 and 9501-9521 of the reference strain D32/00_"HoBi" (AB871953.1) genome, allows detection of virus sequences with a sensitivity of 7.4×10^{-1} copies/μl. The BVDV3 contamination of seven fetal bovine serum samples from South America has been determined. Phylogenetic analysis revealed the similarity of the identified viruses to each other and to the strain BVDV3 D32/00_"HoBi" (Brazilian group). We did not find evidence of the atypical pestivirus circulation among domestic and wild animals on the territory of some Russian regions and the Republic of Kazakhstan. The obtained data confirms the need to continuously update and improve bovine pestivirus diagnostics, and to revise international regulations for fetal serum trade, including strict control of their enforcement.

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PERMISSIVITY OF VARIOUS CELL CULTURES TO LUMPY SKIN DISEASE VIRUS

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Abstract

Lumpy skin disease (LSD) is a transmissible and highly contagious transboundary emergent bovine viral disease that has become especially important for the Russian Federation since 2015 when it entered the Republic of Dagestan from Azerbaijan. In 2016, the infection was found in the Krasnodar Territory and later on in six more regions of the Russian Federation. The infection causes up to 50 % drops in milk productivity, body weight loss, abortions or stillbirths, skin damage, and reproductive disorders in affected livestock up to including a complete loss of bovine fertility and animal deaths due to secondary infections. LSD is caused by a DNA virus of family *Poxviridae*, genus *Capripoxvirus*. The virus isolation, identification and vaccine or diagnostic preparation construction largely depends on the adequate culture system used. This research was aimed at characterization of the cultural properties of an LSD virus isolate detected in internal organ (lung, spleen and lymph nodes) or affected subcutaneous tissue samples from Volgograd region of Russia. In order to isolate the virus, a goatling testicle primary culture (GT), a calf kidney (MDBK) and a rabbit kidney (RK-13/2-03) continuous cell lines were used. In passage 3, the virus titer obtained in cells MDBK and RK-13/2-03 was 4.67 to 5.00 lg $TCID_{50}/cm^3$. Using PCR analysis, a LSD virus genome was detected in the virus-containing culture medium. The obtained LSD virus strain was deposited to the State Collection of Microorganisms of the Federal Research Center for Virology and Microbiology, # 3161. Also, the permissivity of some other cell lines including elk embryo skin (KEL/07), African green monkey kidney (CV-1) and VERO cells, a hybrid line of porcine embryo kidney cells (SPEV TK^-) × porcine spleen splenocytes (A_4C_2/9k), and sheep kidney (ShK), rabbit kidney (RK-13/2-03) and calf kidney (Taurus-1) cells to this LSD virus strain were determined. We found that some continuous cell lines of both homologous (MDBK, Taurus-1, KEL/07, ShK) and heterologous (RK-13/2-03, VERO, CV-1, A_4C_2/9k, SPEV) origin were sensitive to the LSD virus. This work has revealed for the first time ever that LSD virus can proliferate in cells of wildlife species like elk. Also, permissivity of some heterologous continuous cells, RK-13/2-03 and A4C2/9k, to LSD virus was revealed for the first time. The virus culture period until 95 to 100 % CPE depended on the cell substrate selected and the multiplicity of infection. Thus, for MDBK or VERO cells it was 48 hours, and for Taurus-1, SkK, RK-13/2-03 or CV-1 the maximal destructive alterations in the cell monolayers were observed within 48 to 96 hours post infection. With an optimal multiplicity of infection of 0.001-0.00001 $TCID_{50}$ per cell and 2-5 % cattle serum in the maintenance medium the LSDV titers were 6.0 to 6.8 lg $TCID_{50}/cm^3$in the ShK and VERO cells, and 5.8 to 6.6 lg $TCID_{50}/cm^3$ for RK-13/2-03.

Keywords: lumpy skin disease virus, continuous cell lines, embryonic elk skin cell culture, CV-1, VERO, MDBK, Taurus-1, cytopathic effects

Lumpy skin disease (LSD) is an infectious pathology of cattle which is still a cause for significant economic damage to livestock in many countries. In the Russian Federation, the disease was entered the Republic of Dagestan from Azerbaijan in 2015 [1, 2]. At the same time, disease outbreaks were registered in

the Chechen Republic and the Republic of North Ossetia-Alania. In 2016, LSD was found in the Krasnodar Krai, then in six other Russian regions [3, 4]. Lumpy skin disease (infectious nodular dermatitis, pearl disease, vesicular exanthem) is a transmissible, highly contagious transboundary emergent viral disease characterized by fever, skin nodules, the nodular lesions on mucous membranes and viscera, depletion, lymphadenopathy and cutaneous dropsy. LSD is caused by a DNA virus, LSDV, of the *Capripoxvirus* genus (*Chordopoxvirinae* subfamily, *Poxviridae* family) [5-7]. The disease often reduces milk production up to 50 % and leads to body weight loss, abortions and stillbirths, skin damage, reproductive disorders in affected animals, down to total loss of maly fertility, and animal death from secondary infections [8-10]. Immunization is the only effective method to control LSDV infection in endemic regions [11, 12]. A virus vaccine from homologous attenuated Neethling strain or vaccines from heterologous live attenuated strains of sheep or goat pox viruses are used for specific prophylaxis of nodular dermatitis [13-15].

Continuous cell lines are promising for the LSDV culture. They provide production of large amount of uniform virus-containing material which is used in biological, molecular and genetic study of virus. Also continuous cell lines are effective laboratory models for studying how LSDV evolves and for development of disease diagnostics and specific prophylaxis [16, 17]. Success in developing vaccines and diagnostic methods depends largely on the proper choice of the culture system. Therefore, initially it is necessary to determine the sensitivity of cell cultures and their permissivity to a particular virus. In choosing cell systems, we relied on the species culture identity (*Bos taurus, Ovis aries, Capra hircus*), specific cell and tissue tropism of LSDV to dermis, as well as on using homologous cell cultures of lamb testicles LT, fetal bovine testis FBT, calf kidney cells MDBK, etc. [19-21] and heterologous cell lines [9] for these purposes.

A.V. Kononov et al. [22] identified that in cells of homologous origin, i.e. in the subculture of lamb testes (TL) and in continuous cell culture of goatling gonads (GT-04), LSDV isolated from the biomaterial, which was collected in the Dagestan Republic in 2015, was accumulated in titers of 4.5-5.5 lg $TCID_{50}/cm^3$. However, in some cases, there is a need for viruses produced in a heterologous cell system, particularly when the viral antigen is accumulated to produce specific sera. The use of heterologous cell culture systems make it possible to exclude the appearance of antibodies to homologous tissue antigens, which complicates using sera in diagnostic studies or requires additional procedures for antigen purification.

This research first identified that the LSDV can proliferate in cells of wild animals (i.e. of elk) and that heterologous continuous cell lines RK-13/2-03 and A4C2/9k are effective for LSDV culture.

Our aims were the study of cultural properties of a nodular dermatitis virus isolate and optimization of cell cultures the most perspective for its production.

Techniques. Organs (liver, lungs, spleen, lymph nodes) and affected subcutaneous tissue samples were collected from the forcibly killed bulls of the Kalmyk breed (farms of the Volgograd Region, 2016) with typical manifestations of clinical ND symptoms. A 10 % tissue suspensions were prepared in Eagle's medium (MEM, Sigma, USA, HyClone, USA) supplemented with antibiotics (penicillin and streptomycin of 200-1000 IU/ml each, and nystatin of 20 IU/ml). After clarification by centrifugation at 2000 rpm, the suspension was introduced into culture vessels with a pre-formed cell monolayer. At this stage, we used primary goatling testicle cells (TG), as well as continuous cell lines of the bovine kidney (MDBK) and the rabbit kidney (RK-13/2-03) from FRCV&M collection of cell cultures [23]. An hour after adsorption, the suspension was removed, supporting 2 % bovine serum was infused and then it was incubated for 5-6 days at 37±0.5 °C. Ves-

sels with cell cultures were frozen and stored at –40±0.5 °C, then the culture medium was thawed out at room temperature. A 1 cm^3 aliquot of culture liquid was placed on a cell monolayer, with the next reseeding prior to the signs of characteristic viral cytopathic effect (CPE) developed. The state of cells monolayer in CPE tests was evaluated by viewing the culture vessels under the inverted microscope Olympus CKX31 (Olympus Co., Japan).

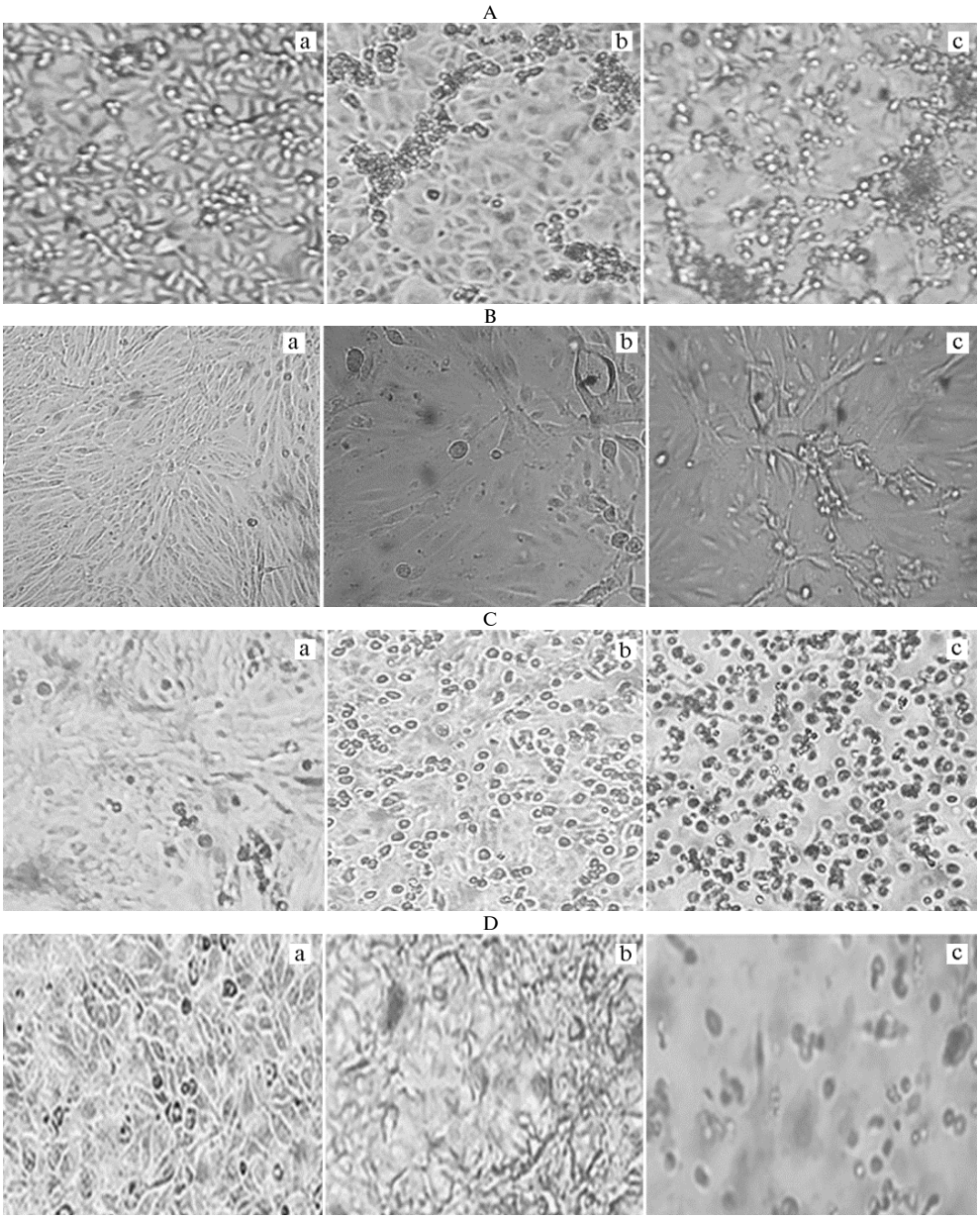
For virus adaptation to homologous and heterologous cell lines, serial passages were used. Cell cultures of elk (*Alces alces*, order *Artiodactyla*) embryo skin (KEL/07), African green monkey kidney (CV-1, VERO), porcine embryo kidney (PEK-66b), hybrid line of porcine embryo kidney cells (РЕКСЫПЭ ТК$^-$) × porcine spleen splenocytes (A_4C_2/9k), sheep kidney (ShK), calf kidney (Taurus-1) were grown in Eagle's medium supplemented with 10 % fetal bovine serum. After formation of a continuous monolayer (24 hours), the growth medium was removed from the vessels and the virus was inoculated at a multiplicity of 0.1-0.00001 $TCID_{50}$ per cell. The virus was allowed for adsorption for an hour at 37.0±0.5 °C. Next, maintenance medium supplemented with 2 % fetal bovine serum was added. The infected cell culture was incubated at 37.0±0.5 °C for 5 days or until 90-100 % cytopathic effect (CPE) developed. Then, the cell culture and the liquid culture were frozen at –50.0 ± 0.5 °C. In the next culture passage, the cells were infected with a thawed virus-containing suspension. Presence of cytopathic changes in the monolayer and a change in the viral titer during the passage indicate permissivity of cells culture to the virus. Viral infectious activity was determined by titration in 1-2-day continuous VERO or ShK cell lines grown in 96-well microplates. Virus titer was estimated by Reed and Muench methods and expressed in lg $TCID_{50}/cm^3$ [24].

Nucleic acids were extracted using RIBO-sorb kit (InterLabService LLC, Russia). Viral genomic DNA was detected by T.R. Bowden et al. [25] with oligonucleotide primers CaPV 074 F1 (5′-AAA ACG GTA TAT GGA ATA GAG TTG GAA-3′), CaPV 074 R1 (5′-AAA TGA AAC CAA TGG ATG GGA TA-3′), and hybridization probe CaPV-074P1 (5′-6FAM-TGG CTC ATA GAT TTC CT-MGB-NFQ-3′). The reaction mixture contained 10 pM of each primer, 3 pM of a fluorescent probe (CJSC Synthol, Russia), 2.5 μl of 10× DNA buffer, 10 mM dNTPs solution mix and 1.5 IU of recombinant Taq DNA polymerase (Thermo Fisher Scientific, USA). Real-time PCR was performed on a thermocycler Rotor Gene 6000 with detecting system (Corbette Research, Australia) as follows: preliminary denaturation at 95 °C for 10 min followed by 45 cycles of amplification (15 s at 95 °C, 1 min at 60 °C) [25].

The data were processed using variational statistics. The table shows the mean (*M*) and standard mean errors (±SEM).

Results. To extract LSDV, the virus-containing material was poured into culture vessels, 3 vessels for each culture, with a pre-formed monolayer of TK, MDBK or RK-13/2-03 cells. First passage did not lead to culture changes, in the second passage, slight changes in cell morphology and their rounding were revealed. In the third passage, the typical cytopathic effect was observed on MDBK and RK-13/2-03 cells (Fig., A) infected initially with a suspension of liver tissue. In this, on day 2 of incubation, in infected culture RK-13/2-03 the cells formed strands, and on day 3 the cells were rounded, while in the control culture such changes were not revealed. The typical changes were in MDBK cell culture. The virus titer in MDBK and RK-13/2-03 cell cultures was 4.67-5.00 lg $TCID_{50}/cm^3$. The isolated strain (deposited to FRCV&M State Collection under accession No. 3161) was further used in the work. While continuous cell lines EES/07 contamination, typical changes were observed (see Fig., B) (virus titer of 4.5-5.5 lg $TCID_{50}/cm^3$). In the TR primary cells culture

during the third passage, the infectious activity of the virus was lower (3.5 lg $TCID_{50}/cm^3$). Homologous and heterologous cell lines MDBK, Taurus-1, ShK, CV-1, VERO, RK-13/2-03, A_4C_2/9k, and SPEV were used to adapt the strain to the continuous cell lines, and to identify sensitive cultures.



Cytopathic effect of bovine lumpy skin disease on continuous cell lines of rabbit kidney RK-13/2-03 (A)**, elk embryo skin KEL/07** (B)**, African green monkey kidney VERO (C), sheep kidney SK** (D)**:** a — control cell culture, b and c — cell culture on day 2 and day 3 (for sheep kidney cells — on day 4) after inoculation (magnification ×150, microscope Olympus CKX31, Olympus Co., Japan).

The character of CPE in different cell cultures was not the same. So, in RK-13/2-03 (see Fig., A), the CPE was similar to that under virus reproduction in the ShK cell culture (see Fig., D). That is, 48 h after inoculation, spindle-shaped cells formed strands, and after 72 h, we observed rounding and detaching of infected cells from the substrate, with lysis and destroying the cell monolayer. In the infected VERO culture (see Fig., C), there was an increased cells rounding, the

formation of inclusions, which are not inherent for normal (uninfected) cells, with further lysis and detaching. The viral infectious activity in these culture systems also varied. The maximum titers were observed in cell cultures Taurus-1 and A_4C_2/9k-7.00, the lg $TCID_{50}/cm^3$, and also VERO and RK-13/2-03-6.67, the lg $TCID_{50}/cm^3$ (Table 1).

1. Propagation of the nodular dermatitis virus in different continuous cell cultures

Cell culture	Passage	Time, h	Titer, lg $TCID_{50}/sm^3$
		Homologous cells cultures	
MDBK	4-6	48	4.67-5.67
Taurus-1	4-6	48-72	6.00-7.00
KEL/07	4-6	72	4.5-5.50
TK	3	144	3.50-4.50
ShK	4	120	4.67
	5-10	72-96	6.0-6.33
	11	48	6.50
		Geterologous cells cultures	
RK-13/2-03	4-7	48-72	5.00-6.67
VERO	4-9	48	5.00-6.67
CV-1	4-11	48-72	5.00-6.67
PEKC	4-6	48-72	4.50-5.50
A_4C_2/9к	4-	48-72	6.00-7.00

Taxonomic attribution of the virus accumulated in cell cultures was confirmed by revealing LSDV genome by real-time PCR. The Ct values were 11.79 for RK-13 (passage 8), 11.91 for Taurus-1 (passage 4), 18.45 and 35.82 for A_4C_2/9K (passage 3) and A_4C_2/9K (passage 3, dilution 10^{-5}), respectively. Samples were considered positive at Ct ≤ 40).

The time required for the development of 95-100 % CPE depended on the cell culture. For MDBK and VERO cell lines, it took 48 hours (see Table 1). For Taurus-1, ShK, RK-13/2-03, and CV-1, the 100 % CPE time varied from 48 to 96 hours.

When determining the optimal virus/cell multiplicity for lines RK-13/2-03, VERO and ShK (Table 2), the growth medium was replaced by the maintenance medium and incubated until the destruction of the cell monolayer completes. With a multiplicity of 0.1-0.01 $TCID_{50}$ per cell, CPE appeared on day 2 of incubation. At 0.001-0.00001 $TCID_{50}$ per cell, the virus titer increased up to 1.50 lg $TCID_{50}/cm^3$, the time for the complete destruction of the monolayer was up to 3-5 days. The infectious titer of virus-containing material was 6.2-6.8 lg $TCID_{50}/cm^3$ when produced by ShK and VERO cells, and 5.8-6.6 lg $TCID_{50}/cm^3$ for RK-13 cells used. To optimize FBS concentration, virus-infected ShK cells were cultured in a maintenance medium without serum and with the addition of 2; 5 and 10 % FBS. The highest virus titer of 6.67 lg $TCID_{50}/cm^3$ was observed at 2-5 % FBS compared to 5.5 lg $TCID_{50}/cm^3$ and 6.0 lg $TCID_{50}/cm^3$ for serum-free medium and 10 % FBS, respectively.

2. Propagation of nodular dermatitis virus (lg $TCID_{50}/cm^3$) in continuous cell cultures depending on multiplicity of infection ($n = 3$, $M \pm m$)

Cell culture	Multiplicity of infection, $TCID_{50}$ per cell					
	0.1	0.01	0.001	0.0001	0.00001	0.000001
ShK	5.3±0.13	6.0±0.19	6.2±0.23	6.7±0.12	6.7±0.23	6.0±0.12
RK-13/2-03	4.7±0.21	4.7±0.00	5.8±0.20	6.0±0.00	6.6±0.18	3.5±0.17
VERO	5.5±0.15	6.0±0.17	6.5±0.26	6.7±0.15	6.8±0.26	6.3±0.17

The development of effective preparations for viral disease prevention and diagnostics depends on the quality of the virus-containing material, which is usually obtained using highly productive cell culture systems and effective methods for culturing virus-infected cells. In the literature, there is information about LSDV propagation in cell cultures of homologous origin, such as kidneys and testes of lambs and calves, as well as in the calves' dermis, with a charac-

teristic cytopathic effect, and in heterologous cell cultures, i.e. rabbit embryonal kidney and skin, VERO. The infectious activity of such cultural virus-containing materials is 4-6 lg $TCID_{50}/cm^3$ [9, 22, 26]. However, these are mostly primary cell cultures. In the present work, we have tested sensitive continuous cell lines which are more technologically suitable. In this, we have shown permissivity of KEL/07 cells of wild elk, a member of *Artiodactyla*, to LSDV. Also, there are reports on experimental infection of Asian buffalo, antelope and giraffe which resulted in clinical symptoms of nodular dermatitis [27, 28]. Given that and also the fact that LSDV is a transmissible virus, one should pay attention to possible LSDV circulation among wild fauna and the formation of enzootic foci in central Russia.

Thus, the continuous cell lines of both homologous and heterologous origin are susceptible to lumpy skin disease virus (LSDV). At optimal MOI of 0.001-0.00001 $TCID_{50}$ per cell and culturing in a maintenance medium with 2-5 % FBS, the virus titers were 6.2-6.8 lg $TCID_{50}/cm^3$ for ShK and VERO continuous cell culture, and 5.8-6,6 lg $TCID_{50}/cm^3$ for RK-13/2-03. As known, cell cultures of heterologous origin not susceptible to viral and prion pathogens of target animals, i.e. cattle and small ruminants, including slow infections, are preferably used in producing vaccine and diagnostic preparations. Our findings indicate that clonal continuous rabbit kidney cell culture RK-13/2-03 seems to be the most prospective for LSDV production.

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DETERMINATION OF SPECIFIC ANTIBODIES TO ENTEROTOXINS OF *Staphylococcus aureus* IN BLOOD AND COLOSTRUM FROM COWS

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Abstract

Mastitis is a widespread infectious disease of the milk cattle causing essential economic damage to livestock production and influencing quality of dairy products all over the world. The main pathogen of cattle mastitis is *Staphylococcus aureus. S. aureus* pathogenicity and steady persistence in the body are due to a set of toxins. The microorganism produces a number of pathogenic factors with an impact on character of the disease. At the moment there is no effective ways to fight against mastitis therefore research of the immune status of cows as per staphylococcus enterotoxins is under consideration. The aim of this study was identification and quantitation of Ig specific to the most widespread staphylococcus enterotoxins (A, B, C, D, E, G, H, I and TSST) in blood and in colostrum of Holstein black and motley cows of 2nd lactation (n = 47, Kaluga region, 2016). Animals were on loose housing keeping and two times a year exposed to immunization by vaccine MASTIVAK (Laboratorios Ovejero S.A., Spain). Anti-enterotoxin immunoglobulins were estimated using indirect enzyme immunoassay. In 53.19 % of blood samples IgG specific to SEH were detected. Antibodies to TSST were found in 4.26 % of samples, at the same time their amount was the lowest in comparison to the antibodies to other enterotoxins found. The analysis of colostrum and milk showed IgA to SEH, SEG and SEI in colostrum. In milk we did not revealed antibodies to enterotoxins. Thus, the antibodies to a wide range of staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and TSST) we have found in blood and colostrum of clinically healthy cows could be indicative of animals' contacts with the staphylococci producing these types of toxins.

Keywords: *Staphylococcus aureus*, mastitis in cows, blood, colostrum, enterotoxins, antibodies against enterotoxins

Mastitis of cows is the main cause of economic losses in the milk production throughout the world [1]. The disease can be caused by 137 different microorganisms [2], most often by *Staphylococcus aureus*, which is detected in both subclinical and chronic infections [3-5]. Effect of antibiotics under mastitis caused by staphylococci is low [6] due to emerged antibiotic resistance of the strains, to also the ability of *S. aureus* to form biofilms and survive in epithelial cells and macrophages [7]. Immune factors of colostrum, milk and blood play an important role in natural immunity and protection against mastitis [8]. In turn, *S. aureus* secretes virulence factors that can suppress the host's immune system. Theas are enterotoxins (SE) and toxic shock syndrome toxin (TSST-1) [9, 10], pore-forming toxins [11], phenol-soluble modulins [12]. Staphylococcal enterotoxins play the most important role in suppressing the host's immune system and

the development of mastitis. The ability of these microorganisms to produce factors that regulate animal immunity stems practical interest concentrated on preventing disease [13]. Vaccines against mastitis have been developed [14, 15], but vaccination does not provide 100 % protection. Thence, information about animal immunity and the antibodies relieving manifestation of the disease is of particular importance. IgG antibodies can pass from blood to other biological fluids and tissues [16], whereas production of secretory IgA is local and concentrates at the site of infection thus reflecting local inflammatory processes [17]. Antibodies are able to prevent the propagation of microbes and to neutralize toxins [18], and, when carried with colostrum and milk from mother to a newborn, are factors of passive vaccination.

In Russia, complex assessment of animal immune status under *S. aureus* infection was not performed until now. This paper is the first to confirm simultaneous detection of antibodies to several toxins of the pathogen in biological fluids of Russian Holstein black-motley cows.

Our objective was to assess titers of antibodies to most common A, B, C, D, E, G, H, I and TSST staphylococci enterotoxins in blood and colostrum of cows.

Techniques. Blood, colostrum and milk samples were collected in 2016 from Holstein black-motley cows (*Bos taurus taurus*) of second lactation (n = 47, 2016) housed in free stall barns on a dairy farm of Kaluga region. Animals were vaccinated twice a year with Mastivak (Laboratorios Ovejero S.A., Spain) containing *Streptococcus agalactiae*, *S. dysgalactiae*, *S. uberis*, *S. pyogenes*, *Staphylococcus aureus*, *Arcanobacterium pyogenes*, *Escherichia coli*, strains Bov-10, Bov-14, Bov-15, Bov-21, J5. Enterotoxins of staphyococci were extracted as described [19], and staphylococcus enterotoxin H (SEH) was received from Gamalei Institute of Epidemiology and Microbiology (Moscow).

Blood samples were collected from tail vein into vacuum vials with Clot Activator and centrifuged to remove formed elements of blood**.** **C**olostrum and mild samples were aseptically collected into sterile flasks from all of udder quarters just after milking. The samples were delivered refrigerated (in thermo containers at +4 °C within 2 hours) or frozen.

Blood IgG to staphylococci enterotoxins A, B, C, D, E, G, H, I and TSST were titrated by indirect ELISA in 96-well plates (Corning, USA). For coating plates, aliquots of the enterotoxins in carbonate buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH 9.0) diluted to a final concentration of 2 μg/ml were absorbed separately, 50 μl per well. To prevent non-specific binding of antigens and antibodies to the microtiter well, 2 % bovine serum albumin (BSA) was used as a blocking agent. Blood serum 1:100 diluents were titrated by 2-fold dilutions in phosphate buffered saline PBST (30 mM KCl, 1 mM KH_2PO_4, 1.38 M NaCl, 160 mM Na_2HPO4, pH 7.5, 0.1 % Tween 20). Mice monoclonal antibodies to enterotoxins serve as a positive control [20]. Signal was detected using horseradish peroxidase-conjugated rabbit anti-cow anti-IgG (Sigma, USA). At each stage, the plates were incubated at 37 °C for 1 hour and then rinsed with PBST. o-Phenylenediamine dihydrochloride (0.4 mg/ml) in citrate-phosphate buffer (1.1 mM Na_2HPO_4, 0.51 % citric acid, pH 5.0, 0.03 % H_2O_2) was used to detect horseradish peroxidase, and 10 % HCl was added as stop solution to terminate the reaction. Absorbance of the reaction products was measured at λ = 490 nm in a microplate spectrophotometer (Bio-Rad, USA).

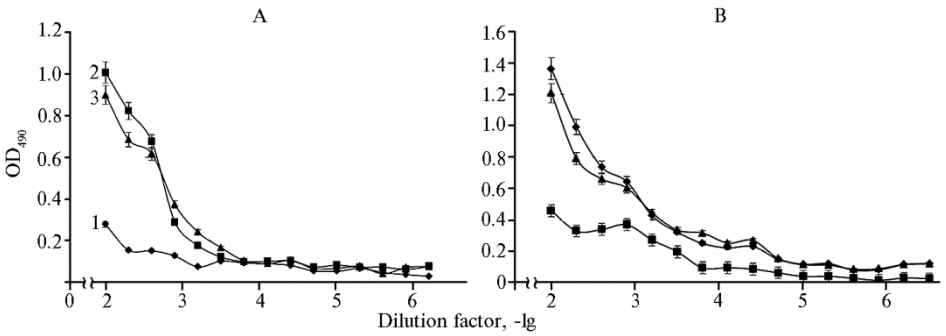
The same protocol was used for titration of IgA to enterotoxins in colostrum and milk. For detection of IgA to toxins, we used horseradish peroxidase-conjugated goat anti-cow anti-IgA (Abcam, USA). The highest dilution that generates OD_{490} signal twice as much as background signal was defined as the titer.

Measurements were made in threefold repetition. Means (M) and standard

errors of means (±SEM) were calculated.

Results. We found SEH in blood of 53.19 % of the cows tested. Importantly, SEH plays crucial role in pathogenesis of mastitis and can cause cell apoptosis in udder of cows [21]. SEH-producing staphylococci make third of the strains isolated from ewes with masititis [22]. In Russia, staphylococci positive for toxin **gene** *seh* have not been isolated from animals with mastitits indicates that animals could have contact with SHE-producing staphylococci. High blood level of anti-SEA and anti-SEC was found in 42.55 % 36.17 % of the cows, respectively, that corresponds to other reported data [23]. Antibodies to SEB, SED, SEE, SEG, SEI and TSST were detected in 10.64, 23.47, 31.91, 29.78, 34.04, and 4.26 % of the animals.

The level of anti-TSST antibodies was the lowest (Fig.), possibly, because the TSSS staphylococcal enterotoxin is rare and plays an insignificant role in bovine mastitis [24]. In colostrum, like in blood, we detected high titers of antibodies to SHE, and also the antibodies to SEG and SEI (see Fig.). IgA to SEG were found in 48.93 % of the population, anti-SEH IgA were present in 53.19 % of the population, and 42.56 % of animals had anti-SEI antibodies. IgA to SEA, SEB, SEC, SED, SEE, and TSST were not revealed.



ELISA titration of enterotoxin H (1)**, enterotoxin A** (2)**, and enterotoxin TSST** (3) **in blood** (A, sample № 7) **and colostrum** (B, sample № 10) **of Holstein black-motley cows** (each measurement made in threefold biochemical repetition; Kaluga Province, 2016 год).

Genes for staphylococcal enterotoxins SEG and SEI, which have genome location, are low expressed unlike genes encoding super antigenic toxins [25] located in phage genomes or in plasmids [25]. Genes *seg* and *sei* were simultaneously revealed in the same *S. aureus* isolates from animals with mastitis [21]. Colostral IgA to enterotoxins may exert preventive effect if staphylococcal infection is caused by strains producing such enterotoxins [25].

Thus, the titers of antibodies detected in blood and colostrum of the cows in our test were 1:800-1:3200 for SHE and 1:800-1:1600 for SEG and SEI. The lowest titers showed anti-TSST and anti-SEB antibodies (1:400 and 1:800, respectively). No reliable data were obtained on the present of milk antibodies to staphylococcal enterotoxins.

Therefore, we detected antibodies to wide range of staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and TSST) in blood and colostrum of clinically healthy Holstein black-motley cows. This indicates that the animals could contact pathogenic strains producing such types of enterotoxins. The fact that milk antibodies were not detected may be due to their low concentration in the samples.

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SPECIES COMPOSITION AND TOXICOLOGICAL CHARACTERISTICS OF FUNGI OF THE GENUS *Aspergillus* ISOLATED FROM COARSE FODDERS

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Abstract

The problem of ensuring the safety of coarse fodders, which annually replenishes the Russian feed production on a large scale, raises concern about the multiple combined contamination with mycotoxins and the extensive spread of toxigenic fungi. Recently, it has been established that *F. sporotrichioides* plays a dominant role among fusarium fungi in these fodders, producing metabolites, the T-2 toxin and diacetoxyscirpenol, which can cause acute poisoning in animals. The purpose of this work, which became the next stage in the study of the main toxin-producing micromycetes of coarse fodders, was the determination of the species composition, occurrence and toxin production in fungi of the genus *Aspergillus* under experimental conditions favourable for the fullest realization of their potential. The objects of mycological analysis were 258 average samples from the production batches of hay and straw harvested in the livestock farms of Bryansk (2011) and Moscow (2013) regions. Isolates with established species affiliation were cultivated on Czapek-Dox agar (CDA), wort agar (WA) and moistened rice grain (RG) for 7 days at 23 °C. The content of sterigmatocystin (STE), emodin (EMO), aflatoxin B_1 (AB_1), ochratoxin A (OA), mycophenolic acid (MPA), cyclopiazonic acid (CPA), ergot alkaloids (EA), deoxynivalenol (DON) and fumonisins (FUM) in extracts of mycelial spore biomass were determined by enzyme immunoassay with certified test systems. To assess the toxin production, 32 isolates of 12 species of *Aspergillus* fungi of hay and straw were used, as well as 27 isolates of *A. flavus* Link, *A. pseudoglaucus* Blochwitz, *A. repens* de Bary isolated earlier from grain fodders. Fungi of the genus *Aspergillus* were found in samples with a frequency of 62.0 % and an infection rate of 1.7-100 %. The obtained isolates belonged to 15 species included in 10 taxonomic groups with the largest species diversity in the *A. glaucus* group (4 species). The most common species were *A. flavus* and *A. niger* van Tieghem (more than 50.0 % of the contaminated samples), followed by *A. versicolor* (Vuill.) Tiraboschi, *A. pseudoglaucus, A. amstelodami* (Mangin) Thom & Church, *A. ochraceus* Wilhelm and *A. wentii* Vehmer (10.6-18.8 %), *A. nidulans* Eidam (6.3 %), the remaining 7 species — *A. candidus* Link, *A. tamarii* Kita, *A. sydowii* (Bain. & Sart.) Thom & Church, *A. fumigatus* Fresenius, *A. repens*, *A. terreus* Thom, *A. chevalieri* (Mangin) Thom & Church were less common (< 5 %). The intensity of formation of the CPA (*A. flavus*) and MPA (*A. pseudoglaucus, A. repens*) was quite comparable in the CDA and RG. Compared to WA, a greater accumulation of the majority of mycotoxins occurred in the RG, i.e. STE (*A. versicolor, A. nidulans*), CPA (*A. flavus*, in all 5 *A. tamarii* isolates CPA could be detected only on this substrate) and EMO (*A. sydowii*). For the biosynthesis of MPA in *A. pseudoglaucus* and *A. repens*, WA was preferred. Testing of fungi on three nutrient media allowes us to establish that a complex of *Aspergillus* fungi which includes 7 species can be associated with the contamination of coarse fodders with STE, CPA and MPA; the source of EMO contamination among the fungi of the genus *Aspergillus* was not found. Only two of the three isolates of *A. sydowii* produced it in small amounts of 120±20 and 245±40 ng/g. The remaining mycotoxins analyzed in the isolates were not detected. The possibility of participation of fungi of other systematic groups in the contamination of fodder with STE, CPA, MPA and EMO is discussed, whereas clusters encoding the biosynthesis of mycotoxins have been found in micromycetes from genetically distinct groups in recent years.

Keywords: hay, straw, fungi of the genus *Aspergillus*, mycotoxins

Micromycetes of the genus *Aspergillus* have the negative impact on ani-

mals not only by mycoses caused by pathogenic species [1, 2], but also via intoxication by fungal metabolic products with a wide spectrum of damage, including neurotoxicity with tremorgenic effect, hepato- and nephrotoxicity [3, 4]. Despite numerous reports on significant fungal contamination of fodder, the risks associated with aspergillotoxicosis are still unclear. In the world literature, information about toxin production of some fungal species of this genus can be found, but as rule, they were obtained for a small number of isolates, under different conditions and often for one or a few closely related toxins [5, 6]. All these make it difficult or impossible to even approximately analyze situations which might arise in animal feeding and care.

Annually, coarse fodders noticeably replenish Russian forage reserve, and it causes concern of experts because of multiple combined mycotoxins contamination [7, 8] and wide spread of *Aspergillus, Penicillinu, Fusarium* and many other fungi, including toxigenic species. Predominance of *F. sporotrichioideds*, which metabolic products are T-2 toxin and diacetoxyscirpenol capable to cause acute toxicosis, has recently been found among fusarium in coarse fodders [9].

We were the first to targetedly seek for toxigenic fungi of the genus *Aspergillus* among species involved in damaging hay and straw in different Russian regions. A new approach to testing isolates on alternative growth media allowed us to confirm possibility of mycotoxins biosynthesis in 7 *Aspergillus* species of 15 identified in the mycobiota. The rear species such as *A. tamarii*, *A. repens* and *A. wentii* is a main focus of interest because information about their toxic potential is quite lacked.

The purpose of this work was to assess species composition of genus *Aspergillus* in coarse fodders and toxigenicity of these isolates in laboratory tests when ensured full realization of their potential.

Techniques. The mycological analysis was performed for 258 bulk samples of hay and straw commercial batches from livestock farms. These were 14 hay samples (cereals, meadow grasses, ryegrass, herbs) and 5 straw samples (the composition was not specified) from Bryansk region (8 areas, 2011), and 230 samples of hay (herbs, cereal, perennial grasses, composed, prairie grasses, timothy grass, bromegrass, lucerne, fescue grass, cereal-legume, vetch, goat's rue, clover) and 9 straw samples (vetch-oats mixture, cereal, wheat, oat) from Moscow region (31 areas, 2013). Inoculum was prepared as described previously [9]. Each batch was cut into 2 cm fragments, and mixed vigorously. The fragments were placed in three Petri dishes, 20 pieces in each per bach, with approximately the same distances between them. Czapek agar supplemented with medical bile (10 %) and antibiotics (penicillin 50,000 IU and streptomycin 100,000 IU per 1 liter of medium). The dishes were placed in a thermostat at 25 25 °C. After 5-7 days, the percentage of the total number of fragments with *Aspergillus* attack was calculated. To isolate pure cultures, the colonies having appearance and features of genus *Aspergillus* were seeded on the same agar medium in Petri dishes, and in 5-7 days, after confirming the purity, reseeded on Czapek Dox Agar (CDA). Species identification was carried out according to identification keys of fungi [10].

Estimation of toxigenicity included preparation of inoculum, substrate, seeding, culture, extraction mycotoxins and their analysis. The 10-day fungi cultures were used to seed on CDA. An approximately equal amount of inoculum, taken from the agar surface using mycological crochet, were placed in three 10 ml vials, a bottom diameter of about 18 mm, with 1 g sterile crushed rice grain prewetted with 1 ml H_2O, and also in three vials with 1.5 ml of CDA or wort agar (WA) (Liofilchem, Italy). The vials were closed with cotton-gauze plugs, which were tightly wrapped with a layer of Parafilm M® (PM-996, Pechiney Plastic Packaging, USA). The vials were kept in the dark for 7 days at 23 °C. Then, acetonitrile:water mixture (v/v 86:16) was added in each vial, and at the begin-

ning and the end of stationary 14-hour extraction the vials were shaken vigorously. The content of sterigmatocystin (STE), emodin (EMO), aflatoxin B_1 (AB_1), ochratoxin A (OA), mycophenolic acid (MPA), cyclopiazonic acid (CPA), ergot alkaloids (EA), deoxynivalenol (DON) and fumonisins (FUM) in extracts were estimated using certified ELISA test systems [11], the lower limit of detection corresponded to 85 % antibody binding. For estimating toxigenicity, 32 isolates of 12 *Aspergillus* species from hay and straw were used, as well as 27 isolates of *A. flavus*, *A. pseudoglaucus*, *A. repens* from the collection of All-Russian Research Institute of Veterinary Sanitary, Hygiene and Ecology which have been isolated earlier from grain forages.

Obtained data were analyzed by descriptive statistics method in Microsoft Excel 2013 software. The tables show the arithmetic mean values (X) and the errors of sample mean (s).

1. Species composition and prevalence of *Aspergillus* fungi in coarse forages (hay and straw) harvested in Bryansk Province (8 regions, 2011) **and Moscow Province** (31 regions, 2013) (n = 160)

Group	Species	Frequency of occurance, %
A. flavus	*A. flavus* Link	56.3
	A. tamarii Kita	3.1
A. niger	*A. niger* van Tieghem	54.4
A. versicolor	*A. versicolor* (Vuill.) Tiraboschi	18.8
	A. sydowii (Bain. & Sart.) Thom & Church	2.5
A. glaucus	*A. pseudoglaucus* Blochwitz	15.6
	A. amstelodami (Mangin) Thom & Church	10.6
	A. repens de Bary	1.9
	A. chevalieri (Mangin) Thom & Church	0.6
A. ochraceus	*A. ochraceus* Wilhelm	15.0
A. wentii	*A. wentii* Vehmer	14.4
A. nidulans	*A. nidulans* Eidam	6.3
A. candidus	*A. candidus* Link	4.4
A. fumigatus	*A. fumigatus* Fresenius	2.5
A. terreus	*A. terreus* Thom	0.7
Не определена	*Aspergillus* spp.	3.8

N o t e. n — number of samples affected by aspergillus.

Results. *Aspergillus* fungi were found in 62.0 % of 258 tested samples with an infection rate from 1.7 to 100 %. Pure cultures of the fungal isolates of this genus were assigned to 15 species of 10 taxonomic groups (Table 1). The highest species diversity was in the *A. glaucus* group with 4 species (*A. pseudoglaucus*, *A. repens*, *A. amstelodami*, *A. chevalieri*), the others comprised 1-2 species. We failed to identify isolates of 13 samples at a species level because they were lost in the early stages of isolation, but according to the pre-estimates the 7 of them belonged to the *A. glaucus* group.

By frequency of occurrence, *A. flavus* and *A. niger* were among the leaders (more than 50.0 % of the affected samples), they were followed by *A. versicolor*, *A. pseudoglaucus*, *A. amstelodami*, *A. ochraceus* and *A. wentii* (0.6-18.8 % of samples), *A. nidulans* (6.3 %), and the rest 7 species, *A. candidus*, *A. tamarii*, *A. sydowii*, *A. fumigatus*, *A. repens*, *A. terreus* and *A. chevalieri* were noticeably more rare (< 5 %). Generally, these results coincided with those obtained earlier in other territories — in the Ryazan Meshcher [12], Tatarstan [13, 14], Dagestan [15], North Ossetia [16] and the Amur Region [17], as well as in the ex-USSR republics — Ukraine [18], Belarus [19], Lithuania [20], Armenia [21, 22], Kazakhstan [23] and Azerbaijan [24, 25]. All surveys reported that *Aspergillus* fungi dominate in the mycobiota of coarse fodders and are represented by many species with a predominance of *A. flavus* and *A. niger*. The similar pattern, although with some differences, was also found for the accompanying species *A. fumigatus*, *A. nidulans*, *A. ochraceus*, *A. versicolor*, *A. candidus*, *A. wentii,* and *A. glaucus*. Among rare species, the researchers found *A. clavatus* [16, 18, 21, 25], *A. flavipes* [22], *A. oryzae* and *A. ustus* [16].

Apparently, the complex of *Aspergillus* species and the observed ratio between them were the result of long-term competitive interrelationships between fungi and formed during the grass stand drying. It is difficult to assume that such a

variety of vegetative plants are subjected to fungal attack so uniformly. It is clear that different species could actively develop on living plants, and those that eventually dominated, during the growing season, could be of secondary importance. In our view, this fact and the suggestion above deserve special attention, since they point to the need for accounting the biosynthetic potential of not only common, but also rarely identified species for the correct prediction of the feed contamination risks.

Toxigenic estimate methodology for this fungi group, according to the available information, was not the subject of special studies. However, in other works, the Czapek-Doks medium was used for testing and producing mycotoxins [26], the assessment of *A. ochraceus* isolates was carried out on rice grains [27, 28], and some fungal species were differentiated by ability of the isolates to accumulate EMO [29], CPA [30, 31] and MPA [32] on WA. In tests on WA, CDA and moistened rice grain (RG), three toxigenic species from the collection (Table 2) under equal conditions (7 days, 23 °C) showed similar production of CPA (*A. flavus*) and mycophenolic acid (MPA) (*A. pseudoglaucus* and *A. repens*) on rice and CDA in all cases, whereas on WA, the CPA production by *A. flavus* isolates was much weaker.

2. Toxigenicity of *Aspergillus* fungi isolated from grain forage on wort agar (WA), **Czapek-Doxa agar** (CDA) **and moistened rice grain** (RG) (23 °C, 7 days)

Species of fungus (number of isolates)	Mycotoxin	Amount of mycotoxin, X±s ng/g substrate		
		WA	CDA	RG
A. flavus (6)	CPA	125±28	1410±280	900±165
		200±80	1780±40	1710±200
		330±55	1190±110	1055±270
		–	93±15	205±83
		200±45	945±245	780±195
		260±78	810±155	1115±180
A. pseudoglaucus (2)	MPA	nd	970±69	1425±95
		nd	775±46	1040±56
A. repens (3)	MPA	nd	975±155	1145±150
		nd	1060±145	1560±190
		nd	2560±335	3005±920

N o t e. CPA — cyclopiazonic acid, MPA — mycophenolic acid; X is the arithmetic mean, s is the sample mean error. A dash indicates that mycotoxin is not found, nd — no detection permormed.

3. Toxigenicity of *Aspergillus* fungi isolated from coarse fodders on wort agar (WA)**, and moistened rice grain** (RG) (23 °C, 7 days)

Species of fungus (number of isolates)	Mycotoxin (n^+)	Amount of mycotoxin, X±s ng/g substrate	
		WA	RG
A. versicolor (3)	STE (3)	1060±150	197860±30560
		160±32	41620±6520
		4050±810	223960±43310
A. pseudoglaucus (4)	MPA (4)	30320±5620	21730±4340
		1750±130	685±44
		1840±45	630±75
		2200±310	940±22
A. wentii (3)	MPA (1)	123±22	–
A. nidulans (3)	STE (3)	–	21460±4200
		–	9480±1830
		–	13570±2330
A. tamarii (5)	CPA (5)	–	3410±680
		–	1920±255
		–	1370±105
		–	2780±550
		–	1760±270
A. sydowii (3)	EMO (2)	–	120±18
		–	245±45
A. repens (2)	MPA (2)	1020±42	480±27
		810±98	595±80

N o t e. STE — sterigmatocystin, CPA — cyclopiazonic acid, MPA — mycophenolic acid, EMO — emodin; n+ — number of isolates producing mycotoxin; X is the arithmetic mean, s is the sample mean error. Dashes mean that mycotoxin is not found.

Given differences in the metabolic response revealed on WA, we continued hay and straw testing on WA and RG (Table 3), of which RG was previously used for growth *Fusarium* from the same objects [9]. The analyzed mycotoxins were AB_1, STE, OA and EA which production by *Aspergillus* fungi is rather well studied [35], MPA, CPA and EMO [29-32] which are less studied, and also FUM and DON which are found only in several strains of *Aspergillus* [33, 34]. For analyses, we selected three to four isolates of five widespread species and *A. nidulans*, and all the isolates obtained (from 1 to 5) were tested for more infrequent species. Pathogenic *A. candidus* and *A. niger* isolated from feed were unable to produce the mycotoxins in question so they were excluded from further study. The choice of two growth medium for testing toxigenicity, as it turned out, was a success. The RG medium allowed for higher accumulation of STE (*A. versicolor*, *A. nidulans*), CPA (*A. tamarii*, only this substrate made it possible to detect CPA in all 5 isolates) and EMO (*A. sydowii*). On the contrary, for the MPA biosynthesis in *A. pseudoglaucus* and *A. repens*, the WA medium was preferable. As per the estimates of *Aspergillus* fungi, the relative error of the mean sample in 3-fold repetition did not exceed 20 % for WA and RG and was quite acceptable.

The tests showed that a complex of *Aspergillus* fungi which comprises 7 species isolated from coarse fodders is capable for biosynthesis of STE, CPA and MPA. *A. flavus* and *A. tamarii*, the two members of the *A. flavus* group, can produce CPA, *A. versicolor* and *A. nidulans* may synthetize STE, and *A. pseudoglaucus*, *A. repens* and *A. wentii* are MPA-producing isolates. Mycotoxicological analysis of hay and straw from the same regions detected an extensive STE, MPA, CPA and EA contamination with incidents of significant accumulated amounts [7, 8]. Potential EMO contaminants of fodder were not found among *Aspergillus* fungi. Only two of three *A. sydowii* isolates produced EMO in small amounts of 120±20 and 245±40 ng/g. *A. fumigatus*, which metabolome contains EA and EMO [36], was represented by a single isolate producing EA in the amount of 220±32 ng/g and not capable of EMO synthesis. OA, FUM and DON were not found in mycelial and spore biomass. CTE was accompanied by trace concentrations of AB_1, 5±3 and 14±1 ng/g, in two *A. versicolor* isolates when grown on RG. *A. amstelodami* and *A. ochraceus*, the members of common forage species, and also *A. terreus* and *A. chevalieri* which are rare in occurrence, did not form any of the studied mycotoxins in amounts exceeding tens of nanograms per 1 g of substrate.

While recognizing the toxigenic potential of *Aspergillus* species for fodder contamination by mycotoxins, it should be noted that, according to the accepted criteria [37], these species basically belong to the weak producers, as the accumulation of toxins, even under the most favorable conditions, did not reach 10,000 ng/g substrate in all *A. pseudoglaucus* isolates and *A. nidulans* and was of next lower order in *A. flavus* and *A. tamarii*. Only one of the frequently occurred species, *A. versicolor,* showed itself as a highly active producer of STE when cultured on RG. Perhaps, fungi of another systematic groups, in particular from the genus *Penicillium*, may participate in forage contamination with the same toxins. Previous studies have indicated that not only representatives of *Aspergillus*, but also fungi of other genera are capable of forming many toxins [38, 39]. Therefore, in the light of modern concepts, definition “aspergillotoxins” becomes more and more conventional. The development of genome analysis shows that clusters encoding the biosynthesis of a particular mycotoxin actually occur in fungi of genetically distant groups [33].

The present paper provides data on the ability of *Aspergillus* fungi, the members of coarse fodder mycobiota, to toxin formation, although not always significant. Nevertheless, it is quite possible that the realization of potential toxygenicity could be enhanced, not only in vivo, during grass growing, but also after

mowing, drying and storing. The study of the causes of abrupt shifts in the metabolic profile in fungi under the influence of habitat conditions noted for certain sanitary species, in particular for *Fusarium graminearum* Schw., remains in focus of mycological research.

Thus, for 7 species of the genus *Aspergillus*, the possibility of participation in extensive contamination of feeds by cyclopiazonic acid, sterigmatocystin and mycophenolic acid with cases of accumulation of significant amounts of mycotoxins has been confirmed. However, the sources of contamination with other toxins, in particular emodin, ochratoxin A, ergoalkaloids, could well be among the representatives of the other identified species, and among those of unidentified taxonomic attribution. Because of the small number of available isolates, we were unlikely to fully appreciate the toxin-forming ability of *A. tamarii*, *A. sydowii*, *A. fumigatus*, as well as *A. terreus* and *A. chevalieri*. It is possible that for *A. amstelodami* and *A. ochraceus*, the selected substrates were not entirely suitable for inducing toxin formation. It would therefore be advisable to extend the search for carriers of toxicity among these species of *Aspergillus* and other micromycetes from the mycobiota of coarse forages and also to continue testing on different media for an exhaustive estimation of the fungal potential of toxigenicity.

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