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CONTENTS

REVIEWS, CHALLENGES

Galochkina V.P., Agafonova A.V., Galochkin V.A. The hypothesis of a specific relationship between peroxisomal, mitochondrial, and cytoplasmic processes in metabolic regulation of highly productive ruminants 223

Yakovlev A.F. Molecular markers in immune response manifestations (review) . . . 235

Glotov A.G., Glotova T.I., Koteneva S.V. Pestiviruses, which contaminate imported fetal bovine serum, may be a cause of the global spreading of viral diarrhea in cattle — a mini review 248

Pobednov Yu.A., Kosolapov V.M. Biology of alfalfa silage making (review) 258

Shamsutdinov Z.Sh., Kosolapov V.M., Shamsutdinova E.Z. et al. About the concept of ecological niche and its role in design of adaptive arid pasture agroecosystems . . 270

GENETIC STRUCTURE OF POPULATIONS

Kulibaba R.A., Liashenko Yu.V., Yurko P.S. Genetic differentiation of Ukranian chicken breeds using various types of molecular genetic markers 282

REPRODUCTIVE BIOTECHNOLOGIES

Abilov A.I., Kombarova N.A., Mymrin V.S. et al. Autoimmunity and the endogenous hormone profiles of bull sires 293

Tkachev A.V., Tkacheva O.L., Rossokha V.I. Cytogenetic status of mares (*Equus caballus*) of Ukrainian riding breed influences their fertility 302

Shatalina O.S. The association between blood group and reproductive performance in cattle 309

Hoseini S.A., Mohammadzadeh S., Kadivar A. Comparison of semen characteristics in Romanov and Lori Bakhtiari rams 318

AQUACULTURE

Lagutkina L.Yu., Ponomarev S.V. Organic aquaculture as promising trend of the fish industry development (review) 326

Adzhiev D.D., Pronina G.I., Ivanov A.A. et al. Functional indicators of poikilothermic aquatic species from natural and artificial water biocenoses 337

Komarova L.V., Kostitsyna N.V., Boronnikova S.V. et al. Genetic structure of natural populations of sterlet (*Acipenser ruthenus* L.) in the catchment basins of the Kama and Ob rivers based on polymorphic ISSR markers 348

NORTHERN REINDEER HERDING

Ilina L.A., Laishev K.A., Yildirim E.A. et al. Comparative analysis of rumen bacterial community of young and adult *Rangifer tarandus* reindeers from Arctic regions of Russia in the summer-autumn period 355

Makarov D.A., Komarov A.A., Ovcharenko V.V. et al. Dioxin and heavy metals contamination of reindeer offal from Russian Far North regions 364

DIETARY ADDITIVES AND BIOACTIVE SUBSTANCES

Nekrasov R.V., Zelenchenkova A.A., Chabaev M.G. et al. Melanine protein-energy additive from *Hermetia illucens* larvae in nutrition of calves 374

Fisinin V.I., Ushakov A.S., Duskaev G.K. et al. Mixtures of biologically active substances of oak bark extracts change immunological and productive indicators of broilers . 385

Sizova E.A., Miroshnikov S.A., Lebedev S.V. et al. Comparative tests of various sources of microelements in feeding chicken-broilers 393

MICROBIOLOGY AND VETERINARY MEDICINE

Selyaninov Yu.O., Egorova I.Yu., Alekseev Ya.I. et al. Phenotypic, biochemical and molecular analysis of *Bacillus anthracis* strains isolated during the outbreaks of anthrax in the Russian Federation, 2014-2016 404

Shakhov A.G., Sashnina L.Yu., Yerina T.A. Use of probiotics Giprolam and Simbiter-2 to correct the vagina biocenosis in down-calving cows 414

VIROLOGY

Pestova Ya.E., Artyukhova E.E., Kostrova E.E. et al. Real time PCR for the detection of field isolates of lumpy skin disease virus in clinical samples from cattle 422

Dubrovskaya O.A., Sereda A.D., Kazakova A.S. et al. Validation of a test system for African swine fever serodiagnosis using immunoblotting 430

Usadov T.R., Morgunov Yu.P., Zhivoderov S.P. et al. Lumpy skin virus isolated in 2015 in Russia from cattle is pathogenic for sheep at experimental infection 438

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THE HYPOTHESIS OF A SPECIFIC RELATIONSHIP BETWEEN PEROXISOMAL, MITOCHONDRIAL, AND CYTOPLASMIC PROCESSES IN METABOLIC REGULATION OF HIGHLY PRODUCTIVE RUMINANTS

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Abstract

The authors believe that the explanation of the accumulated data on metabolic processes in highly productive ruminant animals, which for the time being remains within the framework of the existing physiological and biochemical paradigm, requires an in-depth interpretation on a fundamentally new experimental and conceptual basis, which assumes an analysis of the complex interconnections of the set of objects and their functions that were previously not considered. First, , it is necessary to consider the biochemistry of intracellular compartmentalization from a different point of view based on the strict mutual complementarity of the mitochondrial Krebs cycle and the cytoplasmic glycolysis and gluconeogenesis with a peroxisomal glyoxylate cycle. The possibility of glyoxylate cycle functioning in highly productive ruminants was postulated by the authors for the first time following from experimental data on catalytic activity of isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) (V.P. Galochkina et al., 2012). The presence of these enzymes allows the synthesis of glucose from acetic acid, which comes in large quantities from the contents of the rumen. Ruminants are physiologically hypoglycemic. Phylogenetically, they mainly eat coarse vegetable food which increases the proportion of acetate in the rumen content. Easily hydrolyzed carbohydrates in the rumen content reduce the percentage of acetate and increase the proportion of propionate and butyrate, which results in a decreased pH (M. Oba et al., 2015). Permanent glucose deficiency causes an increase in the somatotropin to insulin level indicating an increase in the metabolically ineffective gluconeogenesis. Simultaneously, the blood concentration of unesterified fatty acidы increases, indicating an increase in lipolysis in fat depots. There is a low ratio of insulin to glucagon with an increase in urea concentration. Milk fat content reduces (F. Piccioli-Cappelli et al., 2014). Peroxysomes are partially capable of beta-oxidation of fatty acids to C 13, which facilitates Krebs cycle and allows changes in its metabolic orientation. The authors consider the glyoxylate cycle as a chance which enables the animal to improve metabolism and intensify productivity. Bicarbon acid oxidation is energetically more effective compared to tricarboxylic acid cycle, since the glyoxylate cycle is a shortened of tricarboxylic acid cycle capable of functioning without limiting isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase reactions (V.P. Galochkina et al., 2011). Secondly, one must considered hypothetical provisions on the leading regulatory role of multifactorial interrelationships between mono- and multimolecular constellations of mono- and polymeric biologically active substances, hormones and enzymes, both temporarily formed and constant. This extensive group of specific agents includes insulin, peroxisomal cysteamine, glyoxylic acid, oxygen, hydroperoxide and D-amino acid oxidases. The theoretical positions stated in the article have passed primary validation in model experiments on intensively fattened bulls with the use of clenbuterol, the agonist of beta-adrenergic receptors.

Keywords: regulation of metabolism, peroxisomes, glyoxylate cycle, D-amino acid oxidase, glyoxylate, cysteamine, insulin, hydroperoxide, oxygen

A large number of organic acids formed in a fore stomach of highly productive ruminant animals and serving as the main source of metabolic energy have a multiple positive effect on the Krebs cycle and on specific features of the pro-

duction and metabolic processes in vivo. Glyoxylate cycle in majority of the living beings plays a role of the main assistant in the main metabolic cycle. Due to the expressed biochemical features of metabolism in highly productive ruminant animals, glyoxylate cycle evolutionally plays a role of the auxiliary link to ensure the improved metabolism and, accordingly, to increase the productivity in animals. It must be understood that organism of highly productive ruminant animals operates in a specific mode of chronic metabolic stresses and requires suitable meeting of the specific metabolic needs for realization of the high productivity potential. It results in a continuing need for the reasoned biologization of all technical feeding and growing aspects of highly productive animals.

Highly proactive animals not only produce more products of better quality, but also consume less nutritional substances for production thereof. Their metabolism is characterized by different speed and different metabolic trend. Highly productive milking cow shall have productive longevity while maintaining its reproductive function. A cow yielding 30 kg of milk daily eliminates at average of 1200 g of fat, 1000 g of protein, and 1400 g. of lactose. Herewith, no more than 10% of the glucose formed in the gastrointestinal tract due to the hydrolysis of feed carbohydrates is used in the metabolic process. The remaining glucose required for synthesis of the milk components and to address all metabolic needs of the organism are synthesized de novo [1, 2].

Recently, many farming units in Russia get an average annual milk yield per stock of 10000 kg per cow and more. Ruminant animals are phylogenetically adapted to consumption of a coarse vegetable food rich in fiber, which is hydrolyzed in the rumen content predominantly formed with acetate. In practice, high-concentrate hydrolyzing rations, hydrolysis products of which change the evolutionally developed fermentation processes are applied. They decrease proportion of acetate and propionate in rumen content and results in decrease of pH and systemic disturbance of acid-base balance [3], acidulation of tissues and decrease of fat biosynthesis in the breast gland. Consequently, syndrome of a low content of fat in milk is an issue of a particular concern in the world, and pertains to so-called nutritional disorders in highly productive cows.

In our view, the existing visions of metabolic processes in highly productive cows do not yield the necessary level of understanding of how to achieve high productivity while maintaining the fat content in milk. A revolutionary new physiologic and biochemical basis for rethinking it shall involve knowledge on glyoxylate cycle in peroxisomal reactions in combination with oxidation of D-amino acids, glycolysis, gluconeogenesis, liposysis and bioenergetic processes in cytoplasm and mitochondria [4, 5]. Before proceeding with presentation of the subject matter of the proposed conceptual approach, we are going to consider the role of some critical sub-cellular organelle, metabolic cycles, and molecular compounds participating in the regulatory processes.

Peroxisome. Being the oldest intracellular subunit and the last one discovered in recent years, it is considered to be the key organelle of intracellular, intercellular, and inter-organ communication, cooperation, and regulation of the biochemical processes [6]. Peroxisome got its name because it always contains enzymes by use of the molecular oxygen for detachment of hydrogen atoms from the organic substrates in the oxidation reactions with formation of hydrogen peroxide [7]. Along with mitochondria, peroxisome serves as the main oxygen utilization center in a cell [8]. Peroxisome, having metabolic systems of formation and decomposition of hydrogen peroxide, generation and quenching of superoxide radicals, may influence on many processes in a cell. Quantity of intracellular hydrogen peroxide defines the intensity of morphogenetic and biochemical processes, and peroxisome serves as the regulator of oxidation-reduction peroxide-

dependent reactions controlling both the speed of biosynthesis and biodegradation [9]. As distinguished from the processes in mitochondria, hydrogen peroxide, rather than high-energy compounds, is formed due to the peroxisomal oxidation. Peroxisome just like the mitochondria performs the biological oxidation function, save that the oxidation process in peroxisome is not associated with generation of the nicotinamide adenine dinucleotide, reduced form (NADN) and adenosine triphosphate (ATP) [10]. If mitochondrial Krebs cycle is publicly recognized as the main metabolic and energetic "tank" utilizing the end products of all principal metabolic flows in vivo and simultaneously performing a function of a regulatory center of such processes, then peroxisomes with their glyoxylate cycle shall be apparently seen as antioxidant "reactors" and "dispatching nods" in vivo. It should be noted than both pro- and antioxidant reactions simultaneously proceed in both most important cell compartments – mitochondria and peroxisome.

Noble Prize Winner C. De Duve was the first to suggest that it was the peroxisomal metabolism that played an important role in development of new biochemical transformation ways. For that end, peroxisome required enzymatic machinery for performance of the metabolic reactions (in addition to electron carrier system) ensuring cooperation with mitochondria [11]. Interoperation between peroxisome and mitochondria is due to the organization of metabolite flows comprising an integral regulated structure specific for this or other tissue, organ, and organism in general [12].

D-amino acid oxidase and production of reactive oxygen forms. Peroxisome is the only sub-cellular organelle where D-amino acid oxydase (DAAO) consume oxygen in the catalytic process of right-handed amino acid hydrolysis and produce hydroperoxide [13]. Oxygen is indispensable for all aerobic organisms and plays the paramount role in generation of high-energy compounds at oxidative phosphorylation. Reactive oxygen forms (ROF), including superoxide anion and H_2O_2, are formed in these reactions, which, inter alia, are required for signal transduction in metabolic pathways, regulating the cell growth and oxidation-reduction status [14]. The oxygen role in vivo is dualistic. ROFs, being natural and absolutely essential metabolites, are involved in a number of natural physiological processes, including killing of microbes and viruses. However, during stress periods of any etiology ROF concentration may drastically grow, thus, causing many pathological states in structures and functions of a cell [15].

Low production of H_2O_2 in astrocyte peroxisome protects neurons from the oxidation stress, whilst high concentration of H_2O_2 is neurotoxic [16, 17]. Inhibition of astrocyte enzyme of D-amino acid oxydase protects neurons from the oxidation loss allowing using the H_2O_2-based neuroprotection machinery [18]. These discoveries show implication of D-amino acid oxydase in control of intracellular concentration of hydrogen peroxide across time and space, and represent opportunity for decoding of the most interesting and novel astrocyte-dependent neuroprotection mechanism. In pursuance of the above-listed factors, it was assumed that for combat with oxidation stress all neurons are linked together in a metabolic complex with astrocytes. The same assumption was made regarding the substrates of peroxisomal oxydase, which could represent an unstable and, rather, temporary structures — unstable non-enzymatic complexes of glyoxylic acid with various nucleophilic agents, including D-amino acids [19]. Hydrogen peroxide plays the key role in a cell signal system, and is deemed to be the predominant candidate to adaptive reaction mediators in astrocytes. Thus, it implies the importance of hydrogen peroxide in maintenance of astrocyte-dependent neuron protection from the oxidation stress and the role of H_2O_2 in in-

duction of astrocyte activation of specific neurotropic transcriptional nuclear factor (Nrf-2). Such results presuppose the availability of specific regulating mechanisms for neuronal cells with involvement of the hydrogen peroxide [20]. The organism constantly requires regulating and neutralization of ROFs with participation of a number of special and indirectly involved enzymes, including DAAO, superoxide dismutase, xanthine oxidase, glutathione peroxidase, hemoxygenase, and etc. [21].

Hypothesis on interoperation of glyoxylate cycle, D-amino acid oxidase and insulin in regulation of the cell metabolism. *Metabolic function of cysteamine.* For a long time, cysteamine was regarded as a traditional antioxidant and standard radio protector. Cysteamine is a thiol-containing product of cysteine decarboxylation with participation of panthotenic acid serving as the primary component of coenzyme A synthesis. In case of deficit of the panthotenic acid in the organism, insulin treatment results in sharp reduction of coenzyme A synthesis [22]. Insulin decreases the degradation speed of coenzyme A and cysteamine synthesis. It is quiet natural since coenzyme A is required for insulin activation of acetate acylation and residues of other fat acids upon synthesis of the fat acids with longer carbon chain [23]. Cysteamine is regarded as an intracellular negative messenger of insulin. To this end, insulin and cysteamine have diametrically opposite effects on a number of metabolic processes. For instance, insulin promotes activity of pyruvate dehydrogenase (PDH), glycogen synthase, hexokinase, phosphorylase, and phosphatase. Cysteamine have an inhibition effect on these enzymes [24]. Insulin inhibits fructose-1,6-bisphosphatase, while cysteamine activates thereof. Decrease of cysteamine concentration has positive effect on the processes flowing under the action of insulin. Krebs cycle, glycolysis, lipogenesis, and synthetic processes are activated [25].

Spontaneously formed nucleophilic cysteamine-glyoxylate complex is a good substrate for D-amino acid oxidase. This complex is formed in the physiological conditions in presence of oxygen [23]. Reaction of oxydase with cysteamine-glyoxylate complex flows significantly faster than with complexes of glyoxylate with acetaldehyde-ammonia, putrescine, aminopropanol, octapamine, ethylendiamine, and ethyl ether of cysteine. Normal physiological amines, including histamine, serotonin, adrenalin, noradrenalin, spermine, spermidine, and cadaverine do not practically react with glyoxylate by D-amino acid oxidase [26, 27]. It is assumed that product of glyoxylate-cysteamine reaction may serve as a metabolic effector which, following formation of a covalent link, may intensify reactivity of enzymes and may modify nucleic acids [28].

Role of glyoxylate acid. Glyoxylate is a quiet toxic intracellular compound for the animals [29]. It controls many reactions: it inhibits mitochondrial carriage of phosphorus, carriage of electrons through cytochrome chain and transfer of mitochondrial substrates, and suppresses activity of enzymes of Krebs cycle. Glyoxylate, having inhibited phosphatase of pyruvate dehydrogenase complex (PDC) (its regulatory subunit), decreases the flow of pyroracemic acid through the pyruvate dehydrogenase complex. It is crucial for the organism to neutralize such highly-reactive hazardous two-carbon intermediate product of peroxisomal reactions. As it was already noted before, the best substrates of peroxisomal oxidase are unstable non-enzymatic glyoxylate complexes with different nucleophilic agents, including the D-amino acids [19, 20].

Inhibitors of D-amino acid oxidase. D-amino acid oxidase is found in peroxisome only. It plays the specific role in the central nervous system due to involvement in maintenance of the cognitive functions, which we associate with animal aggressiveness, temper, and adaptiveness to feeding and keeping conditions. Many studies in activation and inhibition of D-amino acid oxidase are devoted to decoding of the interoperation mechanisms of such enzyme category with

specific receptors, particularly, in the brain tissues [27, 30, 31].

Strong non-specific oxydase inhibitors, including D-amino acid oxidase, involve adenosine diphosphate (ADP), adenosine diphosphate ribose (ADP-ribose), nicotineamide adenine dinucleotide, reduced form (NAADN), nicotineamide adenine dinucleotide phosphate, reduced form (NAADP), diphosphocoenzyme A (intermediary product of CoA synthesis, the most effective inhibitor of such type of inhibitors), which at physiological concentration inhibit under the Flavin Adenine Dinucleotide (FAD) – concurrent mechanism. Weak non-specific inhibitors also include CoA, acetile-CoA, ATP, and etc. [32, 33]. The fact that ADP is an effective inhibitor whereas ATP is not, expressly points out to influence of the energetic cell state on the oxidase activity. Restored nicotinamide coenzyme forms inhibit the oxidase, while the oxidated forms activate it. Accordingly and what is very important, the oxidation-reduction cell systems, are predominantly exposed to effect of O_2 (by all means, along with other substrate-metabolic and cofactor effects) [34, 35].

Insulin. Insulin is one of the important poly-functional and thoroughly studied anabolic hormones. By activation of Na^+-K^+-dependable adenosine triphosphatase it strengthens carriage of both glucose and amino acids to a cell. Along with that, insulin is in charge for biosynthesis and degrading of proteins, growth of muscle mass, depositing and consumption of energetic material in form of lipids and glycogen (23). As we have already noted before, cysteamine has an anti-insulin effect on metabolism. Insulin may be considered to be involved in practically all main processes in vivo: it actively promotes pyruvate dehydrogenase, it advances glycogen synthesis, it activates glycolysis, proteo-synthesis and lipogenesis, at the same time suppressing glycogenolusis and gluconeogenesis, and reducing liposysis. As it infers from the preceding paragraph, cysteamine has positive effect on all the above-listed processes [23].

According to the available information on oxidase inhibition, reactions catalyzed by D-amino acid oxidase are engaged in regulatory insulin system in a cell. Earlier we have considered in detail the possibility of specific induction mechanisms and interrelated operation of peroxisome and insulin in ruminant animals, as apart from such mechanisms in monogastric animals [36, 37]. We believe that studying of the functional activity of insular apparatus, its interoperation with the general hormone status, value and efficiency of metabolic and productive response reaction to practically all influencing factors, serves as the pacing factor for description of the metabolic control mechanisms in animals [36]. Insulin and somatotropin production value and dynamics were studied depending on the qualitative and quantitative type of the animal diet [36]. Based on such studies, we have emerged to a justified opinion that concentration of insulin and somatotropin in blood varies upon strengthening of hydrolysis in rumen and intestinal tract, whereas it is increased in the first one and decreased in the second one. In other words, the increased secretion of insulin induced by the breeding inhibits biosynthesis and secretion of the growth hormone produced by adenohypophysis. However, serious biochemical explanation of the paradox of multidirectional change of concentration of such two anabolic hormones important for the organism ("insulin-somatotropin scissors") is still missing. We have proposed that high concentration of insulin after breeding results in its higher connectivity, and insulin in a bound state does not have any effect on proteolytic activity and is unable to activate somatotropic hormone which, in pursuance thereof, requires participation of a specific protease. Consequently, after feeding most part of somatotropin is in inactive form and is scarcely found. Most probably, such mechanism may partially explain the multidirectional action of such an-

abolic hormones. Insulin and somatotropin are synergic by their effect on somatomedin and are antagonists by their effect on metabolism of glucose and fat acids. Such relations are precisely regulated by "insulin-somatotropin scissors". After feeding, not only accumulation of the plastic and energetic substances in form of the muscle mass is required, but also accumulation of carbohydrates and fats in form of glycogen and deposited lipids. Somatotropin promotes lipolysis and gluconeogenesis, of which from the amino acids. Therefore, after feeding the insulin, having activated the synthesis of glycogen and lipogenesis, presents activation of somatotropin. At that, insulin activates glycolysis for use of the produced energy in synthesis of the muscular proteins.

Tests on bull-calves with various feeding schedules and use of hormones and clenbuterol - agonist of β-adreno receptors. We have compared metabolism of Kholmogory bull calves at different feeding intensity, different levels of dietary protein, lysine, and methionine not digestible in a rumen, upon use of androgenic and estrogenic preparations and betazine thyrostatic and upon use of different dosages of clenbuterol, the agonist of β-adrenoreceptors [36]. The results were conclusive, but unexpected. Thus, use of synthetic clenbuterol anabolic just like the intensive feeding had resulted in suppression of insular apparatus of pancreas, adrenal, and thyroid glands. Compensative strengthening of functions of such glands was noted after withdrawal of the medicines. We have drawn our attention to such paradoxical and unexplainable fact that insulin concentration in blood is reduced, whereas this hormone is primarily in charge for growth of the muscular mass and accumulation of energy sources for maintenance of the growth.

It is a well-known that both clenbuterol and insulin promote biosynthesis of a muscle protein and reduce degrading thereof [36]. Although insulin increases lipogenesis and suppresses lipolysis, clenbuterol reduces deposition of a fat. In other model test at feeding of the bull-calves by exogenous anabolic sex steroids and betazine, activity of all dehydrogenases of citrate cycle was reduced in liver with sharp increase of pyruvate carboxylase activity in combination of the high intensity of growth and significant decrease of concentration of 11-oxycorticosteroids, insulin, and somatotropin in blood [37, 38. Herewith, the most intensive growth and the best results of carcass yield, yield of additional meat in carcass, and content of the protein in meat were found in bull-calves with the lowest basal level of insulin and thyroid hormones in blood. Such direction of metabolic processes and formation of the productive features in animals is absolutely not in line with the existing biochemical paradigm. In our opinion, such facts may have physiological and biochemical explanation only accounting for the processes flowing in peroxisomes. In particular, we believe that ability of such structures to supply the additional acetate-based glucose to the organism, thus resulting in the increased activity of Krebs cycle, and to participate simultaneously in induction of the synthesis and secretion of insulin plays an important role in the considered studies [1, 4, 37].

G.A. Hamilton [23] had shown the ability of peroxisomal oxidase to catalyse formation of H_2O_2 with consumption of molecular oxygen. Besides, it is well-known that reduction of intracellular partial oxygen pressure results in inhibition of oxidation-reduction reactions. Cysteamine-glyoxylate complex serves as an active substrate form of D-amino acid oxidase. Complexes are formed spontaneously and are present in the physiological conditions in concentrations leading to the significant growth of reaction speeds catalyzed by D-amino acid oxidase. Glyoxylate itself is considered as an inhibitor of oxidative metabolism and breath in vitro. It has non-enzymic reaction with oxaloacetate and produces oxalomalonat (keto-isocitrate), which simultaneously serves as an inhibitor of aconitase, NAADP-dependent isocitrate dehydrogenase and α-ketoglutarate dehydrogenase.

Besides, it inhibits mitochondrial carriage of phosphorus and electrons along the cytochrome chain. As we have already noted before, D-amino acid oxidase acidifies glyoxylate in form of glyoxylate-cysteamine complex, and cysteamine performs a function of a negative intracellular insulin messenger. In its turn, insulin may slow down formation of cysteamine by inhibition of CoA metabolism leading in formation of cysteamine. Insulin, having linked with its receptors on membranes, increases hardness of the later and slows down the access of intracellular CoA metabolites and phospho-pantetheine to relevant enzymes — nucleotide pyrophosphatase and alkaline phosphatase located on surface of the membrane exposed to the cytoplasm [23].

Besides, it is known that hypoglucemia is developed in animals with deficit of pantothenate and such animals are substantially more sensitive to insulin than animals sufficiently supplied with such vitamin. It is also known that concentration of CoA does not forego any significant changes upon additional insulin injections. However, the speed of its biosynthesis from the pantothenic acid is sharply decreased (90 % of inhibition in perfused heart). Insulin reduces the CoA degrading and cysteamine synthesis speed. As it was noted before, insulin promotes activity of pyruvate dehydrogenase, glycogen synthase, hexokinase, phosphorylase, and phosphatase. Cysteamine has an inhibition effect on such enzymes. At the same time, insulin inhibits fructose-1,6-bisphosphatase, while cysteamine activates thereof. Since insulin decreases concentration of intracellular oxygen, it shall, in our opinion, decrease the activity of peroxysomal oxydase, thus, leading to accumulation of glyoxylate. At the same time, decrease of the partial oxygen pressure promptly inhibits the activity of Krebs cycle enzymes. Decrease of activity in peroxysomal oxydase results in increased concentration of glyoxylate, which also serves the active inhibitor of Krebs cycle enzymes and cytochrome system enzymes. In our opinion, the resulting metabolic state of a cell shall result in activation of glyoxylate cycle in peroxisomes.

According to G.A. Hamilton [23], H_2O_2, it may have insulin-like effect by free access through membranes. However, this idea was not taken seriously by major part of scientific world, regardless of the firm empirical support of fast phosphorylation of insulin receptors in fat tissue in presence of the hydrogen peroxide. Similar mechanism was confirmed for insulin-like growth factor (IGF). It is notable that in purified plasmatic membranes (as compared to homogenates) hydrogen peroxide does not have an adverse effect. It is assumed that the said insulin-like effect of H_2O_2 may not be intermediated by stimulation of phosphorylation of insulin receptors by hydrogen peroxide. It results in the increased membrane tension and activation of Na^+/K^+-ATP in tissues, including miocytes, which promotes biosynthesis of muscular proteins and growth of muscular mass. The above-listed factors confirm the ability of H_2O_2 to render insulin-like effects. Herewith, activation of receptors by hydrogen peroxide requires a number of cell components [22, 39-41]. We assess these facts in support of our hypothesis, subject to which the processes similar to processes flowing at insulin stimulation may flow under the effect of H_2O_2 in the intensively fed bull-calves at low concentration of insulin in blood and activation of peroxisomal reaction not only in adipocytes, but also in other tissues, including the muscular tissue. These may, in particular, involve the observed strengthening of biosynthesis of the muscular proteins. Besides, even if role of H_2O_2 as an intracellular insulin messenger (the similar function is performed by cysteamine) was not confirmed, its ability to render insulin-like effects was already demonstrated [23, 24].

For ruminant animals, when large quantity of acetic acid is formed at chronic deficit of glucose, it is metabolically important to use part of acetic acid for synthesis of organic acids, from which glucose is formed in furtherance. This

is justified by the need for glyoxylate cycle operation in such animals, which would allow achieving the higher productivity without metabolic disorders in form of ketosis and acidosis, provided sufficient quantity of easily-digestible fiber in a diet of such animals [4]. In complex crosslinks in the clenbuterol test we have seen the action of specific insulin-peroxisome interaction mechanisms. According to our concept, it could be assumed that application of clenbuterol results in activation of glyoxylate cycle with strengthening of peroxisomal β-oxidation of fat acids. Herewith, two molecules of the activated acetate acid with formation of one molecule of succinate, malate, and oxaloacetate are used for one turn of glyoxylate cycle [37].

Glyoxylate cycle. All biochemistry study guides point out to the lack of glyoxylate cycle in higher animals. However, we have demonstrated that key glyoxylate cycle enzymes of isocitrate lyase (EC number 4.1.3.1) and malate synthase (EC number 4.1.3.2) are functioning along with Krebs cycle dehydrogenase and pyruvate carboxylase (key enzyme of gluconeogenesis) in hepatic tissues of bull-calves intensively fed for meat [1, 37]. Therefore, we believe that it is possible to assume that in metabolism securing the high productivity in ruminant animals glyoxylate cycle not only functions by strengthening of metabolite flow through the cycle of tri-carbon acids to terminal oxidation chain, but also renders regulatory effect on the general trend of metabolite flow simultaneously with Krebs cycle dehydrogenase [2, 4]. Two of the above-mentioned cycles, by performance of the intracellular endoecological function, integrate carbohydrate and lipid metabolism regulating the gluconeogenesis, Krebs, and glyoxylate cycles, and electron carriage chains. This demonstrates the quantitative coherence of carbon flows, processes in various sub-cellular compartments and hormone, substrate, cofactor, and enzyme regulation of such processes. In our opinion, glyoxylate and Krebs cycles are two complementary, interchangeable, interrelated, and mutually regulating processes.

Works of the national scientists show functioning of the glyoxylate cycle in laboratory animals — new-born, starving, diabetic, and placed in extreme conditions (stress) [42-45]. In support of our hypothesis, it is principally important that four of the above-described states may be unified by a common consistent pattern – metabolic deficit of glucose, which is also peculiar to ruminant animals with hypoglycemia – physiological norm. The above considered factors allow us to assume that ruminant animals in process of their evolution may have produced and philogenetically fixed the response reaction of organism to chronically lowered concentration of glucose in blood. One of the assumed adaptations is functioning of the glyoxylate cycle is constantly induced by glucose deficit for the additional metabolic support by such essential metabolite. To sum up, it should be concluded that glyoxylate cycle in ruminant animals - first of all (in order of priority, not in order of importance), directly relates to cell endoecology, secondly, relates to growth of metabolic productivity of the tri-carbon acid cycle (upon interoperation thereof with bi-carbon acid cycle) for improvement of the high production substrate support, and thirdly, participates in coordination of the principal intracellular, intercellular, and inter-organ metabolic processes across the time and space.

To conclude, we again draw our attention to potential metabolic role of D-amino acid oxidase in ruminant animals. We have considered the ability of such enzyme to hydrolyse the substrates with the use of molecular oxygen and production of hydrogen peroxide. As it was noted before, reduction of intracellular partial oxygen pressure results in inhibition of the oxidation-reduction reactions, and growth of such pressure results in activation thereof. Accordingly, D-amino acid oxidase may contribute to intracellular regulation at two levels: by decreasing of oxygen concentration and by increasing the concentration of H_2O_2.

Thus, we suggest a hypothesized regulation of the metabolic processes in highly productive ruminant animals accounting for the role of pro-oxidation systems, hormones, cell compartments, related cycles of tri-carbon and bi-carbon cycles. Known laws of biochemical logic allow assuming the existence of the assumed hypothetic chain of metabolic transformations, while the proposed approach allows adjusting the traditional paradigm. Besides, it may contribute to perception of the whole number of received paradoxical and empirical factors pending their interpretation and discovered by us and many authors in considering the specific aspects of metabolism in ruminant animals. Volume of accumulated knowledge allows adjusting the existing physiological and biochemical perspectives at principally new conceptual platform. It is based on subtle entanglement of mitochondrial Krebs cycle, cytoplasm glycolysis and gluconeogenesis processes with peroxisomal glyoxylate cycle. The complex of multi-factor interlinks between the insulin, peroxisomal cysteamine, glyoxylate, Krebs cycle dehydrogenase, oxygen, hydroperoxide, and D-amino acid oxidase should be accounted for understanding of their leading role in regulation of the metabolic processes in highly productive ruminant animals. Naturally, the proposed hypothetic concept requires supplementary understanding, updating, and broad empirical verification.

REFERENCES

1. Galochkina V.P., Solodkova A.V., Galochkin V.A. *Trudy regional'nogo konkursa proektov fundamental'nykh nauchnykh issledovanii*, 2012, 17: 205-214 (in Russ.).
2. Oba M., Mewis J.L., Zhining Z. Effects of ruminal doses of sucrose, lactose, and cornstarch on ruminal fermentation and expression of genes in ruminal epithelial cells. *J. Daire Sci.*, 2015, 98(1): 586-594 (doi: 10.3168/jds.2014-8697).
3. Piccioli-Cappelli F., Loor J.J., Seal C.J., Minuti A., Trevisi E. Effect of dietary starch level and high rumen-undegradable protein on endocrine-metabolic status, milk yield, and milk composition in dairy cows during early and late lactation. *J. Dairy Sci.*, 2014, 97(12): 7788-80 (doi: 0.3168/jds.2014-8336).
4. Galochkina V.P., Solodkova A.V., Galochkin V.A. *Problemy biologii produktivnykh zhivotnykh*, 2011, 4: 5-18 (in Russ.).
5. Galochkin V.A., Agafonova A.V., Galochkina V.P., Cherepanov G.G. *Problemy biologii produktivnykh zhivotnykh*, 2015, 1: 5-25 (in Russ.).
6. Sandalio L.M., Romero-Puertas M.C. Peroxisomes sense and respond to environmental cues by regulating ROS and RNS signalling networks. *Ann. Bot.*, 2015, 116(4): 475-485 (doi: 10.1093/aob/mcv074).
7. Pascual-Ahuir A., Manzanares-Estreder S., Proft M. Pro- and antioxidant functions of the peroxisome-mitochondria connection and its impact on aging and disease. *Oxid. Med. Cell Longev.*, 2017, 2017: 9860841 (doi: 10.1155/2017/9860841).
8. Del Río L.A., López-Huertas E. ROS generation in peroxisomes and its role in cell signaling. *Subcell. Biochem.*, 2013, 69: 231-55 (doi: 10.1007/978-94-007-6889-5_13).
9. Santillo A., Falvo S., Chieffi P., Burrone L., Chieffe Baccari G., Longobardi S., Di Fiore M.M. D-aspartate affects NMDA receptor-extracellular signal-regulated kinase pathway and upregulates androgen receptor expression in the rat testis. *Theriogenology*, 2014, 81(5): 744-751 (doi: 10.1016/j.theriogenology.2013.12.009).
10. De Duve C., Baudhuin P. Peroxisomes (microbodies and related particles). *Physiol. Rev.*, 1966, 46: 323–357 (doi: 10.1152/physrev.1966.46.2.323).
11. Schrader M., Yoon Y. Mitochondria and peroxisomes: are the 'Big Brother' and the 'Little Sister' closer than assumed? *Bioassays*, 2007, 29: 1105-1114 (doi: 10.1002/bies.20659).
12. Camoes F., Bonekamp N.A., Delille H.K., Schrader M. Organelle dynamics and dysfunction: a closer link between peroxisomes and mitochondria. *J. Inherit. Metab. Dis.*, 2009, 32: 163-180 (doi: 10.1007/s10545-008-1018-3).
13. Ohide H., Miyoshi Y., Maruyama R., Hamase K., Konno R. D-Amino acid metabolism in mammals: biosynthesis, degradation and analytical aspects of the metabolic study. *Journal of Chromatography B: Biomedical Sciences and Applications*, 2011, 879(29): 3162-3168 (doi: 10.1016/j.jchromb.2011.06.028).
14. He L., He T., Farrar S., Ji L., Liu T., Ma X. Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. *Cell Physiol. Biochem.*, 2017, 44(2): 532-553 (doi: 10.1159/000485089).
15. Tripathi D.N., Walker C.L. The peroxisome as a cell signaling organelle. *Curr. Opin. Cell Biol.*, 2016, 39: 109-12 (doi: 10.1016/j.ceb.2016.02.017).

16. Trompier D., Vejux A., Gondcaille C., Geillon F., Nury T., Savary S., Lizard G. Brain peroxisomes. *Biochimie*, 2014, 98: 102-110 (doi: 10.1016/j.biochi.2013.09.009).
17. Yamanaka M., Miyoshi Y., Ohide H., Hamase K., Konno R. D-Amino acids in the brain and mutant rodents lacking D-amino acid oxidase activity. *Amino Acids*, 2012, 43(5): 1811-1821 (doi: 10.1007/s00726-012-1384-x).
18. Haruta N., Iizuka H., Ishii K., Yoshihara S., Ichiba H. Alteration in the plasma concentration of a DAAO inhibitor, 3-methylpyrazole-5-carboxylic acid, in the ketamine-treated rats and the influence on the pharmacokinetics of plasma D-tryptophan. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.*, 2011, 87(10): 641-648 (doi: 10.2183/pjab.87.641).
19. Sasabe J., Suzuki M., Imanishi N. Activity of D-amino acid oxidase is widespread in the human central nervous system. *Front. Synaptic Neurosci.,* 2014, 6: 14 (doi: 10.3389/fnsyn.2014.00014).
20. Zhao W.J., Yin M. Advances in the study of D-amino acid oxidase in the central nervous system. *Sheng Li Ko Hsueh Chin Chan [Progress in physiological sciences]*, 2008, 39(1): 64-66 (in Chinese) (PMID: 18357693).
21. Hayes G.R., Lockwood D.N. Role of insulin receptor phosphorylation in the insulinomimetic effects of hydrogen peroxide. *PNAS USA*, 1987, 84(22): 8115-8119 (doi: 10.1073/pnas.84.22.8115).
22. Hamilton G.A. Peroxisomal oxidases and suggestions for the mechanism of action of insulin and other hormones. In: *Advances in enzymology and related areas of molecular biology*. A. Meister (ed.). John Wiley & Sons, 1985, V. 57: 85-178 (doi: 10.1002/9780470123034.ch2).
23. Hamilton G.A., Buckthal D.J., Mortensen R.M., Zerby K.W. Reactions of cysteamine and other amine metabolites with glyoxylate and oxygen catalyzed by mammalian D-amino acid oxidase. *PNAS USA*, 1979, 76(6): 2625-2629 (PMCID: PMC383660 PMID: 37501).
24. Farias R.N. Insulin-membrane interaction and membrane fluidity changes. *Biochim. Biophys. Acta*, 1987, 906(3): 459-468 (doi: 10.1016/0304-4157(87)90020-7).
25. Nishina Y. Structure and reaction mechanism of D-amino acid oxidase. *SEIKAGAKU [Journal of Japanese Biochemical Society]*, 2008, 80(6): 569-578 (in Japanese) (PMID: 18634432).
26. Haruta N., Iizuka H., Ishii K., Yoshihara S., Ichiba H., Fukushima T. Alteration in the plasma concentration of a DAAO inhibitor, 3-methylpyrazole-5-carboxylic acid, in the ketamine-treated rats and the influence on the pharmacokinetics of plasma D-tryptophan. *Proceedings of the Japan Academy. Series B, Physical and biological sciences*, 2011, 87(10): 641-648 (doi: 10.2183/pjab.87.641).
27. Hawkins J., Mahony D., Maetschke S., Wakabayashi M., Teasdale R.D., Bodén M. Identifying novel peroxisomal proteins. *Proteins*, 2007, 69: 606-616 (doi: 10.1002/prot.21420).
28. Salido E., Pey A.L., Rodriguez R., Lorenzo V. Primary hyperoxalurias: disorders of glyoxylate detoxification. *Biochim. Biophys. Acta*, 2012, 1822(9): 1453-1464 (doi: 10.1016/j.bbadis.2012.03.004).
29. Lu J.M., Gong N., Wang Y.C., Wang Y.X. D-Amino acid oxidase-mediated increase in spinal hydrogen peroxide is mainly responsible for formalin-induced tonic pain. *Br. J. Pharmacol.*, 2012, 165(6): 1941-1955 (doi: 10.1111/j.1476-5381.2011.01680.x).
30. Gustafson E.C., Morgans C.W., Tekmen M., Sullivan S.J., Esguerra M., Konno R., Miller R.F. Retinal NMDA receptor function and expression are altered in a mouse lacking D-amino acid oxidase. *J. Neurphysiol.*, 2013, 110(12): 2718-2726 (doi: 10.1152/jn.00310.2013).
31. Khoronenkova S.V., Tishkov V.I. D-amino acid oxidase: physiological role and applications. *Biochemistry*, 2008, 73(13): 1511-1518 (doi: 10.1134/S0006297908130105).
32. Kawazoe T., Park H.K., Iwana S., Tsuge H., Fukui K. Human D-amino acid oxidase: an update and review. *Chem. Rec.*, 2007, 7(5): 305-315 (doi:10.1002/tcr.20129).
33. Bonekamp N.A., Volkl A., Fahimi H.D., Schrader M. Reactive oxygen species and peroxisomes: struggling for balance. *BioFactors*, 2009, 35: 346-355 (doi: 10.1002/biof.48)
34. Saam J., Rosini E., Molla G., Schulten K., Pollegioni L., Ghisla S. O_2 reactivity of flavoproteins dynamic access of dioxygen to the active site and role of H^+ relay system in D-amino acid oxidase. *J. Biol. Chem.*, 2010, 285(32): 24439-24446 (doi: 10.1074/jbc.M110.131193).
35. Galochkina V.P., Galochkin V.A. *Problemy biologii produktivnykh zhivotnykh*, 2007, 2: 84-93 (in Russ.).
36. Galochkina V.P., Galochkin V.A. *Uspekhi fiziologicheskikh nauk*, 2009, 49(1): 66-76 (in Russ.).
37. Fomichev Yu.P., Levantin D.L., Dzyuba N.F., Radchenkov V.P., Butrov E.V., Golenkevich E.K. *Vestnik sel'skokhozyaistvennoi nauki*, 1977, 2(245): 85-92 (in Russ.).
38. Islinger M., Grille S., Fahimi D.H., Schrader M. The peroxisome: an update on mysteries. *Histochem. Cell Biol.*, 2012, 137(5): 547-574 (doi: 10.1007/s00418-012-0941-4).
39. Yang T., Poovaiah B.W. Hydrogen peroxide homeostasis: activation of plant catalase by calcium/calmodulin. *PNAS USA*, 2002, 99(6): 4097-4102 (doi: 10.1073/pnas.052564899).
40. Tsai S.C., Lu C.C., Lin C.S., Wang P.S. Antisteroidogenic actions of hydrogen peroxide on rat Leydig cells. *J. Cell. Biochem.*, 2003, 90: 1276-1286 (doi: 10.1002/jcb.10738).
41. Volvenkin S.V., Popov V.N., Eprintsev A.T. *Biokhimiya*, 1999, 64(9): 1185-1191 (in Russ.).
42. Popov V.N., Volvenkin S.V., Eprintsev A.T., Igamberdiev A.U. Glyoxylate cycle enzymes are present in liver peroxisomes of alloxan-treated rats. *FEBS Lett.,* 1998, 440(1-2): 55-58 (doi: 10.1016/S0014-5793(98)01422-7).
43. Kondrashova M.N., Rodionova M.A. *Doklady AN SSSR*, 1971, 196(5): 1225-1227 (in Russ.).
44. Popov V.N., Volvenkin S.V., Eprintsev A.T., Igamberdiev A.U. *Izvestiya RAN, Seriya biologicheskaya*, 2000, 6: 672-678 (in Russ.).

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MOLECULAR MARKERS IN IMMUNE RESPONSE MANIFESTATIONS
(review)

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Abstract

Genetically controlled immune system is responsible for population heterogeneity on the immune response. This review focuses on molecular mechanisms of cell-mediated and antibody-mediated immune response and molecular markers for genomic selection. Genotypic differences between individuals in terms of tolerance/susceptibility to infectious diseases are characteristic of animal populations (S.C. Bishop et al., 2014). Data massive indicates multiple SNPs associated with high and low immune responses of animals, providing the possibility of calculating the coefficients of genomic breeding values for this attribute. There is a need to assess the dispersion of indirect genetic effects that help to open up new possibilities for the control of infectious diseases through selection. However, it should be noted that the quantitative genetic analysis based on individual animal disease status covers only part of the total genetic variation that affects the dynamics of infectious diseases in populations. Estimation of gene expression patterns in a particular immune response is considered as the most valuable (V.V. Firstova et al., 2010). Study of the major histocompatibility complex (MHC-B) 209296 bp region in birds with high-density SNP chips allowed the authors to determine 45 key genes which affect MHC-B diversity through recombination. The findings extend the understanding of the contribution of recombination to the diversity of MHC-B haplotypes, including the ability to identify hot spots and recombination estimation of recombination frequency (J.E. Fulton et al., 2016). The causative mutations related to the basic genetic variability of innate and adaptive immune responses in chickens are mapped (A. Slawinska et al., 2013). Search for causal mutations responsible for genetic variation in the immune response can be used as an approach to diagnostic tests. E.g., SNP associated with susceptibility to tuberculosis are detected (M.L. Bermingham et al., 2014). Immune response falls into a category of complex and quantitative traits that are under control of multiple genes with a noticeable influence of the environment. Obviously, some genes of common universal action may participate in innate and adaptive immunity. We can assume that such immunity has predominantly additive mode of inheritance (M. Siwek et al., 2015). Breeding for diseases resistance is greatly difficult because of low heritability and lack of estimates for comprehensive genetic assessment of the disease resistance variability. Growing genomic evaluations of the animals has created a basis for the use of molecular markers in breeding to increase animal resistance to diseases. Studies of the genome and the overall adaptive immune response associations in different species of farm animals provide an important starting point for the implementation of such plans. Identification of potential biological pathways and genes associated with immune response can assist in advancing the understanding of the important processes in animal resistance or susceptibility to diseases.

Keywords: immune response, antibodies, genome, single nucleotide polymorphism, SNP, disease, resistance, selection, quantitative traits, receptors, animals, heritability, associations, mutations

Infectious diseases are a big challenge for livestock farming and present a zoonotic threat to human health. Change of the nature of immune response in line with a characteristic of the pathogen effect provides main protection for the animals. Genotypic inter-specie differences in terms of tolerance/susceptibility to infectious diseases were found [1, 2]. However, one issue still pending is the possibility for inclusion of the characteristics of immune response into the selective indices to reduce frequency and severity of diseases in animals.

Over the past decade, genome valuation technique by genome-wide

screening of single nucleotide polymorphisms (SNPs) in animals was developed. The technique allows detecting of up to few millions SNPs, hundreds and thousands of which may serve as variation markers at detection of quantitative traits. Genome selection was successfully implemented in the breeder's service of many countries in the world [3].

Present review summarizes information on candidate gene, quantitative trait locus (QTL), causal mutations engaged in immunity control, and associated single nucleotide polymorphisms (SNPs) due to the genomic selection perspectives for disease resistance in animals. The studies on association of polymorphisms in several genome elements with total adaptive immune response in various species of agricultural animals allow creating the framework for realization of such plans. Identification of the potential genes and biological immune-reactivity mechanisms will help us understanding the formation of the disease tolerance/susceptibility in animals and detecting the markers of immune response traits for inclusion thereof in the genome selection criteria.

Present review compares molecular marker-based methods of animal immune response characterization as the tools for selection of individuals with high tolerance to diseases.

Innate and adaptive immunity are two categories of immune response. Studies of genetic background of immune response are based on identification of relevant QTL (quantitative trait loci) and single mutations in structural candidate genes involved in the immune response control. To search SNPs associations in particular QTL regions and gene candidates, such genes and SNPs found in databases (http://www.ncbi.nlm.nih.gov) are assessed for the relationship between SNPs and variation of qualitative traits. There is an aggregated linear model for evaluation of the relative effects of genes on tolerance/susceptibility to infectious diseases [4]. This model is applicable to study the marker and gene associations with incidence and distribution of diseases. However, traditional quantitative genetic analysis of certain pathology covers only part of the total genetic variation that affects the dynamics of infectious diseases in populations [5]. Although research data confirm applicability of linear model for a probabilistic estimate of susceptibility to the infection, characterization of the parameters assessed by this model is incomplete, and its infectious component is not always linear [6]. Another model is based on the equation for probability of the disease depending on genetically controlled specific susceptibility of the individual and the genotypes of the infected members of the group. These models are good tools to study the variability of quantitative traits, when all genes involved must be assessed simultaneously (e.g. in genomic prediction of breeding value), and to identify genes that facilitate the spread of the infection [7].

The high density SNP chips showed 45 main genes in bird genome, with localization in 209,296 bps region of major histo-compatibility complexes (MHC-B), which promote variability by recombination [8]. SNP genotyping allowed identifying 122 haplotypes by MHC-B, including new recombinant haplotypes due to crossing-over. Besides, evidences of gene duplications and deletion were obtained. It was shown that SNP panel is sufficient for identification of known and new recombinant haplotypes. Perceptions of recombinant contribution to variety of MHC-B haplotypes have been changed, including the opportunity for identification of hot recombination spots and estimation of recombination frequencies [8].

Immune responses refer to the complex quantitative traits and are controlled by a number of genes, manifestation of which depends on the environment. Search for the key mutations in charge for the genetically determined variability of a trait could be considered as an approach to disease tolerance diagno-

sis and as an approach to prevention and treatment of diseases. M. Siwek et al. [9] has studied the adaptive immunity under the effect of hemocyanin and genetic immune response to liposaccharides and lipoteichoic acid. Candidate genes and mutations in chicks were registered with identification of SNP associations in certain QTL regions. At genotyping by chips (Illumina, USA), the most significant SNPs associated by response to hemocyanin were found in gene encoding JMJD6 (Jumonji domain-containing 6 protein, arginine demethylase and lysine hystone) and located on chromosome 18. Four SNPs were located in candidate genes *FOXJ1* (transcription factor 1) on chromosome 18, *EPHB1* (B1 tyrosinase receptor) — on chromosome 9, *PTGER4* (prostangladin E receptor 4) — on Z-chromosome, *PRKCB* (protein kinase C β-isoform) — on chromosome 14. The association with antibody response to liposaccharides was found for all of them. One SNP in *ITGB4* gene (β4 integrin) located on chromosome 18 is also associated with genetic immunity response to liposaccharides. Characteristic of such gene product makes them the candidate for participation in immune response to liposaccharides, in control of T-cell functions and proliferation thereof. Thus, *FOXJ1* gene is involved in regulation of T-cell tolerance and inhibition of spontaneous autoimmune diseases; *PTGER4* gene modulates the immune response by intensification of the production of E2 prostaglandin in case of inflammation; *ITGB4* gene is associated with immune response to lipoteichoic acid which initiates such immune response through recognition of toll-like receptors 2 (TLR) interoperating with macrophages or antigen-presenting dendritic cells.

Therefore, SNPs associated with more significant incidences of immune response s to hemocyanin and lipoteichoic acids are located in QTL regions, which were primarily, based on linkage group analysis, proposed as the principal ones by their effect on the immune response. Similar analysis may merely serve as a preliminary search engine of the key mutations in gene candidates. Several universal genes may participate in formation of the genetic and adaptive immunity. It may be considered that this or other immunity mainly has an additive inheritance type.

QTL associated with immune response was found on chromosomes 9, 4, and 18 in chicks [10]. Additional statistical analysis of QTL, with narrowing of the confidence range, had shown that the selected areas on chromosomes 9, 4, 18 and Z have causal mutations associated with the main genetically determined variability of the innate and adaptive immune responses. Candidate genes associated with lipoteichoic acid antibody synthesis are located on chromosome 9 (genes *EPHB1*, *KLHL6*, *PROCR*) and on chromosomes 18 (genes *ITGB4, UNC13D*, *MAP2K4*, *FOXJ1*, *JMJD6*). The SNP polymorphism association was found of the area of *MAPK8IP3*, *IL9R*, *SOCS1* and *PRKCB* genes on chromosome 14 with the immune response to liposaccharides. The candidate genes for antibody response to lipoteichoic acid are on chromosomes 9 (*KLHL6*), 8 (*FOXJ1*, *ITGB4*, *JMJD6*), and Z (*PTGER4*).

Genome evaluation is a perspective tool for improvement of breeding for economically important qualitative traits in animals, i.e. milk and meat productivity and quality [3, 11, 12]. X. Lu et al. [13] had studied SNPs associated with immune response in 657 pigs. Piglets aged 21 days were vaccinated with a modified live vaccine against the classical swine fever. Blood samples were collected when the animals aged 20-35 days. Blood IFNγ and interleukin-10 (IL-10), their quantitative ratio, and virus-neutralization antibodies of IgG were assessed. Illumina porcine SNP60 BeadChip was used in genotyping. Following quality control, 46079 SNPs were selected for identification of associations based on regression model of each SNP. A total of 10000 iterations were performed for valid results. The 32 SNPs, which accounted for 3.23 to 13.81% of the total phenotype dispersion, were selected at statistically significant level. Phenotype dispersion by

IFNγ, IL-10, IFNγ/IL-10 and IgG comprised 37.52, 82.94, 26.74, and 24.16 %, respectively. It is possible that several significant SNPs are located in areas containing a number of known immunity genes. This study makes the basis for identification of mutations affecting the immune potential in pigs. Associations were found of SNPs in gene *PANE1* (Proliferation Associated Nuclear Element 1, minor histocompatibility antibody) with blood immunological parameters and body weight in young pigs at birth [14]. Further studies identified allele frequency of such gene in domestic pigs and wild boars and its association with reproduction values [15]. Wild pigs did not validly differ from the domestic ones in frequency of polymorphous variants of *PANE1*, however, litter size and weight decreased in sows with C→G replacement in intron 1 of gene *PANE1*.

Modern molecular methods can help to identify high associations of genes and genome areas with the response to vaccines and with tolerance to certain pathogens. This allows researchers to map tolerance-associated genes for further use in genomic selection. Special attention should be drawn to virus diseases. Their causative agents are constantly mutating and evolving that necessities molecular and genetic control of whether the associations involved in the disease tolerance have changed.

Genomic evaluation of the pigs of Korean and Yorkshire crosses in F_2 had shown 46865 SNPs [16]. Regression analysis showed 54 SNPs assumedly associated with neutrophil, lymphocyte, monocyte, eosinophil, basophil, immunoglobulin, insulin, and insulin-like growth factor 1 (IGF1) functions. Each set of SNPs had explained from 24 to 42 % of phenotype dispersion of the traits studied. Several pleiotropic SNPs were found on chromosomes 4, 13, 14, and 15. High density SNP microchips also revealed QTLs for blood components associated with immune response [17, 18].

Genetic factors impact susceptibility to and the course of porcine reproductive and respiratory syndrome (PRRS), a disease with significant economic damage to the livestock [19]. QTLs located on pig chromosome 4 explain most genetic variations in PRRS susceptibility. Genome-wide analysis of 200 pigs with Illumina porcine SNP60 BeadChip has revealed that specific haplotypes could associate with the desired phenotypes, and certain genomic regions were involved in a response to PRRS [19, 20]. The traits associated with the infection response were mostly controlled by the chromosome 4 region, however small effects were due to other chromosomes. These studies also assessed the predictive value of QTL-based genome estimates for pig breeding. Selection for SNP genotypes of QTLs located on chromosome 4 may reduce PRRS effects, including the economic ones. The key role of chromosome 4 mutation rs340943904 in the control of PRRS response is under consideration. The study results will help in development of breeding methods to combat PRRS.

Statistically valid data indicate wide-genome associations of SNPs with susceptibility to infectious diseases. Such associations are identified in cattle in tuberculosis caused by *Mycobacterium bovis*, the disease with significant economic damage and the risk of zoonosis [21-25]. Tuberculosis tolerance loci were revealed with Illumina BovineHD BeadChip. Identification of two new tolerance loci, the *PTPRT* (Receptor-type tyrosine-protein phosphatase T) genes and *MYO3B* (myosine IIIB), were due to use of linear and logistic mixed models, as well as regressions. Informative SNPs explain 21 % of phenotype tolerance to tuberculosis, including 6.2 % dispersion due to *PTPRT* gene and 3.6 % dispersion associated with the assumed *MYO3B* gene variant copies.

Be it repeat that modern methods of quantitative genetic analysis of pathology manifestation in the individual disclose only a part of the genetic variation [26], and selection for disease tolerance is seriously challenged by low heritability

of a trait. Selection for the tolerance by the estimates of direct and indirect selection traits is more effective than the traditional selection methods but necessitates the improved statistical models and the models of indirect genetic effect manifestation. Additionally, modern approach to selection requires relevant methods of data collection and test planning to reliably assess genetic parameters of susceptibility in the host and pathogenicity of the infectious agent [27]. Differences in the infection transfer may be due to inherited physiological and behavioral variations. It should be also noted that improper administration of antibiotics induces the tolerance of pathogens and requires additional measures to control diseases [28, 29].

Canadian researchers had shown that accounting for the immune response traits the animal breeding allows increasing the disease tolerance in dairy cattle. Recording of the cell-mediated reaction and response through antibodies allowed separating the animals into groups with strong, average, and weak immune response [30]. Inheritance ratio of 0.19 to 0.29 indicates perspectives of selection for immune response traits. In turn, higher immunity contributes to a decreased frequency of mastitis, hysteritis, and ketosis [31]. Cows with weak antibody-mediated immunity usually suffer from the most severe mastitis. Immunological parameters should be used in breeding for the improved immunity to reduce the frequency and severity of a number of diseases in agricultural animals. In cows, candidate genes and SNPs associated with antibody-mediated immunity, as well as biological mechanisms involved in control of immune response, were disclosed [32]. With $P < 0.05$ confidence, 4045 out of 54609 of the registered SNPs were associated with humorous immune response. After false alleles with low frequency were excluded, 402 SNPs were obtained. The SNPs, like the genes of major bovine histocompatibility complex (Bola), were mostly located on chromosome 23. So the study of dairy cattle shows numerous SNPs associated with varying capability to produce antibodies, which allows use of this trait in animal breeding.

Many chronic autoimmune diseases may be due to changes in the genes of immune response. Lab based technology was used to characterize in vitro the immune response as related to dairy cattle phenotypes including in vivo immune response phenotyping [33, 34]. Blood lymphocytes of cows different in the strength of immune response were stimulated with concanavalin A to study protein synthesis and gene expression. Production of interleukine 4 (IL-4), IFNγ and *IFNG* gene expression were higher in animals classified as high responders compared to those of low immune status. However the responders did not differ in a number of other traits studied. The authors concluded that the distinct cytokine profiles could be used to define disease resistance phenotypes. The laboratory tests are expected to select animals for immune reactivity in order to reduce incidence and severity of diseases.

Like immune-related QTLs, genes of effector proteins, e.g. cytokines, antiviral interferon, involved in the control of immune mechanisms against certain pathogen may be also mapped.

It was shown that tolerance of mice to infection caused by *Francisella tularensis* subsp. *holarctica* is due to emergence of CD69 molecules at surface of T-helpers and synthesis of IFNγ and IL-17 by splenocytes [35]. HLA-DR (human leukocyte antigen), the late activation marker, appears on the surface of T-helpers (CD69+ and CD45RO+) and cytotoxic lymphocytes under the effect of F1 capsular antigen of *Yersinia pestis* at immunization with a live pestiferous vaccine [36]. Specificities of activation of T and B lymphocytes, expression of their surface markers and synthesis of cytokines as influenced by specific *Y. pestis* and *F. tularensis* antigens are reported [37]. The effects of recombinant producers

of capsular F1 *Y. pestis* KM 277 antigen, the purified product (F1), chemical pestiferous vaccine and protective antigenic complexes of various tularemia agent sub-species on expression of Toll-like receptors 2 and 4 were compared [38]. Recombinant *Y. pestis* KM 277 strain, the capsular F1 antigen, and the chemical vaccine increased mRNA TLR2 expression during the first hours after injection with further enhanced cell proliferation in thymus and spleen resulting in high protective immunity.

Spontaneous production of interferon IFNγ (Th1 T-helper sub-population marker) and interleukin 4 (IL-4) (Th2 T-helper sub-population marker) in blood cell cultures and mixed population of lymphocytes, as well as blood levels of immunoglobulins IgG, IgM, IgA, IgE against capsular F1 antigen were compared to those induced with conconavalin A or antigen of inactivated pestiferous bacteria. The data showed functional activity of Th1 and Th2 cells (production of IFNγ and IL-4) after vaccination [38]. Receptors for IgM which in various periods of ontogenesis is present in cattle blood in soluble and bound forms are described. Qualitative changes in populations of animal cells bearing the receptor proteins IgSF [39], as well as functions of IgSF [40] are described.

TLRs are decisive in genetic immunity against many pathogens [41]. Each TLR identifies certain pathogen and participates in signal transfer to initiate the immune response. The mammals have 13 TLRs (from TLR1 to TLR13) specifically linking the ligands. Number of TLRs varies in different animal species. Toll-like receptors operate in many cell types, including macrophages, antigen-presenting dendritic cells, keratinocytes, and sperm cells. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 on cell membrane recognize molecules specific for pathogens (except for the nucleic acids), while intracellular TLR3, TLR7, TLR8, and TLR9 identify nucleic acids. TLRs are relatively big proteins. Porcine surface and intracellular TLRs consist of 785-856 and 905-1050 amino acids, respectively. A system of TLRs on cell membranes is polymorphous that allows recognition of more pathogens. Pigs have 10 *TLR* genes which are mapped on chromosomes 1, 8, 10, 13, 15, and X. Genetic and functional tests revealed several missence-SNPs in porcine *TLR* genes. *TLR1*, *TLR5* and *TLR6* are involved in production of antibodies after vaccination against *Actinobacillus pleuropneumoniae* [42]. *TLR2* is associated with frequent pneumonias caused by *Mycoplasma hyopneumoniae* [43], *TLR5* is associated with expression of IL-2, IL-10 in mononuclear cells of peripheral blood [44]. SNPs in *TLR2* and *TLR5* cause the amino acid replacements related to differential response to *Salmonella enteric* [45]. Out of 10 of porcine TLS genes, *TLR4* is more frequent. SNPs in *TLR4* are reliably associated with changed transcriptional activity of interferon genes, tumor necrosis factor, interleukins IL-2, IL-4, IL-6, and with lung injuries [46]. In addition, SNPs in TLR4 correlate with the ability of pigs to become agents of infection because of the pathogen in feces [47]. Both humoral and cellular immune responses are effective in African swine fever [48].

Double intramuscular injections of the experimentally inactivated vaccines in chicks against the respiratory mycoplasmosis activate T-helper sub-populations soon after vaccination. Herewith, number of CD4+ T-helpers reaches the maximum values on day 21 [49]. Both histocompatibility gene clusters, MHC-B and MHC-Y, in chicks are located as separate haplotypes [50]. Although many genes of MHC-B are polymorphous, and the system itself is polygenous, the role of only some MHC-B loci is documented, and in most cases associations between MHC-B and disease tolerance are determined only at haplotype level [51]. *BG1* locus has quiet significant effect on development of Marek's disease [52]. Relationship between the genes of MHC-Y cluster discovered recently, and the disease tolerance is established [53]. Several poultry lines inbred for MHC-B, in-

cluding congenic White Leghorn lines, were produced to study the relationships between the MHC-B haplotypes and tolerance to infectious diseases. Haplotypes MHC-B were primarily identified by hemagglutination. Haplotypes including *BG* and *BF* loci were found among homozygotic families [54]. Recently, large-scale sequencing of MHC-B area of 59 kbps confirmed the differences between 14 standard haplotypes. It was proven that mutations, recombinations, and gene conversions have contributed to variation of MHC-B haplotypes [55]. For more accurate differentiation of MHC-B types in several chicken breeds and populations, 101 SNPs were revealed in the main area sequence (GenBank AB268588) containing 45 genes most part of which is involved in control of innate and adaptive immunity.

TLRs participate in activation of macrophages in mammals. *TLR15* expression in birds significantly increases at stimulation by both intact and heat- or formalin-inactivated *Salmonella enterica*, *Escherichia coli*, and *Enterococcus gallinarum*, but does not depend on known TLR agonists and *Rhodococcus equi* [56]. These observations show that several TLR agonists are not ligands of TRL15, which is responsible for recognition of specific gram-negative and gram-positive bacteria causing diseases in chickens.

Crosses of animals with different immune responses to detect QTLs defining such differences may serve an example of functional genomics methods in immune response studies. With the same purpose, the expression of thousands of genes before and after the infection is estimated with oligonucleotide chips via differentiation of the effects for specific antigens [57]. Interpretation of QTL effects is challenged since QTLs are big in size and may contain hundreds of potential candidate genes. Use of microchips allows identification of a large number of differentially manifesting genes, but, unfortunately, does not explain the specificity of their relationships. Cis- and trans-regulation of gene expression initiation still remains a challenge in studying genes operation in a QTL.

Recovery also depends on the effectiveness of immune response [58], and, thus, identification of genes affecting the immune functions may help to identify animals with different recovery speed. The inherited immune characteristics are related to immune-reactive QTL. Thus, the inherited traits, i.e. portion of immunity-related blood cell types and quantity of blood antibodies, are expressed.

T-regulatory cells (Treg), a small sub-population of T-lymphocytes, play an important role in the immune response modulation [59]. Suppressive activity of Treg may have adverse effects. At the same time, these cells are involved in tolerance to own antigens and suppression of autoimmunity in hemotransfusion. This phenomenon gives new perspectives for immune therapy of autoimmune diseases and better acceptance of homotransplants. Data on cellular and molecular mechanisms of immune processes in oncopathologies showed an important controlling and balancing effect of Treg family on the immune system [60]. Molecular and genetic specificities of peripheral Treg cells, micro environmental impact on their differentiation and activity, family self-regulation by nTregs, immune aspects of peripheral tolerance and immune therapy of oncological diseases are now key issues for experiments and clinical tests. Studying mechanisms of immune activation and regulation by naive T-cells, memory T-cells, MHC molecules, and signal proteins will contribute to a progress in anti-tumor vaccine therapy. Peripheral tolerance, i.e. suppression of potentially autoreactive T-and B-cells in peripheral tissues, and participation of Tregs in tolerance formation in malignant tumor are studied [61]. Seven Treg family members differing in immune phenotype and functional characteristics are described [62]. Flow cytometry was used to divide cytotoxic peripheral blood T-lymphocytes

into sub-populations (CD27+, CD28+, CD45RA+, and CD62L+) [64]. This method was also helpful at identification of CD3+CD8+ lymphocyte population during differentiation [63].

Thus, causative mutations and the candidate genes involved in animal immune function are associated with single nucleotide polymorphisms (SNPs) both in QTLs and the candidate genes themselves. Research results evidence on the presence of the key SNPs associated with strong and weak immune response. This data allows calculation of animal breeding value estimates for disease tolerance. Immune responses are usually under control of many genes with various phenotypic effects and under the significant effect of environment. Study of tolerance genes, causal mutations, and molecular markers gives the approach to disease diagnosis, prevention, and treatment at molecular and genetic level. The use of genetically determined characteristics of immune response together with other breeding value estimates will reduce disease incidence and severity in animals.

REFERENCES

1. *Breeding for disease resistance in farm animals*. S.C. Bishop, R.F.E. Axford, F.W. Nicholas, J.B. Owen (eds.). CABI, Wallingford, 2010 (doi: 10.1079/9781845935559.0000).
2. Bishop S.C., Woolliams J.A. Genomics and disease resistance studies in livestock. *Livest. Sci.*, 2014, 146: 190-198 (doi: 10.1016/j.livsci.2014.04.034).
3. Yakovlev A.F., Smaragdov M.G. *Zootekhniya*, 2011, 5: 2-4 (in Russ.).
4. Anche M.T., Bijma P., De Jong M.C. Genetic analysis of infectious diseases: estimating gene effects for susceptibility and infectivity. *Genet. Sel. Evol.*, 2015, 47: 85 (doi: 10.1186/s12711-015-0163-z).
5. Lipschutz-Powell D., Woolliams J.A., Bijma P., Doeschl-Wilson A.B. Indirect genetic effects and the spread of infectious disease: are we capturing the full heritable variation underlying disease prevalence? *PLoS ONE*, 2012, 7(6): e39551 (doi: 10.1371/journal.pone.0039551).
6. Lipschutz-Powell D., Woolliams J.A., Doeschl-Wilson A.B. A unifying theory for genetic epidemiological analysis of binary disease data. *Genet. Sel. Evol., 20*14, 46: 15 (doi: 10.1186/1297-9686-46-15).
7. Meuwissen T.H.E., Hayes B.J., Goddard M.E. Prediction of total genetic value using genome-wide dense marker maps. *Genetics*, 2001, 157: 1819-1829.
8. Fulton J.E., McCarron A.M., Lund A.R., Pinegar K.N., Wolc A., Chazara O., Bed'Hom B., Berres M., Miller M.M. A high-density SNP panel reveals extensive diversity, frequent recombination and multiple recombination hotspots within the chicken major histocompatibility complex B region between *BG2* and *CD1A1*. *Genet. Sel. Evol.,* 2016, 48: 1 (doi: 10.1186/s12711-015-0181-x).
9. Siwek M., Slawinska A., Rydzanicz M., Wesoly J., Fraszczak M., Suchocki T., Skiba J., Skiba K., Szyda J. Identification of candidate genes and mutations in QTL regions for immune responses in chicken. *Anim. Genet.*, 2015, 46(3): 247-254 (doi: 10.1111/age.12280).
10. Slawinska A., Siwek M. Meta and combined QTL analysis of different experiments on immune traits in chickens. *J. Appl. Genet.*, 2013, 54: 483-487 (doi: 10.1007/s13353-013-0177-6).
11. Gray K.A., Cassady J.P., Huang Y., Maltecca C. Effectiveness of genomic prediction on milk flow traits in dairy cattle. *Genet. Sel. Evol.,* 2012, 44*:* 24 (doi: 10.1186/1297-9686-44-24).
12. Yakovlev A.F. *Genetika i razvedenie zhivotnykh*, 2014, 2: 3-6 (in Russ.).
13. Lu X., Liu J., Fu W., Zhou J., Luo Y., Ding X., Liu Y., Zhang Q. Genome-wide association study for cytokines and immunoglobulin G in swine. *PLoS ONE,* 2013 (doi: 10.1371/journal.pone.0074846).
14. Huang H., Deng H., Yang Y., Tang Z., Yang S., Mu Y., Cui W., Yuan J., Wu Z., Li K. Molecular characterization and association analysis of porcine PANE1 gene. *Mol. Biol. Rep.*, 2010, 37(5): 2571-2577 (doi: 10.1007/s11033-009-9775-0).
15. Yudin N.S., Aitnazarov R.B., Knyazev S.P., Bekenev V.A., Podoba Yu.V., Berdibaeva A.B., Voevoda M.I. *Vavilovskii zhurnal genetiki i selektsii*, 2014, 18(2): 258-262 (in Russ.).
16. Lee Y.M., Alam M., Choi B.H., Kim K.S., Kim J.J. Whole genome association study to detect single nucleotide polymorphisms for blood components (immunity) in a cross between Korean native pig and Yorkshire. *Asian Austral. J. Anim.*, 2012, 25(12): 1674-1680 (doi: 10.5713/ajas.2012.12503).
17. Lim H.T., Lee J.B., Jung E.J., Ko M.S., Lee J.H., Jeon J.T. QTL analysis of white blood cell, platelet and red blood cell-related traits in an F2 intercross between Landrace and Korean native pigs. *Anim Genet.*, 2011, 42(6): 621-626 (doi: 10.1111/j.1365-2052.2011.02204.x).
18. Lu X., Liu J.F., Gong Y.F., Wang Z.P., Liu Y., Zhang Q., Mapping quantitative trait loci for T lymphocyte subpopulations in peripheral blood in swine. *BMC Genet.*, 2011, 12: 79-88 (doi: 10.1186/1471-2156-12-79).

19. Boddicker N.J., Bjorkquist A., Rowland R.R., Lunney J.K., Reecy J.M., Dekkers J.C. Genome-wide association and genomic prediction for host response to porcine reproductive and respiratory syndrome virus infection. *Genet. Sel. Evol.*, 2014, 46(1): 18 (doi: 10.1186/1297-9686-46-18).
20. Boddicker N.J., Waide E.H., Rowland R.R.R., Lunney J.K., Garrick D.J., Reecy J.M., Dekkers J.C.M. Evidence for a major QTL associated with host response to porcine reproductive and respiratory syndrome virus challenge. *J. Anim. Sci.*, 2012, 90: 1733-1746 (doi: 10.2527/jas.2011-4464).
21. Bermingham M.L., Bishop S.C., Woolliams J.A., Pong-Wong R., Allen A.R., Mc Bride S.H. Genome-wide association study identifies novel loci associated with resistance to bovine tuberculosis. *Heredity*, 2014, 112: 543-551 (doi: 10.1038/hdy.2013.137).
22. Bishop S.C., Doeschl-Wilson A.B., Woolliams J.A. Uses and implications of field disease data for livestock genomic and genetics studies. *Frontiers in Genetics*, 2012, 3: 114 (doi: 10.3389/fgene.2012.00114).
23. Kirkpatrick B.W., Shi X., Shook G.E., Collins M.T. Whole-genome association analysis of susceptibility to paratuberculosis in Holstein cattle. *Anim. Genet.*, 2011, 42: 149-160 (doi: 10.1111/j.1365-2052.2010.02097.x).
24. Bermingham M.L., Bishop S.C., Woolliams J.A., Pong-Wong R., Allen A.R., McBride S.H. Genome-wide association study identifies novel loci associated with resistance to bovine tuberculosis. *Heredity*, 2014, 112: 543-553 (doi: 10.1038/hdy.2013.137).
25. Kirkpatrick B.W., Shi X., Shook G.E., Collins M.T. Whole-genome association analysis of susceptibility to paratuberculosis in Holstein cattle. *Anim. Genet.*, 2011, 42: 149-160 (doi: 10.1111/j.1365-2052.2010.02097.x).
26. Lipschutz-Powell D., Woolliams J.A., Bijma P., Doeschl-Wilson A.B. Indirect genetic effects and the spread of infectious disease: are we capturing the full heritable variation underlying disease prevalence? *PLoS ONE*, 2012, 7(6): e39551 (doi: 10.1371/journal.pone.0039551).
27. Proshin S.N., Kosyakova G.P., YAkovlev A.F. Immunotsitokhimicheskie markery pro-liferatsii limfotsitov krovi pri leikoze korov. *Voprosy normativno-pravovogo regulirovaniya v veterinarii*, 2015, 2: 90-93
28. Bishop S. Opportunities for incorporating genetic elements into the management of farm animal diseases: policy issues. In: *Commission on Genetic Resources for Food and Agriculture*. M. de Jong, D. Gray (eds.). Rome, FAO, 2002: 36-39.
29. Lee C.-R., Cho I.H., Jeong B.C., Lee S.H. Strategies to minimize antibiotic resistance. *Int. J. Environ. Res. Public Health,* 2013, 10(9): 4274-4305 (doi: 10.3390/ijerph10094274).
30. Wagter L., Mallard B.A. *Method of identifying high immune response animals. University of Guelph assignee. US20020051789A1. US Application. US Pat., inventors. 2007, No. 7.*
31. Thompson-Crispi K.A., Sewleam A., Miglior F., Mallard B A. Genetic parameters of adaptive immune response traits in Canadian Holsteins. *J. Dairy Sci.*, 2012, 95: 401-409 (doi: 10.3168/jds.2011-4452).
32. Thompson-Crispi K.A., Sargolzaei M., Ventura R., Abo-Ismail M., Miglior F., Schenkel F., Mallard B.A. A genome-wide association study of immune response traits in Canadian Holstein cattle. *BMC Genomics*, 2014, 15: 559-568 (doi: 10.1186/1471-2164-15-559).
33. Martin C.E., Paibomesai M.A., Emam S.M., Gallienne J., Hine B.C., Thompson-Crispi K.A., Mallard B.A. Cytokine profiles from blood mononuclear cells of dairy cows classified with divergent immune response phenotypes. *J. Dairy Sci.*, 2016, 99(3): 2364-2371 (doi: 10.3168/jds.2015-9449).
34. Fleming K., Thompson-Crispi K.A., Hodgins D.C., Miglior F., Corredig M., Mallard B.A. Variation of total immunoglobulin G and β-lactoglobulin concentrations in colostrum and milk from Canadian Holsteins classified as high, average, or low immune responders. *J. Dairy Sci.,* 2016, 99(3): 2358-2363 (doi: 10.3168/jds.2015-9707).
35. Firstova V.V., Pavlov V.M., Gorbatov A.A., Kombarova T.I., Karaulov A.V., Dyatlov I.A. *Immunologiya*, 2014, 3: 147-150 (in Russ.).
36. Firstova V.V., Bakhteeva I.V., Titareva G.M., Zyrina E.V., Ivanov S.A., Kiseleva N.V., Kopylov P.Kh., Anisimov A.P., Dyatlov I.A. *Problemy osobo opasnykh infektsii*, 2010, 1(103): 56-59 (in Russ.).
37. Firstova V.V., Bakhteeva I.V., Titareva G.M., Zyrina E.V., Ivanov S.A., Anisimov A.P. *Meditsinskaya immunologiya*, 2009, 11(4-5): 336-337 (in Russ.).
38. Shchukovskaya T.N., Smol'kova E.A., Shmel'kova T.P., Klyueva S.N., Bugorkova S.A. *Epidemiologiya i vaktsinoprofilaktika*, 2011, 6(61): 78-83 (in Russ.).
39. Ezdakova I.Yu. *Veterinarnaya meditsina*, 2007, 1: 11-12 (in Russ.).
40. Ezdakova I.Yu. *Identifikatsiya i kharakteristika biologicheskikh svoistv belkov supersemeistva immunoglobulinov zhivotnykh. Avtoreferat doktorskoi dissertatsii* [Identification and characterization of animal proteins of immunoglobulin superfamilies. DSci. Thesis]. Moscow, 2010 (in Russ.).
41. Clop A., Huisman A., van As P., Sharaf A., Derdak S., Sanchez A. Identification of genetic variation in the swine toll-like receptors and development of a porcine TLR genotyping array. *Genet. Sel. Evol.*, 2016, 48: 28 (doi: 10.1186/s12711-016-0206-0).
42. Shinkai H., Arakawa A., Tanaka-Matsuda M., Ide-Okumura H., Terada K., Chikyu M. Genet-

ic variability in swine leukocyte antigen class II and Toll-like receptors affects immune responses to vaccination for bacterial infections in pigs. *Comp. Immunol. Microbiol. Infect. Dis.*, 2012, 35: 523-532 (doi: 10.1016/j.cimid.2012.05.003).
43. Uenishi H., Shinkai H., Morozumi T., Muneta Y., Jozaki K., Kojima-Shibata C., Suzuki E. Polymorphisms in pattern recognition receptors and their relationship to infectious disease susceptibility in pigs. *BMC Proceedings*, 2011, 5(Suppl. 4): S27 (doi: 10.1186/1753-6561-5-S4-S27).
44. Yang X., Murani E., Ponsuksili S., Wimmers K. Association of *TLR5* sequence variants and mRNA level with cytokine transcription in pigs. *Immunogenetics*, 2013, 65: 125-132 (doi: 10.1007/s00251-012-0662-9).
45. Shinkai H., Suzuki R., Akiba M., Okumura N., Uenishi H. Porcine Toll-like receptors: recognition of *Salmonella enterica* serovar *Choleraesuis* and influence of polymorphisms. *Mol. Immunol.*, 2011, 48(9-10): 1114-1120 (doi: 10.1016/j.molimm.2011.02.004).
46. Yang X.Q., Murani E., Ponsuksili S., Wimmers K. Association of *TLR4* polymorphism with cytokine expression level and pulmonary lesion score in pigs. *Mol. Biol. Rep.*, 2012, 39(6): 7003-7009 (doi: 10.1007/s11033-012-1530-2).
47. Kich J.D., Uthe J.J., Benavides M.V., Cantro M.E., Zanella R., Tuggle C.K. *TLR4* single nucleotide polymorphisms (SNPs) associated with *Salmonella* shedding in pigs. *J. Appl. Genet.*, 2014, 55(2): 267-271 (doi: 10.1007/s13353-014-0199-8).
48. Sereda A.D., Kazakova A.S., Imatdinov A.R., Kolbasov D.V. Humoral and cell immune mechanisms under African swine fever (review). *Agricultural Biology*, 2015, 50(6): 709-718 (doi: 10.15389/agrobiology.2015.6.709eng).
49. Obukhovskaya O.V., Stegnii B.T. *Aktual'nye voprosy veterinarnoi biologii*, 2015, 3: 27-31 (in Russ.).
50. Miller M.M., Goto R.M., Taylor R.L., Zoorob R., Auffray C., Briles R.W. Assignment of Rfp-Y to the chicken major histocompatibility complex/NOR microchromosome and evidence for high-frequency recombination associated with the nucleolar organizer region. *PNAS USA*, 1996, 93(9): 3958-3962 (doi: 10.1073/pnas.93.9.3958).
51. Hofmann A., Plachy J., Hunt L., Kaufman J., Hala K. V-src oncogene-specific carboxy-terminal peptide is immunoprotective against Rous sarcoma growth in chickens with MHC class I allele B-F12. *Vaccine*, 2003, 21(32): 4694-4699 (doi: 10.1016/S0264-410X(03)00516-4).
52. Goto R.M., Wang Y., Taylor R.L., Wakenell P.S., Hosomichi K., Shiina T. BG1 has a major role in MHC-linked resistance to malignant lymphoma in the chicken. *PNAS USA*, 2009, 106(39): 16740-16745 (doi: 10.1073/pnas.0906776106).
53. Miller M.M., Taylor J.R. Brief review of the chicken major histocompatibility complex — the genes, their distribution on chromosome 16 and their contributions to disease resistance. *Poultry Sci.*, 2016, 95(2): 375-392 (doi: 10.3382/ps/pev379).
54. Briles W.E., Bumstead N., Ewert D.L., Gilmour D.G., Gogusev J., Hála K. Nomenclature for chicken major histocompatibility (B) complex. *Immunogenetics*, 1982, 15(5): 441-447 (doi: 10.1007/BF00345903).
55. Hosomichi K., Miller M.M., Goto R.M., Wang Y., Suzuki S., Kulski J.K. Contribution of mutation, recombination, and gene conversion to chicken MHC-B haplotype diversity. *J. Immunol.*, 2008, 18: 3393-3399 (doi: 10.4049/jimmunol.181.5.3393).
56. Nerren J.R., He H., Genovese K., Kogut M.H. Expression of the avian-specific toll-like receptor 15 in chicken heterophils is mediated by Gram-negative and Gram-positive bacteria, but not TLR agonists. *Vet. Immunol. Immunop.*, 2010, 136(1-2): 151-156 (doi: 10.1016/j.vetimm.2010.02.017).
57. de Koning D.-J., Carlborg O., Haley C.S. The genetic dissection of immune response using gene-expression studies and genome mapping. *Vet. Immunol. Immunop.*, 2005, 105(3-4): 343-352 (doi: 10.1016/j.vetimm.2005.02.007).
58. Lunney J. *Understanding genetic disease resistance. U.S. National Hog Farmer. April 26, 2011.* Available http://www.nationalhogfarmer.com/health-diseases/understanding-genetic-disease-resistance-0415. Accessed April 11, 2018.
59. Glazanova T.V., Rozanova O.E., Bubnova L.N. *Meditsinskaya immunologiya*, 2014, 16(5): 409-416 (in Russ.).
60. Baldueva I.A., Novik A.V., Karitskii A.P., Kuleva S.A., Nekhaeva T.L., Danilova A.B., Protsenko S.A., Semenova A.I., Komarov Yu.I., Pipia N.P., Slavyanskaya T.A., Avdonkina N.A., Sal'nikova S.V., Belyaev A.M., Sepiashvili R.I. *Allergologiya i immunologiya*, 2015, 16(4): 354-358 (in Russ.).
61. Mougiakakos D., Choudhury A., Lladser A., Kiessling R., Johansson C.C. Regulatory T cells in cancer. *Adv. Cancer Res.*, 2010, 107(57): 117-122 (doi: 10.1016/S0065-230X(10)07003-X).
62. Kakita N., Kanto T., Itose I., Kuroda S., Inoue M., Matsubara T., Higashitani K., Miyazaki M., Sakakibara M., Hiramatsu N., Takehara T., Kasahara A., Hayashi N. Comparative analyses of regulatory T cell subsets in patients with hepatocellular carcinoma: a crucial role of CD25-FoxP3-T cells. *Int. J. Cancer*, 2012, 131(11): 2573-2583 (doi: 10.1002/ijc.27535).
63. Kudryavtsev I.V., Borisov A.G., Krobinets I.I., Savchenko A.A., Serebryakova M.K. *Meditsinskaya immunologiya*, 2015, 17(6): 525-538 (doi: 10.15789/1563-0625-2015-6-525-538) (in Russ.).

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PESTIVIRUSES, WHICH CONTAMINATE IMPORTED FETAL BOVINE SERUM, MAY BE A CAUSE OF THE GLOBAL SPREADING OF VIRAL DIARRHEA IN CATTLE — A MINI REVIEW

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Abstract

Pestiviruses are an important cause of economic losses in the dairy and beef industry. Diseases caused by them are common around the world with varying prevalence associated with the features of regional strategy of livestock including in Russia (A.G. Glotov et al., 2002; M.I. Gulyukin et al., 2013; J.F. Ridpath, 2010). The bovine viral diarrhea virus is considered as a prototype member of the genus *Pestivirus*, *Flaviviridae* family. Two distinct viruses designated as BVDV1 and BVDV2 cause the disease in cattle. A candidate member of the genus is BVDV3, the atypical and not classified pestivirus which shows high similarity to BVDV1 and BVDV2. The BVDV3 presence in the cattle population can compromise BVDV control or eradication (F.V. Bauermann, 2013). This virus requires special attention. BVDV was isolated from commercial lots of fetal bovine serum used for cell culture and biologicals, and is dangerous because of possible spread to new regions (H. Schirrmeier et al., 2004). Viruses of this genus are contaminants of fetal serum, continuous cell line cultures, human and animal vaccines, interferons, trypsin, embryos, stem cells, etc. (B. Makoschey et al., 2003; S.Q. Zhang et al., 2014). Because of globalization and rapid development of cell biotechnology in veterinary and human medicine, the demand for fetal bovine serum, which is a by-product of beef industry, is annually increasing (G. Gstraunthaler et al., 2013). OIE has established product quality standards and regulations according to which all the cell cultures intended to use must be tested for the absence of the virus and its RNA in some passages. Blood serum including fetal serum must be free of the virus and also of the specific antibodies thereto (OIE, 2015). These requirements should also apply to BVDV3. The lack of fetal bovine serum production in Russia creates the possible risk of lots from foreign manufacturers of questionable quality. Special scholar publications report on cases of contamination of different cell cultures and sera by noncytopathic BVDV strains in Russia (S.V. Alekseenkova et al., 2013). The live vaccines prepared using low-quality raw materials can be a potential source of virus for susceptible animals, and contaminated diagnostic antigens can cause false results of the study. Thence, more strict control is extremely important to prevent biological contamination of vaccines and other biologicals.

Keywords: pestiviruses, cattle, bovine viral diarrhea viruses, atypical pestivirus, fetal bovine serum, contamination

Pestiviruses are an important cause of economic losses in the dairy and beef industry. Diseases caused by them are common around the world, including Russia, with varying prevalence associated with the regional features of the cattle breeding strategy [1-3]. Viral diarrhea is caused by mucous membrane diseases in the bovine animals (BVDV — Bovine Viral Diarrhea Virus) and is considered to be a prototype member of the genus *Pestivirus* (*Flaviviridae*) family. Two distinct viruses designated as BVDV1 and BVDV2 cause the disease in bovine animals. The first one is common all over the world (recently, 21 subtypes — from 1a to 1u — are described) [4-7], and the second one is characterized by the limited

spread, in particularly, in USA and Canada [8, 9], South America (Brazil, Uruguay) [10, 11], in several European countries (Germany, Slovakia, Italy) [12-14], Asia (South Korea, Japan) [15, 16], and Mongolia [17]. The BVDV2 is divided into five subtypes (from 2a to 2e) [18]. A candidate member of the genus is a non-officially classified virus having several names (BVDV3, Hobi-like pestivirus, an atypical pestivirus) and showing high similarity to BVDV1 and BVDV2. The BVDV3 presence in the cattle population can significantly mitigate the effectiveness of BVDV control and pathogen eradication. The BVDV3 was for the first time secreted in 2004 from the fetal calf serum made in Brazil [21], and in furtherance was found in bovine animals in South America [22], Asia [23-25] and Europe [26, 27]. Representatives of *Pestivirus* genus are known as serum contaminants, continuous cell culture lines, vaccines for medical and veterinary purposes, interferon, trypsin, and other medicines for biotechnical researches and techniques, embryos, stem cells, etc. [19, 20]. Although BVDV as a biological product contaminant is well known since 1960s [28], role of the atypical virus is elucidated to a lesser extent.

Specific feature of the studied virus group is its ability to cause persistent fetal infection by only non-cytopathogenic biotype. Fetal infection takes place on day 40-125 of intrauterine growth, when fetal immune system is not formed yet [1]. It results in birth of immune tolerant calves serving as persistent source of pathogen for non-immune animals. Concentration of virus in blood of such individuals is high starting from the intrauterine growth, and they produce it during the entire life with all discharges and excreta. No specific antibodies are produced in persistently infected animals [3].

We have summarized information on contamination by fetal bovine serum (FBS) pestivirus. This fact should be accounted for due to the threat of pestivirus spread with the lots of imported FBSs, especially due to the growth of FBS consumption in cell technologies, biotechnology, pharmaceutics, and medicine.

Fetal serum. FBS is the most known and widely used additive to breeding grounds for initiation and increase of cell culture growth speed in mammals due to high concentration of biological substances in fetal blood [28]. There are still no other universal and effective cell growth stimulators. FBS is a natural mixture of factors required for cell sticking in substrata, their active growth, and proliferation [29]. In Russian scholar publications FBS is called a fetal calf serum, however during the last years such term was revised by several authors since serum is not produced in embryo period, but rather in the later fetal period.

The serum is produced in aseptic conditions from the fetal blood of randomly selected pregnant beef cows meant for slaughter [29]. Since animals of both sexes are freely pastured together in large-size fleets, cows often become pregnant. Cows are specially settled for production of fetal serum in Hungary, Baltic States, and, possibly, Czech Republic [30]. Usually, biomaterial is a 6-month foetus, but in fact foetus aged 3-months may be used. Usually, blood is collected by cardiocentesis, while in Uruguay, Brazil, and Australia blood in collected by centesis of umbilical or jugular vein [30]. Each lot of commercial fetal serum includes the material collected from different farms. As a result, the entire lot may be contaminated if it contains serum of infected animals [31].

The highest demand for FBS is in USA and Europe, where the largest share of FBS is produced, however, with the use of raw material supplied by Brazil, Argentina, Central America states, South Africa, Australia, and New Zealand [28]. Main exporters of finished products for cell culture at production of vaccines and preparations are USA, New Zealand, and Australia. Serum for research purposes is mainly supplied from the South America, South Africa, and

Brazil. Thus, in 2007 Brazil was the second beef meat producing and exporting country. Consequently, during the year 70 % of serum used in the European medical industry was from Brazil [32].

FBS market. Cell cultures are widely used in biopharmaceutical industry, growth of which, in its turn, promotes FBS production and sales. Since 2013 biopharmaceuticals became the largest and, according to forecasts, rapidly growing segment of cell culture market [33]. It is expected that by 2019 the global market of the cell culture based products (culture medium, serum, and reagents) in biotechnology, pharmacy, and medicine would grow by up to USD 4.1 billion [34]. Due to studies of human stem cells and their use for treatment of various malfunctions, it is expected that the market of products for cell cultures will grow up to USD 14.8 billion by 2019 (as compared to 2014, when such market volume comprised USD 6.0 billion) [35]. Nearly 500000 l of fetal bovine serum is produced annually all over the world requiring up to 1000000 calves. FBS sales grow [28], while the entire market is under control of several producers. For instance, in 2014 it was three American companies, the Thermo Fisher Scientific, Life Technologies Co. and Sigma-Aldrich, the aggregate share of sales of which comprised 80 %; buyers were mainly large biopharmaceutical companies. The first two of the above-listed firms accounts to 60 % of FBS market in USA and in the world [30].

In this context, it is important that FBS buyers around the world have reviewed their relations with the suppliers and have determined the strategy to lower the risks of possible FBS contamination by endotoxins, mycoplasmas, PrPsc, and viruses (in particular, pestiviruses) accounting for the qualitative and quantitative, geographic, and seasonal changes in FBS lots [36]. Regardless of the fact that veterinary specialists during studies select foetus only from animals suitable for human consumption, it is widely known that FBS is a potential source of many viruses. FBS contamination by bovine pestiviruses is known since 1960s, provided that BVDV is most prevalent due to its ability to trans-placental transmission with further persistence in immunologically immature foetus [37]. Due to the risk of virus contamination, it was strongly recommended to inactivate serum in addition to the direct virus testing with the use of validated and effective methods [38]. However, in recent years persistence of contamination was reported even after the recommended procedures.

To ensure FBS quality, representative samples of the unified lots are usually tested for sterility (bacteria, fungus), endotoxins, immunoglobulins, viruses, biochemical values and electrophoretic profiles. Afterwards, it is sterilized by filtration and may be treated by γ-rays or high temperatures. These procedures, as well as ultimate freezing, ensure the additional risk-free safety [39]. Premium quality values of FBS are low concentration of immunoglobulin, absence of viruses, and endotoxins. However, serum lots not always pass necessary testing or it is insufficiently effective. Following the protocol that combined cell culture method and detection of RNA pestiviruses, B. Makoschey at al. [37] had shown that 4 of 7 FBS lots were contaminated by infectious BVDV1 of non-cytopathogenic biotype.

H. Xia at al. [40], for the first time having demonstrated the contamination of commercial FBS of different geographic origin not only by BVDV1 and BVDV2, but also by the emergent BVDV3, had suggested that such viruses are much more widely spread than it was earlier assumed. Analysis of 33 FBS lots from 10 producers by reverse transcription polymerase chain reaction (RT-PCR) allowed detecting BVDV1 in 29 lots from 11 countries, BVDV2 in 11 lots from South America, and BVDV3 in 13 lots from America, Australia, Brazil, Canada, and Mexico. S.Q. Zhang at al. [31] had established that Chinese FBS medicines

from different regions of the country were contaminated by minimum one type of pestivirus (including BVDV1 and BVDV2).

BVDV3 virus. Although the origin and emergent properties of HoBi-like-virus are unknown, according to one of the hypothesis it originates from the South America, from where it was spread to other countries and other continents with contaminated biological products, such as fetal serum and vaccines [23]. As per F.V. Bauermann at al. [41], over 30 % FBS lots from the South America tested in the Europe were contaminated by such virus.

After PCR analysis of 26 archival FBS lots (years 1992-2013), having passed filtration and γ-raying [27], all of them was found to contain minimum one type of bovine pestivirus. BVDV1 was in 2 lots, BVDV2 was in 10 lots, and HoBi-like-virus was in 15 lots. Seven lots were produced in the South America, one lot was from Australia, and 7 lots had undefined origin. Based on results of philogenetic analysis, the identified virus was referred to the Brazil group. This virus was brought in Italy with FBS [2]

Upon examination of 90 series of commercial serum made in USA and pre-packed in Europe, the authors have reported that no virus was found and, thus, have concluded on no cross-country circulation thereof [42]. Nevertheless, part of lots contained BVDV: BVDV1 was found in 19 and BVDV2 was found in one lot out of 20 positive series based on results of the phylogenic analysis. Such fact implies the contamination possibility of FBS marked as US product, provided pre-sales treatment and packing, as well as probability of improper marking following such treatment and mixture with samples from other geographical regions of the world. It is alarming since lots marked as made in USA or Australia are present at FBS market, i.e. in countries, which according to the official data are free from the atypical bovine pestivirus.

Spread of atypical bovine pestivirus (as apart from BVDV1 and BVDV2) is possibly limited by several regions. As it was noted before, Hobi-like-virus was primarily secreted and characterized in 2004 in Germany by analysis of FBS lot collected in Brazil and pre-packed in Europe [21]. Isolate called D32/00_'HoBi' was considered to be prototype for the Brazil group of pestiviruses. Afterwards, several authors have identified its genetically varying subtypes with regional spread, in particular Thai subtype [42]. Afterwards, it was hypothesized on existence of the third, Indian group of strains [25]. There is an assumption on existence of the fourth group of virus secreted outside the Indian region, in particular in Italy [27]. Therefore, four genetic groups of BVDV3 (3a-d) are recently identified.

Available data confirm the need for constant updating and advancing the bovine virus identification methods, and for development of the rules of international trade in FBS and animals. In the past years, several technologies for lowering the quantity or inactivation of FBS viruses were tested. According to the Directive of the European Medicines Agency (EMEA), United Kingdom, BVDV was included in the list of viruses which could be used for quality verification of the inactivation procedure [30]. Impulse treatment of FBS by UV rays with wave length of 355 and 266 μm has a good effect [43]. In this respect, a device to perform 14 minute treatment with red LED (Light Emitting Diode, $\lambda = 627$ μm) and methylene blue in ultimate concentration of 1 μm effective against BVDV are proposed for use [41]. Nevertheless, risk of FBS contamination by pestiviruses remains real, and even weak contamination upon use of FBS as an additive to growing medium may result in infection of the cell cultures [30].

FBS use. Because of high FBS contamination by viruses, it is impossible to exclude the use of its virus-containing lots in large-scale production of vaccines [37]. Therefore, the virus must be inactivated in each lot under strict

control of the effectiveness of the procedure by laboratory tests. To this end, guidelines and rules of inactivation methods and relevant tests were developed. Recently, EME rules, which are applicable to production of medicines for veterinary and medicinal purposes, presuppose compulsory treatment by the approved methods [38]. BVDV test shall be one of the primary contamination assessment methods before and after the inactivation of virus. The ultimate lot of medicine shall be free from virus and antibodies [30].

Upon use of FBS in production of veterinary medicines, virus identification protocol presupposes at least three transits in sensitive cell culture and immunohistochemistry analysis with referent anti-BVDV monospecific antiserum (polyclonal or containing the pool of monoclonal antibodies). If virus is detected, titrating from the serum lot is performed to confirm that concentration of virus is sufficient for control of the performed inactivation with the use of validated tests and does not exceed 10^5-10^6 TCD_{50}/ml. Moreover, for testing purposes, it is proposed to use PCR with electrophoretic separation of products and in real time mode. Sensitivity and specificity of additional methods shall not be less than in standard tests. Besides, the producer shall be able to determine whether the detected RNA is infected. There is also a directive presupposing the methodology which should be used at suspicion to contamination of the finished serum lot by nuclein acids of virus [30] to identify whether virus infection factors are encoded by such consequences. To this end, double testing involving detection of virus by PCR (predominantly by semi-quantitative with internal control) and detection of contamination by virus in other tests are applied. Only laboratory tests are used upon identification of viral contamination of the live vaccines. To this end, the end product is inoculated in sensitive cells with conduction of at least three blind passages with further immunoperoxidase painting of monolayer, as well as by testing by immunofluorescence or PCR methods. In case of negative test results in vitro with confirmation thereof in PCR, no in vivo test is conducted, save for the exclusive circumstances.

Production of biological preparations for medical detection of virus requires methods to effectively detect both virus biotypes. Besides, immunofluorescent staining of cell culture monolayer with fluorescein-tagged antibodies (FA) is recommended. Direct PCR method is deemed to be less suitable for detection of infectious virus. At detection of contamination, it is estimated by quantitative methods (value shall be less that the established value for virus inactivation by valid methods). Upon detection of BVDV, serum shall be repeatedly tested with continued treatments until the negative result.

Cows used as donors of biomaterial for FBS production, shall be clearly established, and their state shall be fixed subject to EMEA regulations [37]. It is not recommended to carry out vaccination in such herds for prevention of any influence of post-vaccination antibodies.

Regardless of the numerous attempts to govern FBS safety, falsifications of products, from which honest suppliers and large number of serum consumers have suffered, take place [36]. From 2003 to 2011 one firm added bovine serum albumin, water and growth additives to FBS made in USA. Some lots of such serum (143 series, 280000 l) could be still sold around the world under other trademarks or trade names. In this regard, it is suggested that FBS market is not governed quiet effectively and falsification remains possible [28]. In this case, in addition to improper medicine composition, the producer could not be identified. Falsified product may contain BSA of mature animals from USA and/or mixture of fetal serum obtained from other sources in Canada, Argentina, Brazil, or Mexico. This recently reported illegal practice may significantly influence on the results and validity of scientific tests with cell and tissues cultures [28] and dis-

credit the global FBS market [30]. An issue of falsification of the geographical origin of FBS deserves special attention. As early as in 1994, it was reported on the sale of nearly 30000 l of serum from New Zealand around the world, however according to the official data only 15000 l of high quality products were annually prepared in this country [43]. Accordingly, the consumer may get a product, which by its geographical origin is not compliant with the official requirements and is produced in the region where infection status of donor fleets is less favorable and value of FBS is far lower [30].

It was suggested that several companies allow mixing the serum during production and transportation [40]. It results in breach of the equipment cleaning regulation or in infected lot among the lots from different producers. Moreover, FBS lots may be erroneously tagged by the country of origin different from the real one [39].

To ensure quality, it is necessary to take special care at confirmation of validity of the supplier information and to exercise duly care at operation with all suppliers of the preparation. Qualitative supply means that all values, including the history of origin, shall be properly documented, fully transparent, and confirmed by an independent auditor. Besides, other important information shall be also available. For instance, at detection of virus tests results depend on the inoculate volume, used cell culture line, number of passages, specificity of antibodies used in immunofluorescence test, correct selection of primers or probes for RT-PCR. It is also useful to indicate virus detection threshold in commonly used international units (for instance, number of infected particles or genome copies).

World Organization for Animal Health (OIE, France) issues a clear regulation subject to which all cell cultures prior to the targeted use shall be tested for BVDV in several passages. Blood serum, including fetal serum, shall be free from viruses, and also from specific antibodies [46, 47]. Nevertheless, cases of cell culture and serum contamination by BVDV strains of non-cytopathogenic type were described in Russia [48-50]. As a result, cultural live vaccines may become a source of viruses for sensible animals, and contaminated diagnostic antibodies may be a reason of invalid test results. Therefore, improved control aimed at prevention of biological contamination is highly important in production of vaccines and other biologicals [48].

Thus, because of globalization and modern cell biotechnologies in veterinary and medicine, a demand for fetal bovine serum (FBS) is annually increased. FBS contamination is still relevant and results from the increased demand, availability of unfair producers and sellers, incompliant marking of the products and lack of the unified FBS control methodology. In the extension of FBS market, existence of atypical pestiviruses requires special attention. They have been detected in the commercial blood serum pools used for cell culture and production of biological medicines, and represent a hazard due to ability to spread in new regions. Lack of FBS production in Russia opens the way for doubtful goods quality from unreliable manufacturers.

REFERENCES

1. Ridpath J.F. Bovine viral diarrhea virus: global status. *Veterinary Clinics of North America: Food Animal Practice*, 2010, 26(1): 105-121 (doi: 10.1016/j.cvfa.2009.10.007).
2. Glotov A.G., Glotova T.I., Petrova O.G., Nefedchenko A.V., Tatarchuk A.T., Koteneva S.V., Vetrov G.V., Sergeev A.N. *Veterinariya*, 2002, 3: 17-21 (in Russ.).
3. Gulyukin M.I., Yurov K.P., Glotov A.G., Donchenko N.A. *Voprosy virusologii*, 2013, 6: 13-18 (in Russ.).
4. Simmonds P., Becher P., Bukh J., Gould E.A., Meyers G., Monath T., Muerhoff S., Pletnev A., Hesse R.R., Smith D.B., Stapleton J.T., ICTV Report Consortium. ICTV virus Taxonomy profiles: *Flaviviridae. J. Gen. Virol.*, 2017, 98: 2-3 (doi: 10.1099/jgv.0.000672).

5. Vilcek S., Durkovic B., Kolesarova M., Paton D.J. Genetic diversity of BVDV: consequences for classification and molecular epidemiology. *Preventive Veterinary Medicine*, 2005, 72: 31-35 (doi: 10.1016/j.prevetmed.2005.08.004).
6. Pecora A., Malacari D.A., Ridpath J.F., Perez Aguirreburualde M.S., Combessies G., Odeyn A.C., Romera S.A., Golemba M.D., Wigdorovitz A. First finding of genetic and antigenic diversity in 1b-BVDV isolates from Argentina. *Res. Vet. Sci.*, 2014, 96(1): 204-222 (doi: 10.1016/j.rvsc.2013.11.004).
7. Deng M., Ji S., Fei W., Raza S., He C., Chen Y., Chen H., Guo Al. Prevalence study and genetic typing of bovine viral diarrhea virus (BVDV) in four bovine species in China. *PLoS ONE*, 2015, 10(7): e0134777 (doi: 10.1371/journal.pone.0134777).
8. Evermann J.F., Ridpath J.F. Clinical and epidemiologic observations of bovine viral diarrhea virus in the northwestern United States. *Vet. Microbiol.*, 2002, 89(2-3): 129-139 (doi: 10.1016/S0378-1135(02)00178-5).
9. Carman S., Van Dreumel T., Ridpath J., Hazlett M., Alves D., Dubovi E., Tremblay R., Bolin S., Godkin A., Anderson N. Severe acute bovine viral diarrhea in Ontario, 1993-1995. *J. Vet. Diagn. Invest.*, 1998, 10(1): 27-35 (doi: 10.1177/104063879801000106).
10. Silveira S., Weber M.N., Mysena A.C., da Silva M.S., Streck A.F., Pescador C.A., Flores E.F., Weiblen R., Driemeier D., Ridpath J.F., Canal C.W. Genetic diversity of Brazilian bovine pestiviruses detected between 1995 and 2014. *Transbound. Emerg. Dis.*, 2017, 64: 613-623 (doi: 10.1111/tbed.12427).
11. Maya L., Puentes R., Reolyn E., Acuca P., Riet F., Rivero R., Cristina J., Colina R. Molecular diversity of bovine viral diarrhea virus in Uruguay. *Arch. Virol.*, 2016, 161(3): 529-535 (doi: 10.1007/s00705-015-2688-4).
12. Tajima M., Frey H.R., Yamato O., Maede Y., Moennig V., Scholz H., Greiser-Wilke I. Prevalence of genotypes 1 and 2 of bovine viral diarrhea virus in Lower Saxony, Germany. *Virus Res.*, 2001, 76(1): 31-42 (doi: 10.1016/S0168-1702(01)00244-1).
13. Novácková M., Jacková A., Kolesárová M., Vilcek S. Genetic analysis of a bovine viral diarrhea virus 2 isolate from Slovakia. *Acta Virologica*, 2008, 52(3): 161-166.
14. Luzzago C., Lauzi S., Ebranati E., Giammarioli M., Moreno A., Cannella V., Masoero L., Canelli E., Guercio A., Caruso C., Ciccozzi M., De Mia G.M., Acutis P.L., Zehender G., Peletto S. Extended genetic diversity of bovine viral diarrhea virus and frequency of genotypes and subtypes in cattle in Italy between1995 and 2013. *BioMed Res. Int.*, 2014, 2014: Article ID 147145 (doi: 10.1155/2014/147145).
15. Oem J.K., Hyun B.H., Cha S.H., Lee K.K., Kim S.H., Kim H.R., Park C.K., Joo Y.S. Phylogenetic analysis and characterization of Korean bovine viral diarrhea viruses. *Vet. Microbiol.*, 2009, 139(3-4): 356-360 (doi: 10.1016/j.vetmic.2009.06.017).
16. Yamamoto T., Kozasa T., Aoki H., Sekiguchi H., Morino S., Nakamura S. Genomic analyses of bovine viral diarrhea viruses isolated from cattle imported into Japan between 1991 and 2005. *Vet. Microbiol.*, 2008, 127(3-4): 386-371 (doi: 10.1016/j.vetmic.2007.08.020).
17. Ochirkhuu N., Konnai S., Odzaya B., Gansukh S., Murata S., Ohashi K. Molecular detection and characterization of bovine viral diarrhea virus in Mongolian cattle and yaks. *Arch. Virol.*, 2016, 161(8): 2279-2283 (doi: 10.1007/s00705-016-2890-z).
18. Giangaspero M., Harasawa R. Characterization of genotypes among bovine viral diarrhea virus type1 strains according to palindromic nucleotide substitutions in the genomic 5′-untranslated region. *J. Virol. Methods*, 2014, 195: 34-53 (doi: 10.1016/j.jviromet.2013.10.003).
19. Bauermann F.V., Ridpath J.F., Weiblen R., Flores E.F. HoBi-like viruses: an emerging group of pestiviruses. *J. Vet. Diagn. Invest.*, 2013, 25(1): 6-15 (doi: 10.1177/1040638712473103).
20. Glotov A.G., Glotova T.I. Atypical bovine pestiviruses (review). *Agricultural Biology*, 2015, 50(4): 399-408 (doi: 10.15389/agrobiology.2015.4.399eng).
21. Schirrmeier H., Strebelow G., Depner K., Hoffmann B., Beer M. Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. *J. Gen. Virol.*, 2004, 85: 3647-3652 (doi: 10.1099/vir.0.80238-0).
22. Weber M.N., Mosena A.C.S., Simoes S.V.D., Almeida L.L., Pessoa C.R., Budaszewski R.F., Silva T.R., Ridpath J.F., Riet-Correa F., Driemeier D., Canal C.W. Clinical presentation resembling mucosal disease associated with “HoBi”-like pestivirus in a field outbreak. *Transbound. Emerg. Dis.*, 2016, 63(1): 92-100 (doi: 10.1111/tbed.12223).
23. Mao L., Li W., Zhang W., Yang L., Jiang J. Genome sequence of a novel Hobi-like pestivirus in China. *J. Virol.*, 2012, 86(22): 12444 (doi: 10.1128/JVI.02159-12).
24. Haider N., Rahman M.S., Khan S.U., Mikolon A., Gurley E.S., Osmani M.G., Shanta I.S., Paul S.K., Macfarlane-Berry L., Islam A., Desmond J., Epstein J.H., Daszak P., Azim T., Luby S.P., Zeidner N., Rahman M.Z. Identification and epidemiology of a rare HoBi-like pestivirus strain in Bangladesh. *Transbound. Emerg. Dis.*, 2014, 61: 193-198 (doi: 10.1111/tbed.12218).
25. Mishra N., Rajukumar K., Pateriya A., Kumar M., Dubey P., Behera S.P., Verma A., Bhardwaj P., Kulkarni D.D., Vijaykrishna D., Reddy N.D. Identification and molecular characterization of novel and divergent HoBi-like pestiviruses from naturally infected cattle in India. *Vet. Microbiol.*, 2014, 174: 239-246 (doi: 10.1016/j.vetmic.2014.09.017).
26. Decaro N., Lucente M.S., Mari V., Uttenthal A., Polak M.P., Stehl K., Alenius S., Shan H.,

Yin H., Belák S. Atypical pestivirus and severe respiratory disease in calves, Europe. *Emerg. In-Infect. Dis.*, 2011, 17(8): 1549-1552 (doi: 10.3201/eid1708.101447).
27. Giammarioli M., Ridpath J.F., Rossi E., Bazzucchi M., Casciari C., De Mia G.M. Genetic detection and characterization of emerging HoBi-like viruses in archival foetal bovine serum batches. *Biologicals*, 2015, 43(4): 220-224 (doi: 10.1016/j.biologicals.2015.05.009).
28. Gstraunthaler G., Lindl T., van der Valk J. A plea to reduce or replace fetal bovine serum in cell culture media. *Cytotechnology*, 2013, 65(5): 791-793 (doi: 10.1007/s10616-013-9633-8).
29. Gstraunthaler G. Alternatives to the use of fetal bovine serum: serum-free cell culture. *ALTEX*, 2003, 20(4): 275-281.
30. Flatschart R.B., Caldas L.A., Almeida D.O., dos Santos N.C., Boldrini L.C., Granjeiro J.M., Folgueras-Flatschart A.V. The Impact of BVDV presence on fetal bovine serum used in the biotechnology industry. In: *Advances in medicine and biology*. Nova Biomedical, New York, 2016. V. 95: 75-95.
31. Zhang S.Q., Guo B.T.L., Wang F.X., Zhu H.W., Wen Y.J., Cheng S. Genetic diversity of bovine viral diarrhea viruses in commercial bovine serum batches of Chinese origin. *Infection, Genetics and Evolution*, 2014, 27: 230-233 (doi: 10.1016/j.meegid.2014.07.021).
32. Häusl P. Fetal Bovine Serum running short. *European Biotechnology — Life Sciences and Industry Magazine*, 2008. Available http://www.european-biotechnology-news.com. No date.
33. *Market Research Reports. Cell Culture Market by Equipment — Global Forecast to 2018*. Available http://www.rnrmarketresearch.com. No date.
34. *BCC Research. Global Markets for Media, Sera and Reagents in Biotechnology. 2015*. Available http://www.bccresearch.com. No date.
35. *Market Research Reports. Cell Expansion Market by Product — Forecast to 2019*. Available http://www.reportlinker.com. No date.
36. Davis D., Hirschi S.D. Fetal bovine serum: what you should ask your supplier and why. *BioProcess. J.*, 2014, 13(1): 19-21 (doi: 10.12665/J131.DavisHirschi).
37. Makoschey B., van Gelder P.T., Keijsers V., Goovaerts D. Bovine viral diarrhoea virus antigen in foetal calf serum batches and consequences of such contamination for vaccine production. *Biologicals*, 2003, 31(3): 203-208 (doi: 10.1016/S1045-1056(03)00058-7).
38. EMA. *Guideline on the use of bovine serum in the manufacture of human biological medicinal products*. 2013. Available http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/06/WC500143930.pdf. Accessed April 09, 2018.
39. Siegel W., Foster L. Fetal bovine serum: the impact of geography. *BioProcess. J.*, 2013, 12(3): 28-30 (doi: 10.12665/J123.Siegel).
40. Xia H., Vijayaraghavan B., Belák S., Liu L. Detection and identification of the atypical bovine pestiviruses in commercial foetal bovine serum batches. *PLoS ONE*, 2011, 6(12): e28553 (doi: 10.1371/journal.pone.0028553).
41. Bauermann F.V., Harmon A., Flores E.F., Falkenberg S.M., Reecy J.M., Ridpath J.F. In vitro neutralization of HoBi-like viruses by antibodies in serum of cattle immunized with inactivated or modified live vaccines of bovine viral diarrhea viruses 1 and 2. *Vet. Microbiol.*, 2013, 166(1-2): 242-245 (doi: 10.1016/j.vetmic.2013.04.032).
42. Bauermann F.V., Flores E.F., Falkenberg S.M., Weiblen R., Ridpath J.F. Lack of evidence for the presence of emerging HoBi-like viruses in North American fetal bovine serum lots. *J. Vet. Diagn. Invest.*, 2014, 26(1): 10-17 (doi: 10.1177/1040638713518208).
43. Daryany M.K.A., Hosseini S.M., Raie M., Fakharie J., Zareh A. Study on continuous (254 nm) and pulsed UV (266 and 355 nm) lights on BVD virus inactivation and its effects. *Journal of Photochemistry and Photobiology B: Biology*, 2009, 94(2): 120-124 (doi: 10.1016/j.jphotobiol.2008.10.009).
44. Ceylan C., Severcan F., Ozkul A., Severcan M., Bozoglu F., Taheri N. Biophysical and microbiological study of high hydrostatic pressure inactivation of Bovine Viral Diarrhea virus type 1 on serum. *Vet. Microbiol.*, 2012, 154(3-4): 266-271 (doi: 10.1016/j.vetmic.2011.07.026).
45. Hodgson J. To treat or not to treat: that is the question for serum. *Bio/Technology*, 1995, 13(4): 333-343 (doi: 10.1038/nbt0495-333).
46. OIE. *Manual of diagnostic tests and vaccines for terrestrial animals. P. 2, S. 2.4. Ch. 2.4.8. Bovine viral diarrhoea*. Paris, France, 2015.
47. Flatschart R.B., Caldas L.A., de Oliveira Almeida D., Correeia dos Santos N., da Cunha Boldrini L., Granjeiro M.J., Flatschart A.V.F. The impact of BVDV presence on fetal bovine serum used in the biotechnology industry. In: *Advances in Medicine and* Biology. L.V. Berhardt (ed.). Nova Science Publishers, Inc., 2016, V. 95, Chapter 4: 75-93.
48. Alekseenkova S.V., Yurov G.K., Gal'nbek T.V., Kalita I.A., Yurov K.P. *Rossiiskii veterinarnyi zhurnal*, 2013, 1: 15-18 (in Russ.).
49. Uryvaev L.V., Ionova K.S., Dedova A.V., Dedova L.V., Selivanova T.K., Parasyuk N.A., Mezentseva M.V., Kostina L.V., Gushchina E.A., Podchernyaeva R.Ya., Grebennikova T.V. *Voprosy virusologii*, 2012, 5(57): 15-21 (in Russ.).
50. Koteneva S.V., Maksyutov R.A., Glotova T.I., Glotov A.G. Identification of the bovine atypical pestivirus in biological samples. *Agricultural Biology*, 2017, 52(6): 1259-1264 (doi: 10.15389/agrobiology.2017.6.1259eng).

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BIOLOGY OF ALFALFA SILAGE MAKING
(review)

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Abstract

Alfalfa dry matter is characterized by the less content of sugar, celluloses and hemicelluloses and more quantity of pectin in comparison to grasses (P. Mc-Donald et al., 1970). The high level of pectin provides increased rate of feed fermentation in a rumen (E.F. Annison, et al., 1962). This leads to improved assimilation of alfalfa silage dry matter by cattle, despite the low energy level unlike to cereal grasses silage (M. Grabov, 2016). As a result, the nutrients intake and productivity of cows increase. However, there are some particularities in qualitative alfalfa silage- and haylage-making, such as absence of abundant *Enterobacteriaceae* bacteria on the alfalfa plants (R.A. Shurchno et al., 2008), unlike cereal grasses (Yu.A. Pobednov et al., 2015). Thereof the basic kind of alfalfa silage and haylage spoilage is butyric (putrid) fermentation. With due regard to this fact, the main principle of alfalfa conservation is based on the known rule of G.W. Wieringa (1963), which tells about increasing of clostridium bacteria sensitivity to active acidity (pH) of feed when dry matter content in plants rises. This allows providing feed preservation under significantly higher pH, than at ensiling the freshly-cut mass (F. Weissbach, 2012). However, fodder must reach fast acidification with determined pH value to eliminate a butyric fermentation at each dry matter content. But this condition especially difficult for performance at alfalfa ensiling, because plants contain much weak-bound water even at 35 % dry matter content, in contrast to cereal grasses and red clover. At weak acidification, it can lead to intensive proteolysis (X.S. Guo et al., 2012) with ammonia accumulation and an increase in buffer capacity of feed. As a result, pH of alfalfa silage does not decline to necessary level for elimination the clostridium bacteria growth during the long period and it causes to accumulation a butyric acid and the products of putrid decay of the proteins. It is possible to reduce the intensity of proteolysis by increased feed acidification with addition of liquid organic acids or inoculants of lactic acid bacteria combined with sugar. Another way is ensiling of alfalfa wilted to ≥ 40 % dry matter content followed by application of the lactic acid bacteria-based inoculants. At this level of dehydration, the content of sugar in dry matter increases 1.6 times (Yu.A. Pobednov et al., 2016), and addition of the bacterial inoculants leads to increasing a degree of feed acidification as well as storage and feed-out stability (F. Weissbach, 2012). Application of enzymes in ensiling alfalfa wilted to ≥ 40 % dry matter is one more advanced method of this forage crop conservation (A.A. Anisimov, 2006). Another effective approach of alfalfa silage-making is using enzyme additives combined with lactic acid bacteria (M. Grabov, 2016).

Keywords: alfalfa, proteolysis, dry matter content, acidification, lactic acid bacteria-based inoculants, enzymes, silage quality

Animals fed with poor silage experience deficit of nutritious matter, which negatively affects their productivity, health, meat and milk production [1]. The problem with production of the high quality feed is particularly acute at procurement of silage and haylage from alfalfa. It is more often applied in the agricultural practice since for many farming units production of the feed with content of up to 22 % raw protein in dry matter (DM) is a real way to increase the economic stability [2]. Besides high levels of raw protein, alfalfa is characterized by the less content of pectin, as well as presence of special structured fiber [3]. High pectin fermentation results in accelerated alfalfa fermentation in a ru-

men. Thus, digestibility of pectin and pectin acid in sheep reaches 90 % [4]. Alfalfa does not follow the known rule that animal consumption of bulky feed dry matter correlates to its nutrient value. Nutritional value of the dry matter in the diet with alfalfa silage is less that in the diet with grass silage (6.9 vs. 7.1 mJ/kg for net energy of lactation), however its daily consumption by cows is higher, 22.1-23.2 and 20.3-21.2 kg [5]. This results in increased consumption of nutrients and animal productivity.

At the same time, high content of raw protein in alfalfa is often associated with its main unfavorable technological attribute of a non-silage culture [6]. Having compared buffering capacity of various plant species containing raw protein, calcium, and magnesium, the researchers have found that the richer the plants in the said compounds, the higher their buffer capacity [7]. However, it is mineral compounds, and not the nitrogenous matter, that play the main role in improvement of buffer capacity. Alfalfa is rich in mineral substances with expressed properties of bases [8, 9]. High buffer capacity of alfalfa entails accumulation of significantly larger quantity of lactic acid than it is required for acidification of grasses and clover, which in the context of sugar deficit is hard to ensure at regular silage making even in sun-cured form. This fact increases the interest in use of lactobacillus medicines at alfalfa silage making.

Sugar deficit and buffer capacity of the biomass leads to necessity for sun-wilting of alfalfa up to ≥ 45 % of DM (10). Chemical content of plants may be improved by their wilting in mowing. Thus, after 6-hour mowing of alfalfa up to 35.1 % of DM, amount of sugars increased 1.3 times from 4.82 to 6.24 % [11]. Simultaneously, sugar-buffer ratio to cured mass increased from 1.0 to 1.4. Consequently, rapid dehydration of alfalfa provides transferring thereof from the category of non-ensilaging plants to the category of hardly-ensilaging plants. However, in alfalfa, unlike the clover and grasses, the procedure has no detectable effect on ensilaging results [12].

Earlier it was erroneously suggested than plants cured up to ≥ 35 % DM are successfully ensilaged regardless of sugar content in them [13]. This suggestion still does not have an experimental confirmation [14, 15]. The concept of silage capacity of vegetable feed should also be clarified. In Russia it is still associated with chemical composition of the ensilaged mass and with misconception that wilting to up to 40 % DM does not have negative effect on the intensity of lactic-acid fermentation [16]. Meanwhile, even poor wilting up to 30 % DM noticeably retains development of lactobacillus resulting in slowing down the speed of feed acidification and, consequently, occurrence of the negative microbiological processes in the feed [15]. Nature of the later is totally dependent on the plant species and the extent of wilting. Because of that, even high concentration of sugar in cured grasses does not warrant production of high quality silage.

Ensilaging also depends on the factors that promote the plant ability to rapid and quiet strong acidification. The most important of them is quantitative and qualitative composition of epiphytic microflora. It is usually favorable at ensilaging of maize, 1 g of which at the middle dough stage contains ≥ 10^5 CFU of lactobacillus [7]. In the above count they are represented by only highly active hormone-enzyme *Lactobacillus plantarum* [17] well-adapted to bulk fermentation with relatively high content of dry matter. Silage from the chopped maize, regardless of the significant DM content, is rapidly acidified at the middle dough stage to active acidity (pH) ≤ 4.2 that depresses development of all unfavorable bacterium. In perennial herbs, mainly alfalfa, the composition of epiphytic microbes association is often unfavorable [18], but determines not only the dominant type of feed damage, but also ways to avoid spoiling. It is established [19] that alfalfa is not characterized by the presence on coli group usual in large

number in grasses [17]. Mainly, alfalfa silage is damaged due to butyric (septic) fermentation [15]. It leads to the main conservation principle of alfalfa (rule of G.W. Wieringa) that is based on a fact that Clostridia sensitivity to feed acidity significantly increases along with increase of the dry matter in the green biomass [20]. It allows production of the oil acid free silage from the cured alfalfa at significantly higher pH as compared to ensilaging of new-mown grasses.

It should be noted that such rule applies only to ensilaging of high-protein leguminoze grasses, but becomes fully irrelevant at use of sugar-contained cured grasses with other composition of epiphytic microbial flora requiring application of other conservation methods. As apart from the high-protein leguminoze grasses, butyric fermentation is often neither eliminated, nor becomes more manifested (secondary fermentation) in ensilaging of grasses in cured form [14, 15]. This is due to intensive development of coli forms – enterobacterium against the background of slow feed acidification. Enterobacteria, because of ineffective sugar fermentation in grasses, lead to sugar deficit in the ensilaged mass and the butyric fermentation. Not only Clostridia, but also enterobacterium shall be suppressed to stop the butyric fermentation in the silage from cured grasses. Regardless of the wilting extent, grasses shall be acidified to pH $\leq$ 4.3 for 3 days [21] to exclude growth of enterobacteria. Accounting for the limited lactobacillus fermentation at beginning of ensilaging, the required acidification speed and extent may be achieved only with addition of osmotolerant lactobacillus strains. In lack of them, even easily ensilaging grasses cured up to $\geq$ 30 % DM are not such in fact [22]. Thus, in the global practice the term "fermentability" is recently used in lieu of "ensilage capacity" [23], the term that is based on the chemical composition of plants and defines their potential ability to acidification.

Note that the rule of G.W. Wieringa [20] is not fully correct for alfalfa. According to the rule, it could be concluded [7] that at sugar-buffer ratio of 0.5-10 the alfalfa shall be cured up to 37-41 % DM. However, it is impossible to produce qualitative feed from alfalfa by spontaneous ensilaging, provided such quantity of dry matter. Green alfalfa mass rich in protein and pectin contains a lot of poorly bound water [24]. Because of acidification delay, plant protein-egrading enzymes remain highly active for a long time [25]. During the first 2 days of ensilaging, proteolysis in alfalfa comprises 25 μmol of amino acids/h per 1 kg of DM [26]. Wilting up to 35 % DM does not reduce activity thereof. It should be highlighted that protein of clover and grasses is hydrolyzed during ensilaging to significantly lesser extent that alfalfa protein [27].

Due to the rapid sugar consumption, amino acids are subjected to oxidative de-amination with high ammonium production [28] that improves buffer capacity of feed in ensilaging. Otherwise, alfalfa, which due to preliminary wilting acquires the properties of hardly-fermented plants, is again transformed into the non-ensilaging crop within the first 2 days of ensilaging. That is why alfalfa, unlike hardly-fermentable perennial grasses and clover, is usually not ensilaged, but hay-laid by wilting up to $\geq$ 45 % DM.

It is considered that haylage, as compared to silage, is preserved not due to active acidification, but due to formation of the physiological dryness in cured grasses that warrants lack of access of moisture germs contained in the plants [29, 30]. Besides, even A.A. Zubrilin [31] warned of an obvious mistake to count only dry matter in the green mass at determination of the extent of development of this or other micro germ group. It is known that several products, for instance, jelly contains a lot of water, but it is inaccessible for many microbes. Recently, at determination of micro germ development extent, activity of contained water (Aw). Aw (is expressed in dimensionless units of the scale from 0 to 1) —

is a quantity of non-bound micro germ accessible water in product) is estimated [32]. Even at 45-50 % DM, Aw in plants does not fall below 0.95 [7], whereas pH is not standardized only at Aw of no more than 0.85 [33]. It is significantly lower than it is noted in grasses, even if such grasses are cured up to 60 % DM. To this end, it could be concluded that wilting of alfalfa up to ≥ 45 % DM is not only associated with creation of physiological dryness in plants, but also with the necessity for assurance of the high preservation of feed in the context of weak acidification. In fact, feed acidification extent and accumulation of the fermentation acids are standardized at haylaying of the sugar-containing grasses testified in support of such assumption.

According to G. Pahlow at al. [21], at conservation of sugar-containing grasses with DM ≥ 45 %, it should be acidified during 3 days to pH ≤ 4.5, and shall accumulate in the DM of at least 3.5 % acetic acid. The latter is required for suppression of yeast fungi development, being the main initiators of aerobic damage of haylage when removed from the storage reservoirs [7]. Just like at ensilaging of cured grasses, it could be achieved only with involvement of lactobacillus. However, medicines based on the heterofermentative lactobacillus strains should be used at preparation of haylage from grasses producing, along with the lactic acid, significant quantity of acetic acid [34]. Accordingly, ensilaging process also flows in the green mass cured up to ≥ 45 % DM.

Regardless of affiliation with non-ensilaging cultures, alfalfa (at ensilaging in small insulated vessels) in the laboratory tests is preserved for a long time even in newly mown form without fermentation and damage thereof. It is explained by presence of the secondary vegetable metabolites with antimicrobial action [35]. It includes several free non-protein amino acids [36], saponines, many phenol compounds, alkaloids [37] and other compounds. Majority of the secondary metabolites are cancerogenous and, thus, hazardous to animals [38]. The role of the secondary vegetable metabolites in preservation of alfalfa silage is so high that results of the laboratory tests could not be transferred in production environment without relevant adjustment. Presence of the secondary metabolites in alfalfa and other legumetized species is also due to the high aerobic stability of produced silage and haylage [35, 39]. However, it is impossible to preserve alfalfa silage and haylage in production environment without proper acidification. It is alfalfa inability to acidify during a continuous time to the extent eliminating development of butyric bacteria mainly causing feed damage. Thus, in one of the lab-based alfalfa ensilaging tests with 39.9 % DM after 3, 7, 15, 30 and 60 days of ensilaging the pH value was 5.85; 5.54; 5.17; 4.85, and 4.57, respectively [11]. It means that acidification had reached value eliminating the butyric fermentation only after 2 months of ensilaging. High preservation ability of feed in the laboratory tests was assured by the secondary vegetable metabolites; however in the production environment such silage would have become useless during the said storage term.

To increase the alfalfa fermentation ability, it is required to suppress proteolysis in process of plant dehydration in the field and their further ensilaging. Such opinion, in particular, is supported by X.S. Guo at al. [40]. In the field it is achieved by the intensive wilting of alfalfa in mowing (within 2-4 hours after mowing content of DM in the green mass achieves 35 % and more) [11]. Proteolysis intensity at ensilaging may be reduced by fast switching of pH to the limits of proteolytic enzyme activities [25, 41], which is hard to achieve at spontaneous fermentation of the cured mass due to its buffer capacity and lack of sugar. The situation becomes more challenged because the optimal proteolytic pH in alfalfa is lower than in clover, by 6.0 and 6.5 [42]. It means that alfalfa shall be acidified faster and more intensively than the clover to avoid proteolysis.

At low alfalfa wilting (≤ 35 % DM), stability of the feed is ensured only at its rapid acidification to pH ≤ 4.6, which could not be reached even with the use of lactobacillus. It is established that administration of lactobacillus medicines at ensilaging of alfalfa (36 % DM) did not result in notable increase of the feed acidification (pH 4.34-4.40 against 4.42 in regular silage) [43]. Several researchers believe that qualitative silage could be produced at ensilaging of alfalfa even with the use of lactobacillus biologicals in combination with sugar additives [44]. However, their results show that such approach relates to significant loss of nutrient compounds (up to 30 %) and is useless in practice. According to other data, effect of molasses with lactobacillus medicines on reduction of proteolysis at ensilaging of alfalfa has been growing with increase of the dry matter content from 20 to 37 % [45]. Use of molasses had resulted in increase of the acetic acid in feed that evidences on activation of unfavorable microbial flora.

To elevate sugar content, the cured alfalfa ensilaging with fermentative additives is considered. Thus, in favorable weather conditions Ferkon fermentative medicine upon ensilaging of alfalfa cured up to 30 % DM was as effective as formic acid [46]. However, at detailed analysis, both medicines were ineffective. Just like at administration of Ferkon, and at introduction of 0.5 % muratic acid, pH of feed comprised 4.72-4.70 and accumulation of ammonium in dry matter of silage was 0.43-0.41 %. It means that neither of the used medicines ensured creation of the active acidification that is able to prevent butyric fermentation. Upon ensilaging of clover meadow cured to the same content of dry matter and Ferkon medicine, formic acid, and content of ammonium in the feed comprising accordingly 0.09 and 0.10 %, pH of silage was 4.14 and 4.37. There is a report on the use of common method for satisfactory ensilaging of alfalfa-grass mixture cured up to 35-40 % DM, with share of alfalfa from 50 to 75 % [47]. At ensilaging of net alfalfa with Ferkon and formic acid, positive results were obtained only at wilting to up ≥ 40 % DM [12]. In such a case, silage produced in the production environment is acidified to pH of 4.47 and 4.33, which ensured its high storage stability.

L. Kung at al. [48] had achieved good results by ensilaging of alfalfa with the same content of dry matter upon the use of heterofermentative *Lactobacillus buchneri* with β-glucanase, α-amilase, xylanase and galactomanase. Such ensilaging (along with better preservation and quality of feed) had resulted in notable increase of its aerobic stability. It was certain [35, 39] that haylage and silage from the cured legumetized grasses are quiet resistant to aerobic fall, elimination issue of which arises only at preparation of silage from the maize at middle dough stage [49, 50], sorgo [51] and haylage from grain crops and grasses [52, 53].

Alfalfa and lactobacillus-based ensilaging technology shall be used since even in the southern regions of Russia it is often impossible to cure the mass to the haylage moisture [54] resulting in low quality feed. In Volgograd Region, more than half of the prepared haylage is annually associated with the low grade feed [55]. At favorable wilting conditions, when content of DM in alfalfa rapidly goes up to value ≥ 45 %, stimulation of lactobacillus fermentation by the use of the lactic fermentating agents provides significant effect. According to O.M. Kurnaeva [56], haylaying of alfalfa in the productive environment with introduction of Litosil lactobacillus medicine had reduced accumulation of ammonium in the feed from 26.4 to 10.1 mg%, with loss of DM from 17.6 to 13.2 %, and had ensured stability of haylage at storage. At haylaying of alfalfa (with up to 50 % DM) in tranches, feed pH following 6 and 12 months of storage remained high and comprised accordingly 5.21 and 5.06 [56]. For this reason, accumulation of the oil acid in the natural feed kept growing: after 6 and 12 months of storage it comprised 0.06 and 0.11 %. During the entire storage term the oil acid was not

accumulated in the haylage where, due to administration of Litosil medicine, feed pH had rapidly decreased to the extent limiting the growth of butyric acid bacteria. Upon haylaying of the same mass in roll under films, feed pH after 1 and 6 months of storage comprised accordingly 5.08 and 5.32, while content of butyric acid comprised 0.11 and 0.21 %, accordingly. Use of Litosil in this case also ensured production of the feed free from butyric acid. Consequently, even upon wilting up to 50 % DM warranted preservation of feed both at storage in tranches and rolls is ensured only upon use of lactobacillus medicines.

Upon reduction of DM in alfalfa, instability of silage at storage is significantly increased. To solve the problem, we must first of all increase the lactobacillus-butyric acid bacteria competition, which to a significant extent is promoted by alfalfa wilting up to ≥ 40 % DM. In this case, lactic acid is accumulated more than in ensilaging of newly mown alfalfa [15]. At slow acidification, it is important that butyric acid bacteria are present in plants as spores [18]. The dryer the plants, the slower the spores of butyric acid bacteria are growing; and lactobacillus (even at low activity) manage to acidify the feed to the required pH value. Thus, the alfalfa ensilaging mechanism cured up to ≥ 45 % DM is applied recently [33]. This is also associated with the requirement of DM preservation within the range from 40 to 50 % (i.e. the acceptable deviation from the recommended value is no more than 5 %) [7], which significantly challenges haylaying of alfalfa in the production context and accounts for the feasibility of use of lactobacillus fermentations accelerating acidification of the mass and increasing the feed stability at storage and removal.

It is understood that upper wilting limit of the ensilaged alfalfa mass is determined by its technical properties, namely the compression ability. At placing of alfalfa for storage in tranches, content of DM shall not exceed 50 %, provided careful grinding of plants and their absolute air insulation [7]. The upper wilting limit is related not only to the ability of the mass to rapid acidification under the effect of biological preparations to the state excluding development of butyric acid bacteria. The more the green mass is cured, the more effective is application of lactobacillus medicines at wilting. Thus, at ensilaging of alfalfa cured up to 41.1 % DM by lactobacillus medicine Ecosyl, the feed is acidified to pH 4.45 vs. to 4.64 in regular silage [57]. Herewith, it is pH at beginning of ensilaging process suppressing the proteolysis that is important rather than final active acidity of the feed [58]. It is indicated by reduction of the share of nitrogen ammonia in the total feed nitrogen (with 11.2 to 7.6 %) in the silage prepared by lactobacillus fermentation.

At less dry matter in the green mass, effectiveness of Ecosyl use is notably decreased. For instance, at ensilaging of alfalfa with 38.1 % of DM, pH of the trial and control feed comprised 4.73 and 4.53, accordingly. Share of nitrogen ammonia in the total nitrogen had decreased from 12.7 to 9.4 %. At ensilaging of alfalfa with 22.6 % DM, pH in control and test sample comprised 5.23 and 4.93; share of nitrogen ammonia in the total nitrogen was 17.2 and 14.5 %. The above data testifies that upon increase of the quantity of DM in the ensilaged alfalfa from 22.6 to 41.1 %, share of nitrogen ammonia in the total nitrogen of feed had practically decreased in two times — from 14.5 to 7.6 %, and pH had decreased from 4.93 to 4.45. Accordingly, ensilaging of alfalfa cured up to ≥ 40 % DM with lactobacillus not only promotes acceleration, but also more acidification of feed that improves its stability at storage.

According to some researchers, use of lactobacillus is the main reason of ammonia growth in the dry matter of alfalfa silage [59]. Such conclusion is based on the assumption that at sugar deficit in the ensilaged green mass, lactic bacteria uses carbon skeleton of amino acids for energetic metabolism. It hap-

pens in cases when use of lactobacillus medicines does not promote any more intensive acidification of the feed. Thus, in one of alfalfa ensilaging tests (Taisia variety) with 31.3 % DM by regular way and with introduction of Biotrof preparation, pH in finished silage comprised accordingly 5.06 and 5.02, ammonia content in the dry matter comprised 0.54 and 0.62 % [11]. At the same time, at ensilaging of alfalfa Pastbischnaya 88 variety rich in sugar, even at regular ensilaging of plants with 24.5 % DM the feed was acidified to pH 4.70. Biotrof preparation did not have any significant effect on the acidification extent of the finished silage (pH 4.68), but had accelerated such process. Dry matter of the control silage sample contained 0.17 % of oil acid, while it was not found in the trial sample. Intensification of acidification had resulted in accumulation of ammonia in dry matter of feed from 0.30 to 0.26 %. Accordingly, at development of the effective technology of ensilaging of the cured alfalfa by lactobacillus medicine it is important to account for the possible significant role of the kind differences in plants.

Interesting results were obtained by Hungarian researchers [60] at ensilaging of alfalfa (32.3 % DM) upon use of maize flour, in which 89 % of starch was hydrolyzed to simple sugars. Maize flour was added accounting for 1.0 % to the green mass. According to authors, it promoted production of high quality feed with good ratio of lactic and acetic acids. At the same time, addition of cellulosolythic enzymes and their combinations with lactobacillus at ensilaging of alfalfa with 34 % DM was ineffective just as in the above-described tests [61]. Ensilaging of alfalfa cured up to 33.40 and 53.00% DM with addition of tannin was rested with positive results [62]. Importantly, in this case the effect of tannin use for protheolysis limitation had increased by increasing the content of dry matter in the green mass. Disadvantage of the above method is that high tannin dosages (≥ 2.0 % of DM) reduce digestibility of the nutrient components in the feed.

The most important property of alfalfa silage produced with addition of lactobacillus medicine is its high productive action validly exceeding the productive action of spontaneous fermentation silage. At difference of total 0.3 kg in consumption of DM rations with alfalfa silage produced in regular manner and with addition of lactobacillus, the difference in the average daily milk yield was 0.8 kg (40.7 vs. 39.9 kg) at higher milk fat (3.43 vs. to 3.37 %) in animals of the test group [48]. The literature sources yet lack clear explanation of such case. Some researchers associate it with increase of the mass of rumen microbial flora, which serves a source of complete protein for high productive cows [63, 64].

Let us also consider the factors which, besides decrease of protheolysis in alfalfa green mass cured up to ≥ 40 % DM, may be the reason for more intense acidification of the feed at addition of lactobacillus enzymes. First of all, it promotes more homofermentative type of lactobacillus fermentation in the silage [65, 66]. Lactic acid which is dissociated to ions more than other fermentation acids [7, 67] promotes higher acidity in the feed. Important condition of successful ensilaging of alfalfa in deeply cured form is creation of sugar both at rapid wilting of plants up to ≥ 40 % DM [11, 15], as well as in fermentation process of the cured mass in anaerobic conditions. It is due to content of up to 5 % of starch [23] in the dry matter of alfalfa which is hydrolyzed under the effect of fermentation acids to simple sugars. Besides, alfalfa contains saponine ($C_{27}H_{37}O_{16}$) that is fermented into sapogenin, glucose, and unknown tart substance [68]. It may happen that another important message is that up to 2 % of apple acid is accumulated in the dry matter of alfalfa cured up to ≥ 40 % DM, which, according to certain authors [69], may be fermented by lactobacillus.

Thus, alfalfa is characterized, along with sugar deficit, by very high buffer capacity exceeding 1.6-2.3 times the buffer capacity of other fodder plants.

The higher the buffer capacity, the more fermentation acids are neutralized, which strongly suppresses feed acidification, especially during the first ensilaging phase. At high protein and pectin contents, alfalfa biomass, even when wilted up to ≥ 35 % dry matter (DM), still contains significant amount of weak-bound water which at slow acidification leads to high proteolytic activity and protein hydrolysis. It is accompanied by high accumulation of ammonium in silage and further increase in buffer capacity. Thence, acidification of wilted alfalfa to the pH suppressing clostridia may take more time, even in use of lactobacilli or their combinations with enzymes, resulting in feed damage. In this, it is required i) to reduce proteolysis in the ensilaged green mass, ii) to slow down growth of butyric acid bacteria during the first phase of ensilaging with intensification of lactobacillus fermentation, and iii) to increase the pH to that critical for butyric acid bacteria. To this end, alfalfa mass shall be rapidly wilted to ≥ 40 % DM and subjected to ensilaging with the use of osmotolerant lactobacillus strains.

REFERENCES

1. Laptev G.Yu., Novikova N.I., Il'ina L.A., Yyldyrym E.A., Soldatova V.V., Nikonov I.N., Filippova V.A., Brazhnik E.A., Sokolova O.N. Dynamics of mycotoxin accumulation in silage during storage. *Agricultural Biology*, 2014, 6: 123-130 (doi: 10.15389/agrobiology.2014.6.123eng).
2. Chub O. *Zhivotnovodstvo Rossii*, 2015, 10: 55-56 (in Russ.).
3. Mak-Donal'd P., Edvards R., Grinkhaldzh Dzh. *Pitanie zhivotnykh* [Animal feeding]. Moscow, 1970 (in Russ.).
4. Ennison E.F., L'yuis D. *Obmen veshchestv v rubtse* [Rumen metabolism]. Moscow, 1962 (in Russ.).
5. Grabov M. *Tsenovik*, 2016, 5: 43 (in Russ.).
6. Albrecht K.A., Beauchemin K.A. Alfalfa and other perennial legume. In: *Silage science and technology, Agronomy Monograph 42.* D.R. Buxton, R.E. Muck, J.H. Harrison (eds.). ASA, CSSA, and SSSA, Madison, 2003: 633-664 (doi: 10.2134/agronmonogr42.c14).
7. Vaisbakh F. *Problemy biologii produktivnykh zhivotnykh*, 2012, 2: 49-70 (in Russ.).
8. Sosnowski J., Jankowski K., Wiśniewska-Kadźajan B., Jankowska J., Kolczarek R. Effect of the extract from *Ecklonia maxima* on selected micro- and macroelements in aerial biomass of hybrid alfalfa. *J. Elementol.*, 2014, 19(1): 209-217 (doi: 10.5601/jelem.2014.19.1.608).
9. Hancock D.W., Buntin G.D., Ely L.O., Lacy R.C., Heusner G.L., Stewart R.L. *Alfalfa management in Georgia.* Athens, 2005.
10. Pobednov Yu.A., Kosolapov V.M., Bondarev V.A., Akhlamov Yu.D., Mamaev A.A., Klimenko V.P., Otroshko S.A., Shevtsov A.V. *Silosovanie i senazhirovanie kormov (rekomendatsii)* [Silage and hay making]. Moscow, 2012 (in Russ.).
11. Pobednov Yu.A., Mamaev A.A., Ivanova M.S. V sbornike nauchnukh trudov: *Zhuchenkovskie chteniya II* [II Zhuchenko Readings. Iss. 11(59)]. Moscow, 2016. Vypusk 11(59): 180-188 (in Russ.).
12. Anisimov A.A. *Vash sel'skii konsul'tant*, 2006, 4: 28-30 (in Russ.).
13. Vasin V.G., Zotikov V.I., Vasina A.A. *Proizvodstvo kormov dlya molochnykh kompleksov* [Feed production for dairy commercial farms]. Orel, 2012 (in Russ.).
14. Weissbach F., Honig H. Über die Vorhersage und Steurung des Gärungsverlaufs bei der Silierung von Grünfutter aus extensiven Anbau. *Landbauforschung Völkenrode*, 1996, 1: 10-17.
15. Pobednov Yu.A. *Teoreticheskie predstavleniya i sposoby konservirovaniya kukuruzy i trav na osnove regulirovaniya mikrobiologicheskikh protsessov* [Theoretical aspects and methods for preserving maize and herbs based on the regulation of microbiological processes]. St. Petersburg, 2017 (in Russ.).
16. Bondarev V.A., Kosolapov V.M., Klimenko V.P., Krichevskii A.N. *Prigotovlenie silosa i senazha s primeneniem otechestvennykh biologicheskikh preparatov* [Domestic biologicals in silage and hay making].Moscow, 2016 (in Russ.).
17. Fehrmann E., Műller Th. Jaresverlauf des epiphytischen Mikrobenbesatzes auf einen Graslandstandort. *Das Wirtschaftseigene Futter*, 1990, 36(1): 66-78.
18. Shmidt V., Vetterau G. *Proizvodstvo silosa* [Silage making]. Moscow, 1975 (in Russ.).
19. Shurkhno R.A., Norina O.S., Tagirov M.Sh., Naumova R.P. *Doklady Rossiiskoi akademii sel'skokhozyaistvennykh nauk*, 2008, 6: 23-26 (in Russ.).
20. Viringa Dzh. *Materialy 8-go Mezhdunarodnogo lugopastbishchnogo kongressa (11-21 iyulya, 1960 g., g. Reding, Angliya)* (perevod s angliiskogo) [Proc. 8th Int. Grassland Congress, 11-21 July, 1960, Reading. England]. Moscow, 1963: 334-343 (in Russ.).
21. Pahlow G., Weissbach F. New aspects of evaluation and application of silage additives. *Landbauforschung Völkenrode*, 1999, 206: 141-158.

22. Pobednov Yu.A. *Problemy biologii produktivnykh zhivotnykh*, 2009, 3: 89-100 (in Russ.).
23. *Proizvodstvo grubykh kormov. Kniga 1* /Pod redaktsiei D. Shpaara [Coarse fodder production. Book 1. D. Shpaar (ed.)]. Torzhok, 2002 (in Russ.).
24. Pobednov Yu.A. *Adaptivnoe kormoproizvodstvo*, 2016, 2: 21-37 (in Russ.).
25. Charmley E., Veira D.M. Inhibition of proteolysis at harvest using heat in alfalfa silages: effect on silage composition and digestion by sheep. *J. Anim. Sci.*, 1990, 68(3): 758-766 (doi: 10.2527/1990.683758x).
26. McKersie B., Buchanan-Smith J. Changes in the levels of proteolytic enzymes in ensiled alfalfa forage. *Canadian Journal of Plant Science*, 1982, 62(1): 111-116 (doi: 10.4141/cjps82-017).
27. Purwin C., Pysera B., Fijałkowska M., Antoszkiewicz Z., Piwczynski D., Wyzlic I., Lipinski K. The influence of ensiling method on the composition of nitrogen fractions in red clover, alfalfa and red fescue silage. *Proc. XVI International Silage Conference*. Hämeenlinna, 2012: 256-257.
28. Ulit'ko V.E., Pykhtina L.A., Desyatov O.A. *Povyshenie produktivnogo deistviya kormov pri proizvodstve moloka i myasa v Srednevolzhskom regione* [Increasing feed effect on milk and meat production in the Middle Volga region]. Ul'yanovsk, 2016 (in Russ.).
29. Li S.S., Pshenichnikova E.N., Kroneval'd E.A. *Vestnik Altaiskogo gosudarstvennogo agrarnogo universiteta*, 2014, 2(112): 98-102 (in Russ.).
30. Makarov S.A. *Mezhdunarodnyi nauchno-issledovatel'skii zhurnal*, 2016, 3(45-3): 109-112 (doi: 10.18454/IRJ.2016.45.035) (in Russ.).
31. Zubrilin A.A. *Konservirovanie zelenykh kormov* [Green forage preserving]. Moscow, 1938 (in Russ.).
32. Gorelikova G.A. *Osnovy sovremennoi pishchevoi biotekhnologii* [Fundamentals of modern food biotechnology]. Kemerovo, 2004 (in Russ.).
33. Pobednov Yu.A. *Problemy biologii produktivnykh zhivotnykh*, 2016, 2: 42-54 (in Russ.).
34. Driehuis F., Oude Elferink S.J.W.H., Van Wikselaar P.G. Fermentation characteristics and aerobic stability of grass silage inoculated with *Lactobacillus buchneri,* with or without homofermentative lactis acid bacteria. *Grass Forage Sci.,* 2001, 56(4): 330-343 (doi: 10.1046/j.1365-2494.2001.00282.x).
35. Weissbach F. Consequences of grassland de-intensification for ensilability and feeding value of herbage. *Landbauforschung Völkenrode***,** 1999, 206(Sonderheft): 41-53.
36. Sagiyan A.S. *Enantiomerno chistye nebelkovye aminokisloty. Sposoby polucheniya* [Enantiomeric pure non-protein amino acids — synthesis, isolation, purification technique]. Moscow, 2010 (in Russ.).
37. Luckner M. *Vtorichnyi metabolizm u mikroorganizmov, rastenii i zhivotnykh* [Secondary metabolism in microorganisms, pants, and animals]. Moscow, 1979 (in Russ.).
38. Heldt H.-W. *Biokhimiya rastenii* [Plant biochemistry]. Moscow, 2014 (in Russ.).
39. Davies D.R., Fychan R., Jones R. Aerobic deterioration of silage: causes and controls. *Proc. Alltech's 23rd Annual Symposium «Nutritional Biotechnology in the Feed and Food Industries»*. Nottingham, 2007: 227-238.
40. Guo KH.S., Cheng W., Tao L., Zhu Yu., Zhou H. Contribution of endo — and exopeptidases to formation of nonprotein nitrogen during ensiling of alfalfa. *Proc. KHVI International Silage Conference*. Hämeenlinna, 2012: 58-59.
41. McKersie B.D. Effect of pH on proteolysis in ensiled legume forage. *Agron. J.*, 1983, 77(1): 81-86 (doi: 10.2134/agronj1985.00021962007700010019x).
42. Tao L., Guo X.S., Zhou H., Undersander D.J., Nandety A. Short communication: characteristics of proteolytic activities of endo- and exopeptidases in alfalfa herbage and their implications for proteolysis in silage. *J. Dairy Sci.*, 2012, 95(8): 4591-4595 (doi: 10.3168/jds.2012-5383).
43. Filya I., Muck R.E., Contreras-Govea F.E. Inoculant effects on alfalfa silage: fermentation products and nutritive value. *J. Dairy Sci.*, 2007, 90: 5108-5114 (doi: 10.3168/jds.2006-877).
44. Shifer K., SHtainkhefel' O., Nad' B. *Novoe sel'skoe khozyaistvo*, 2007, 4: 74-78 (in Russ.).
45. Hashemzadeh-Cigari F., Khorvash M., Chorbani G.R., Taghizadeh A. The effects of wilting, molasses and inoculants on the fermentation quality and nutritive value of lucerne silage. *S. Afr. J. Anim. Sci.*, 2011, 41(4): 377-388 (doi: 10.4314/sajas.v41i4.8).
46. Kosolapov V.M., Bondarev V.A., Panov A.A., Akhlamov Yu.D., Udalova E.V., Isaenkov N.I., Anisimov A.A., Otroshko S.A., Klimenko V.P. *Tekhnologiya silosovaniya vysokobelkovykh mnogoletnikh bobovykh trav s polifermentnym preparatom Ferkon (rekomendatsii)* [Silaging of high-protein perennial legumes using multi enzyme preparation Ferkon — recommendation]. Moscow, 2008 (in Russ.).
47. Smitt K.-O., Pratz H. Mit Luzerne die Futtergrudlage. *Rheinische Bauer Zeitung*, 1996, 5: 20.
48. Kung L.J., Taylor C.C., Lynch M.P., Neylon J.M. The effect of treating alfalfa with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for lactating dairy cows. *J. Dairy Sc.*, 2003, 86: 336-343 (doi: 10.3168/jds.S0022-0302(03)73611-X).
49. Ranjit N.K., Taylor C.C., Kung L. Effect of *Lactobacillus buchneri* 40788 on the fermentation, aerobic stability, and nutritive value of maize silage. *Grass Forage Sci.*, 2002, 57: 72-81 (doi: 10.1046/j.1365-2494.2002.00304.x).
50. Kristensen N.B., Sloth K.H., Højberg O., Spliid N.H., Jensen C., Thøgersen R. Effects of micro-

bial inoculants on corn silage fermentation, microbial contents, aerobic stability, and milk production under field conditions. *J. Dairy Sci.*, 2010, 93: 3764-3774 (doi: 10.3168/jds.2010-3136).
51. Tabacco E., Righi F., Quarantelli A., Borreani G. Dry matter and nutritional losses during aerobic deterioration of corn and sorghum silages as influenced by different lactic acid bacteria inocula. *J. Dairy Sci.*, 2011, 94: 1409-1419 (doi: 10.3168/jds.2010-3538).
52. Shah A.A., Xianjun Y., Zhihao D., Siran W., Tao S. Effect of lactic acid bacteria on ensiling characteristics, chemical composition and aerobic stability of king grass. *Journal of Animal & Plant Sciences*, 2017, 27: 747-755.
53. Randby A.T., Gismervik K., Andersen A., Skaar I. Effect of invasive slug populations (*Arion vulgaris*) on grass silage: I. Fermentation quality, in-silo losses and aerobic stability. *Anim. Feed Sci. Tech.*, 2015, 199: 10-19 (doi: 10.1016/j.anifeedsci.2014.09.026).
54. Marchenko F.Yu., Zabashta N.N., Golovko E.N. *V sbornike nauchnykh trudov Severo-Kavkazskogo nauchno-issledovatel'skogo instituta zhivotnovodstva* [In: Scientific papers of North-Caucasian Research Institute of Animal Husbandry]. Krasnodar, 2016: 182-188 (in Russ.).
55. Amerkhanov Kh.A., Tyapugin E.A., Simonov G.A., Tyapugin S.E. *Effektivnost' vedeniya molochnogo skotovodstva v usloviyakh Evropeiskogo Severa Rossii* [Effective dairy cattle farming in the European North of Russia]. Moscow, 2001 (in Russ.).
56. Kurnaev O.M. Vplyv tekhnologiï zagotivli sinazhu na vtraty syrogo proteïnu ta iogo fraktsiinyi sklad uprodovzh zberigannya. *Kormi i kormovirobnitstvo. Mizhvidomchii tematichnii naukovii zbirnik (Vinnitsya)*, 2010, 66: 274-280.
57. Moran J.P., Owen T.R. The effect of bacterial inoculant on the fermentation of lucerne silage. *Proc. KHI International Silage Conference*. Aberystwyth, 1996: 166-167.
58. Fijałkowska M., Pysera B., Lipiński K., Strusińska D. Changes of nitrogen compounds during ensiling of high protein herbages — a review. *Ann. Anim. Sci.*, 2015, 15(2): 289-305 (doi: 10.1515/aoas-2015-0008).
59. Ilyaletdinov N.K., Akhmediev A.N. *Izvestiya AN SSSR. Seriya biologicheskaya*, 1979, 3: 427-434 (in Russ.).
60. Rigy E., Zsédely E., Tóth T., Schmidt J. Ensiling alfalfa with hydrolyzed corn meal additive and bacterial inoculant. *Acta Agronomica Óvariensis*, 2011, 53(2): 15-23.
61. Lynch J.P., Jin L., Lara E.C., Baah J., Beauchemin K.A. The effect of exogenous fibrolytic enzymes and a ferulic acid esterase producing inoculant on the fibre degradability, chemical composition and conservation characteristics of alfalfa silage. *Anim. Feed Sci. Tech.*, 2014, 193: 21-31 (doi: 10.1016/j.anifeedsci.2014.03.013).
62. Tabacco E., Borreani G., Crovetto G.M., Galassi G., Colombo D., Cavallarin L. Effect of chestnut tannin on fermentation quality, proteolysis, and protein rumen degradability of alfalfa silage. *J. Dairy Sci.*, 2006, 89(12): 4736-4746 (doi: 10.3168/jds.S0022-0302(06)72523-1).
63. Kurtoglu V., Coskum B. Effect of bacterial adding alfalfa silage on milk yield and milk composition of dairy cattle. *Revue Mèd. Vèt.*, 2003, 154(12): 755-762.
64. Mohammed R., Stevenson D.M., Beauchemin K.A., Muck R.E., Weimer P.J. Changes in ruminal bacterial community composition following feeding of alfalfa ensiled with a lactic acid bacterial inoculant. *J. Dairy Sci.*, 2012, 95(1): 328-339 (doi: 10.3168/jds.2011-4492).
65. Silva V.P., Pereira O.G., Leandro E.S., Da Silva T.S., Ribeiro K.G., Mantovani H.C., Santos S.A. Effects of lactic acid bacteria with bacteriocinogenic potential on the fermentation profile and chemical composition of alfalfa silage in tropical conditions. *J. Dairy Sci.*, 2016, 99(3): 1895-1902 (doi: 10.3168/jds.2015-9792).
66. Tao L., Zhou H., Zhang N., Si B., Tu Ya., Ma T., Diao Q. Effects of different source additives and wilt conditions on the pH value, aerobic stability, and carbohydrate and protein fractions of alfalfa silage. *Anim. Sci. J.*, 2017, 88(1): 99-106 (doi: 10.1111/asj.12599).
67. Lück E. *Chemische Lebensmittelkonservirung*. Berlin, Heidelberg, NY, Tokyo, 1985.
68. CHukanov N.K., Popenko A.K. *Mikrobiologiya konservirovaniya trudnosilosuemykh rastenii* Microbiology of preservation of plants which are hard to be silage]. Alma-Ata, 1986 (in Russ.).
69. McDonald P. *Biokhimiya silosa* [Silage biochemistry]. Moscow, 1985 (in Russ.).

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ABOUT THE CONCEPT OF ECOLOGICAL NICHE AND ITS ROLE IN DESIGN OF ADAPTIVE ARID PASTURE AGROECOSYSTEMS

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Abstract

The concept of ecological niche occupies the central position in modern ecology (Eu. Odum, 1975). The concept of ecological niche may to a certain extent explain how different species can normally function and produce, growing side by side with each other, and absorbing water and mineral resources within a certain ecotope. In the context of the traditional concept of ecological niche, the community can be imagined as extensive *n*-dimensional hyper space within which each specific population evolves in such direction to correspond to own part of this space (G. Huthinson, 1957). The niche is characterized by its position and the response to the factors within the hyperspace of this community. In recent years, along with the traditional concept of niche, there was a concept of neutralizm which is actively developed by Stephen Hubbel and his supporters (G. Bell, 2001; J. Whitfield, 2002). According to this concept, species coexist thanks to similarity, but not distinctions, as a result of similarity on demographic characteristics, i.e. the similar specific speed of population growth and speed of settling of the released site. A number of authors have tried to unite within one model the neutralistic and niche mechanisms of functioning of species in community (D. Gravel et al., 2006). Now even more often ecologists speak about two types of communities (A.M. Gilyarov, 2010). Communities of the first type are organized according to the principle of a discrepancy of types on different ecological niches. Their existence is possible only because their niches are differing. Communities of the second type are organized and capable to coexist very long if are ecologically identical due to the same probability of an individual of different species to reproduce, die out, and occupy free spaces. It is supposed that if species long live in the same place, then they already are definitely rather close ecologically. We created multispecific, multi-tiered agroecosystems consisting of shrubs, semishrubs and grasses for arid conditions of Central Asia based on the traditional concept of a divergence of species in different ecological niches. For formation floristic and cenotic full-member multispecific pasture agroecosystems, we used fodder shrubs, typical for the southern deserts (*Haloxylon aphyllum*, *Aellenia subaphylla*), semi-shrubs (*Eurotia ceratoides*), draft semishrubs (*Kochia prostrata*, *Salsola orientalis*, *Camphorosma lessingii*, *Artemisia diffusa*), xerofitic perennial grasses (*Poa bulbosa*, *Carex pachystylis*) as the members of typical zonal flora. Multispecific shrubs-semishrubs-grassy pasture agrophytocenosis which were formed using zonal dominant species of fodder plants provided for a rapid restoration of biodiversity and the fodder efficiency lost under land degradation. The spring-summer and autumn-winter pasture agroecosystems created from mix of fodder shrubs, semishrubs, draft semishrubs and perennial grasses with different rhythmic of development, different type of root system, different drought resistance and heat resistance are more durable and productive than natural pasture ecosystems of the Central Asian deserts. Along with ecological advantages, multispecific pasture agroecosystems are much more various on structures of forages, they are better eaten and more stoutly satisfy physiological needs of animals for nutrients.

Keywords: natural pastures, constructed pastures, pasture agrophytocenosis, ecological niche

The ecological niche concept as a focus of modern ecology [1] was first suggested upon the attempt to formulate the role of a species in the community [2]. An ecological niche puts together plants, animals, and microorganisms to a sustainable unit which fits species living under specific environmental conditions. The concept of ecological niche may to a certain extent explain how different species can normally function, growing "side by side" and using water and nutrients within a certain ecotope. The interest of researchers to the concept of ecological niche has grown enormously over the last 60 years.

Purpose of present article is to justify construction perspectives of high productive everlasting multispecies pastture agricultural ecosystems in arid regions of the Central Asia.

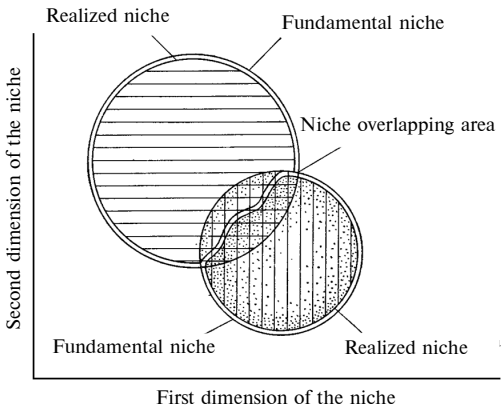

Fig. 1. The balance between the realized and fundamental niches for two species. Outer periphery limits (in two dimensions) the areas of the niche space where both species may potentially exist. At that, they push out each other from the part of overlapping area of fundamental niches. The realized niche is a real area occupied by any specie in a relevant space [7].

The term "niche" was introduced by J. Grinell [cit. ex 3] for denotation of the smallest unit of specie outspread; Ch. Elton [4] had defined it as a place of an organism in biotic environment. It was assumed that ecological niches in rare species are not overlapped. E. Pianka [2] and Yu. Odum [1] have developed a concept of competitive exclusion. According to G.F. Gause [5], an ecological niche is a space which could not be occupied by any two species for the unlimited long time. As per G.E. Hutchinson [6], a definition of ecological niche should account for all physical, chemical, and biological variables of the environment. Ecological niche of any specie can be represented as a part of a multidimensional space, separate axis of which correspond to factors required for normal existence. The niche with n significant dimensions is described as *n-dimension* space or hyperspace by stepwise addition of one dimension. G.E. Hutchinson had introduced the term of fundamental niche (covers all multitude of optimal conditions in which any specie kind may exist in the absence of competition) and realized niche (actual set of conditions in which any specie usually exists) (Fig. 1). The realized niche is less than or equal to the fundamental one [3]. The ecological niches are discrete (fundamental niches are not overlapped), and species wealth of any community depends on the total occupied space and average size of each niche. The ecological niche of a species can be established through the distribution function of the activity of using a resource along its gradient [7]. Hyperspace of niches of any single specie involves parts of hyperspaces of other species. If overlapping is insignificant or if resources are overabundant, then species with overlapping niches may co-exist in practically separated niches. If niches are overlapped to a greater extent and if resources in the overlapping area are not enough to meet the demands of a species, then the more adapted species will limit the abundance of the less adapted species. As a result, a competitive exclusion may occur in the overlapping points. In extensive *n-dimension* hyperspace occupied by a community, each species population evolves to correspond to its part of the space. The niche of the species is due to its position and the response to the factors within the hyperspace occupied by the community [3].

In 2000s, new approaches to study structure of communities appeared [8, 9]. The neutralism concept actively developed by S.P. Hubbell [10-12] and other researchers [13, 14] was an impulse. S.P. Hubbell had proposed neutral hypothesis subject to which various tree species coexist due to their similarity, rather than due to differences. To achieve stable coexistence, plants shall be similar, to a maximum extent, by its demographic characteristics, shall have similar specific population growth speed and occupation speed of a vacated area. The neutral theory was exemplified in tropic rain forests (Barro Colorado Island, Panama Canal area), where no notable separation of the ecologic niches between tree species exists [15]. It allows for coexistence of the species and does not require their compulsory deviation by different ecologic niches. Species of a single community may coexist due to similarity of their ecologic properties [16]. Herewith, neutrality is expressed at the level of individuals rather than species [13, 14]. According to S.P. Hubbell [11], the principal mechanism of high wealth of species in tropic forests is plant seedling elimination depending on the stand density. Such mechanism is considered as applicable to temperate forests [17, 18]. I. Volkov et al. [19] have mathematically substantiated the Hubbell's model. However, it was assumed [20, 21] that neutrality concept could be applied in particular cases and had no status of a universal theory. There were attempts [23, 24] to integrate neutral and niche mechanisms of species functioning. For instance, P.B. Adler et al. (24) had proposed a model linking the neutrality concept with traditional opinions on the niche structure of the community. The model is based on idea of P. Chesson [25] considering the neutrality as an extreme case of more general theory accounting primarily for the species deviations to different niches.

The past two decades had demonstrated the role of ecological niches in maintenance and preservation of plant biodiversity [26], optimization of the population and cenotic diversity [27] in forest successions [28, 29]. The concept of ecological niches is applicable in phytoindication [30], at differentiation of the ecologicsl niches of grasses in the area of desert wooden plant [31], for maintenance of the species and intra-species population diversity of *Artemisia* genus [32], for stable development of feed production in arid regions [33], in studying invasive plant ecology [34, 35], at estimation of allelopathy of invasive plants [36] and competitiveness of invasive species [34], at assessment of cenotic stress on agrophytocenosis [37], an also at studying population diversity of pine marten [38] and morphological diversity of mammals [39].

Ecologists mostly discuss two types of communities [40], the niche assembled communities, and dispersal assembled communities, at probable appearance of a species at a particular location with further survival, regardless of similarity with neighbors. It is assumed that if species coexist in the same place, they are ecologically close.

An example of the approach corresponding to the traditional concept of ecological niches is the studied herein formation of pastture agricultural ecosystems based on dispersal of phytocenotically balanced mixtures of feeding plants different in ecologic and biological sense in the context of Karnabchul desert. It ensures successive recovery of biodiversity and feeding productivity of the pasture agricultural ecosystems in area of degraded lands. Earlier [33, 41], we have empirically demonstrated that in such conditions the fullest mastering of the fundamental ecological niche and its resources could be reached at modeling by type of the natural zonal biogeocenotical structures. They represent a mixture of ecologically and biologically diversified feeding shrubs, sub-shrubs, suffrutices, and grasses. Usually, integrity of species, ecotypes, and cultivars of fodder crops, which suite to the zonal biogeocenose types [2] and are ecobiologically and phy-

tocenotically compatible, gives synergic effect [41].

Upon use of the external niche resources, L.G. Ramensky [42] had distinguished interoperation of species by type of additions. K.A. Kurkin [43] had developed a principle of ecological niche differentiation by type of complementarities in species during formation of narrow pasture ecosystems. At construction of pasture agricultural ecosystems in the arid regions of the Central Asia and Russia, fluctuation type is important. Optimal productivity of such pastures requires use of fodder plants growing in different conditions but having in the ecosystems the same function, with mutual replacement upon environment changes.

N.T. Nechaeva [44] indicates that in different *Carex* species (*Carex physodes*, *C. subphysoides*, *C. pachystylis*), co-growing in foothill semi-desert Turkmenistan the yields in different years are similar in quality and quantity. Such communities are created by type of ecologically functional aggregations of close species, when two and more species are involved in cenose as an ecologic sum of types, in which such representative of community (specie) is distinguished and which is more adapted to certain environment [45]. Variability of environment factors causes response at biocenotic level. Arid territories require multicomponent mixtures of replaceable fodder plants to stabilize productivity of the ecosystems. Thus, mixtures of *Bassia prostrate*, *Salsola* sp., and *Artemisia* sp. with bulbous bluegrass and ephemeral plants are good on piedmont and foothills in the Central Asia [46].

Seasonal complementary type is based on combination in the pasture agricultural ecosystems of the species, ecotypes, and genus of feeding plants with different growth and development rhythms (long-sustained vegetating, short-term vegetating, and ephemeral phenorhythmotypes). Species vegetating all year around or during its most part may involve feeding shrubs such as *Haloxylon aphyllum*, *Salsola paletzkiana*, *Haloxylon persicum*, *Aellenia subaphylla*, *Ephedra strobilacea*; semi-shrubs — *Kochia prostrata*, *Salsola orientalis*, C*amphorosma lessingii*, *Salsola gemmascens*, and battle sansola as annual species. Short-term vegetating types of feeding plants growing in spring and at beginning of summer involve *Astragalus*, *Calligonum*, dry sansolas, several longstanding grains, ephemeroids (*Poa bulbosa*) and ephemeral plants (*Bromus tectorum*, *Malcolmia grandiflora*, *Eremopyrum orientalis*, *Leptaleum filifolium*, etc.).

Phenological diversity of species promotes longstanding increase in the pasture feed. Combination of ephemeroids of *Poa bulbosa* and *Carex pachystylis* vegetating from February until May, and annual sansola genus (*Climacoptera lanata*, *Gamanthus gamacarpus*, *Halimocnemis villosa*) vegetating from May to the end of November is an example of high seasonal productivity of pastures in piedmont of the Central Asia [47].

Successive complementary type presupposes replacement of less sustainable species by more sustainable that ensures rapid achievement of the maximum productivity by less sustainable species. It is reasonable to select high productive species that are relatively resistant to a set of abiotic stresses. Species forming from the second year of life sufficiently high feeding mass crops in the complex of pasture agricultural ecosystems involve *Kochia prostrata, Camphorosma lessingii, Aellenia subaphylla*, and *Sansola orientalis*. During 3-6 years biomass yield increases, and then during 6-12 years their productivity becomes flat with further stepwise decreasing thereof. In this, *Camphorosma lessingii*, followed by *Kochia prostrata*, *Aellenia subaphylla* and *Sansola orientalis* give place to *Haloxylon* sp., *Salsola paletzkiana*, *Salsola Richteri*, and *Ephedra strobilacea* without any changes in the upper productivity threshold of the agrophytocenose (at least 6-8 c/ha in dry mass). Ephemeral plants and annual *Sansola* sp. may successively replace species of longstanding pasture agricultural ecosystems.

Tier-based plant stand complementarity ensures mastering of various ecological niches and intensified use of eco-resources in arid areas of the Central Asia and Russia [47]. This principle applies at ecologic recovery of degraded landscapes by use of the mixture of various forms and species of fodder plants. Ecologically and biologically diversified species get different ecologic niches during formation of the pasture ecosystems and further can interact temporally and spatially.

Functional differentiation of ecological niches is essential in productivity and sustainability of agro-ecosystems and may become a sufficient reserve for recovery of degraded lands and increase in pasture productivity. Feeding shrubs and semi-shrubs which are unable to produce grassy turf sustainable for pasture are usually used upon creation of the pasture agricultural ecosystems on degraded lands. *Poa bulbosa* and *Carex pachystylis* (or each of these species) may form grassy sod preventing pasture digression. One more example of functional complementation is the use of condensed moisture by xerohalophilous shrubs (*Haloxylon* sp.) at sesquialteral or double maximum hygroscopicity of soil.

Until the middle of XX century, restoration of biodiversity and productivity of desert pastures was deemed impossible. However, such technology was proposed later [41, 46]. There is an opinion that all зкуыуте arid ecosystems are secondary antropogenous [48]. Because of overpasture, burning out, and plowing, they lost certain species, become simplified and cenotically incomplete. The floristic composition of grass stand and horizons of arid plant communities became poorer. In such communities ecological niches remain free, and resources of heat, moisture, and mineral nutrition are not fully used. Thus, the fodder productivity of such communities is always lower compared to floristically full phytocenoses [49].

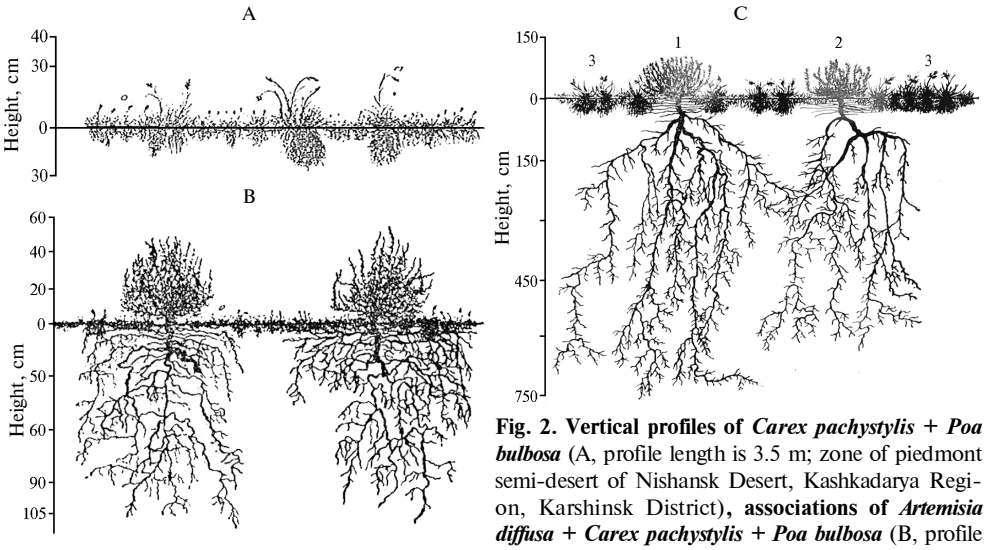

Fig. 2. Vertical profiles of *Carex pachystylis* + *Poa bulbosa* (A, profile length is 3.5 m; zone of piedmont semi-desert of Nishansk Desert, Kashkadarya Region, Karshinsk District)**, associations of *Artemisia diffusa* + *Carex pachystylis* + *Poa bulbosa*** (B, profile length is 3 m; zone of absinthic-ephemeral Karnabchul desert, Samarkand Region, Sovetobadsky District) **and spring-summer suffrutex-grassy ecosystem aged 8 years** (C, profile length 3.5 m; Samarkand Region, Sovetobadsky District): 1 — *Kochia prostrata*, 2 — *Camphorosma lessingii*, 3 — *Poa bulbosa*.

Floristic and cenotic deficiency and availability of unused ecologic niches in the existing ephemeroid and ephemeroid-absinthic pasture ecosystems we showed in our past studies [33, 41]. Ecological niches of ephemeroid sedge-poeceae communities are small (Fig. 2, A), those of ephemeroid-absinthic communities are somewhat larger (see Fig. 2, B). These natural phytocenoses use resources available in small amount in air and soil. Constructed multispecies

and multitier pasture ecosystems with zonal typical fodder shrubs, semi-shrubs and suffrutices which roots deeply penetrate into the soil ensure new ecological niches and consumption of moisture and nutritional reserves from significantly more soil layers (see Fig. 2, C).

In spring-summer suffrutex-grassy ecosystems for southern deserts we used semi-shrub *Eurotia ceratoides*, 25 % + suffrutices *Kochia prostrata*, 30 % + *Camphorosma lessingii*, 25 % + *Poa bulbosa*, 20 %. The pasture ecosystem created on disturbed natural forage lands in Karnabchul Desert consisted of two synusiae. The first one was *Eurotia ceratoides*, *Kochia prostrate*, and *Camphorosma lessingii.* The above-ground heights of the plants were 75-78 cm, 70-75 cm, and 57-60 cm, respectively. The number of generative and vegetative shoots per shrub was up to 27 and 38 for *Eurotia ceratoides*, and up to 24 to 41 for *Kochia prostrate*, with of generative and vegetative shoot length of 50-75 cm and 10-24 cm, respectively. In *Camphorosma lessingii*, shoot number was much lower — 38-42, of which 9-11 shoots were generative (32-48 cm), and the remaining ones were vegetative (5-12 cm). Suffrutices aged 6 years had formed powerful, deeply penetrating root system. Roots of *Kochia prostrata* reached a depth of 750 cm and were spread horizontally to 355 cm. The depth and width of roots of *Camphorosma lessingii* were 700 cm and 250 cm, respectively (see Fig. 2, C).

Ephemeroids *Poa bulbosa*, *Carex pachystylis*, together with ephemeral plants *Bromus tectorum*, *Boissiera purnilio* and *Trigonella grandiflora* were the grassy synusia of the suffrutex-grassy pasture ecosystem. The height of plants averaged 18-20 cm. The depth of *Poa bulbosa*, *Bromus tectorum*, and *Boissiera purnilio* roots reached up to 30 cm, with some root depth of 45 cm. *Poa bulbosa* and *Carex pachystylis* plants formed a dense grassy sod. The roots of the synusia main occupied the 0-20 cm soil layer.

In the *Kochia—Camphorosma* pasture ecosystem aged 3 years ephemeral plants became numerous. They appeared in the spring and summer due to germination of the seeds from the soil and those brought from outside. After seeding *Kochia prostrata* and *Camphorosma lessingii*, single *Bromus tectorum* and *Boissiera purnilio* plants were found in the first and second years, and they grew in number starting from the third year of life. A total of 17 ephemeral species of 7 families were in the ecosystem. They were mainly represented by grasses (7-10 species), crucifers (2-4 species), and leguminous (2-4 species) plants. Nutritionally valuable ephemeroids, ephemeral plants, etc. (12-17 species), including *Bromus tectorum*, *Boissiera pumilio*, *Poa bulbosa*, *Malcolmia turkestanica* and *Trigonella grandiilora* which can form the base of the pasture grassy layer were insignificant in number.

Starting from the 2-3 year of life and in the next 6 years, fodder productivity of the ecosystem increased (Fig. 3), with the maximum at years 5-6. Sharp increase of the feeding mass happened from year 2 when pasture agro-ecosystem is recommended for use.

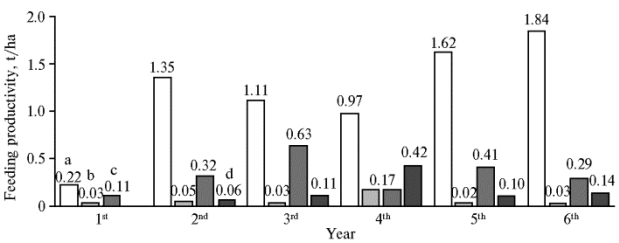

Fig. 3. Feeding productivity of spring-summertime suffrutex-grassy pasture ecosystem: a — *Kochia prostrata*, b — *Eurotia ceratoides*, c — *Camphorosma lessingii*, d — *Poa bulbosa* (Karnabchul Desert, Samarkand Region, Sovetobadsky District, 2002-2007).

Characteristic feature of the created spring-summer pasture ecosystems is rapid growing during spring. By the mid-April, from 0.50 to 0.65 t/ha of dry

feeding mass were accumulated depending on the meteorological conditions. Productivity of the natural pastures by this period did not exceed 0.01 t/ha for dry matter in dry years, and 0.3 t/ha in favorable wet periods. By the mid-May ecosystem accumulated from 50 to 80 % of the yield at the end of summer. Intensive growth of *Kochia prostrata* feeding mass was mainly by the end of June (60-80 %) followed by slowing down. Until the mid-summer (end of June) green biomass of *Camphorosma lessingii* was slowly growing with further notable growth. Such complementation ensures a uniform distribution of herbaceous plants in spring-summer pasture ecosystems, which allows animals to eat well

Shrub *Haloxylon aphyllum*, 15 % + semi-shrub *Aellenia subaphylla,* 20 % + suffrutices *Artemisia diffusa*, 35 % + *Salsola orientalis,* 30 % were used in fall-winter shrub-grassy pasture ecosystems. This agricultural ecosystem consisted of five synusiae different in aboveground and underground spheres (Fig. 4).

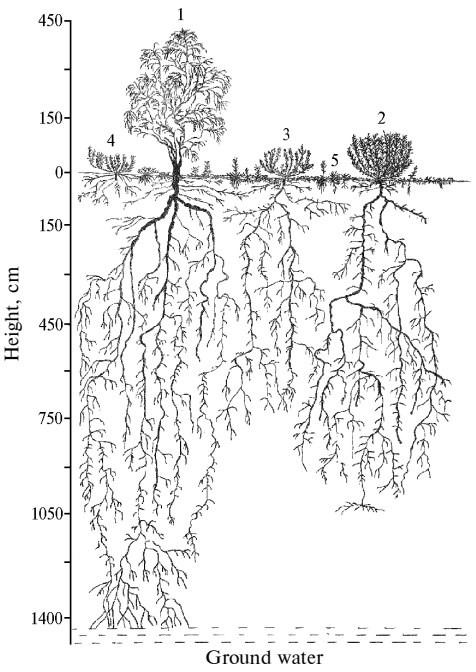

Fig. 4. Vertical profile of fall-winter pasture shrub-suffrutex-grassy ecosystem aged 7 years: 1 — *Haloxylon aphyllum*, 2 — *Halothamnus subaphylla*, 3 — *Salsola orientalis*, 4 — *Artemisia diffusa*, 5 — *Ephemeretum* (profile length 4.5 m; Karnabchul Desert, Samarkand Region, Sovetobadsky District).

At age of 7 years, *Haloxylon aphyllum* of the first synusia, had reached 400-450 cm in height with head diameter of 320-350 cm. Powerful root system penetrated into the soil up to 14 m and spread horizontally, depending on the mechanical composition of soil layers, up to 2.5-5.0 meters. Roots of *Haloxylon* sp. reaching the soil layers of capillary wetting by ground waters (at depth of 10-12 m), formed numerous small roots. Height of *Aellenia subaphylla* plants of the second synusia was 120-140 cm. Rooting system was powerful and penetrated into soil up to 750 cm. In synusia of *Salsola orientalis* the shrub height and width were 50-60 cm and 40-45 cm, respectively. Dimensions of its roots were up to 700 cm in depth and up to 200 cm in the horizontal direction. *Artemisia diffusa* formed the fourth synusia with height of plants of 35-40 cm at diameter of bushes of 40-45 cm. Depth of the roots of *Artemisia diffusa* was up to 110 cm, size in the horizontal direction was up to 150 cm. Ephemeroid *Poa bulbosa* and ephemeral plants *Bromus tectorum*, *Malcolmia turkestanica* prevailed in the fifth synusia. Their height was 20-25 cm. Roots of *Poa bulbosa* and *Bromus tectorum* are filaceous and form dense grassy sod at soil surface (0-5 cm). Main part of roots (up to 85 %) was at the 0-15 cm soil layer.

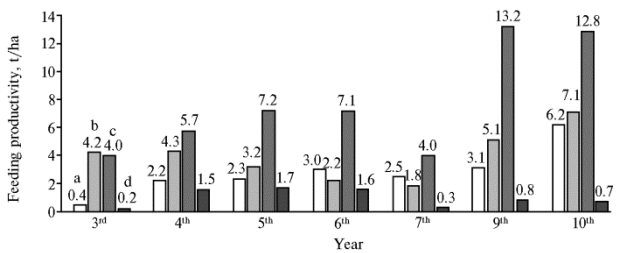

Fig. 5. Pasture productivity of fall-winter shrub-suffrutex-grassy ecosystem: a — *Haloxylon aphyllum*, b — *Aellenia subaphylla,* c — *Salsola orientalis*, d —*Artemisia diffusa* (Karnabchul Desert, Samarkand Region, Sovetobadsky District, 2004-2011).

Created pasture ecosystem of fall-winter use had high and stable feeding productivity (Fig. 5), which as early as within 3 years was twice higher than in the natural desert-like pastures (0.3 t/ha). Feeding productivity growth was sustainable until 9-10 years of life due to *Haloxylon aphyllum*, *Sansola subaphylla*), suffruticulose (*Salsola orientalis, Artemisia diffusa* typical for the Karnabchul Desert with participation of the naturally growing *Poa bulbosa*. Maximum yield of the fall-winter pasture ecosystem was at the age of 9-10 years (2.2-2.6 t/ha of dry matter). The portion of fodder plants in total yield of the ecosystem depended on the species and living forms of the plants. The maximum yield was from *Salsola orientalis* (11.6-59.4 % depending on the plant age and meteorological conditions of the year). Yield of *Aellenia subaphylla* and *Artemisia halophila* significantly varied.

Aellenia subaphylla and *Salsola orientalis* can rapidly accumulate green mass during the first years of life (2-4 years), while *Haloxylon aphyllum* displays this ability significantly slower. Depending on the age and meteorological conditions, *Haloxylon aphyllum* made 3.6 to 28.1 % of yield of the pasture ecosystem. Presence *Salsola orientalis*, *Artemisia halophila* and *Aellenia subaphylla* makes the pastures excellent for grazing sheep in fall-winter period. In its turn, *Haloxylon aphyllum* not only gives enough fodder for animals during fall and winter, but also protects them from strong winds at low winter temperatures.

Therefore, the ecological niche concept is important for improvement of the theoretical principles and methods to adaptively construct floristically and cenotically complete pasture agro-ecosystems in arid areas of the Central Asia. Accelerated ecological reclamation by creation of arid pasture ecosystems which are based on staged, seasonal, successive, functional, and fluctuation differentiation of the ecologic niches ensures recovery of biodiversity and growth of feed productivity. We have realized this approach in the Central-Asian region by seeding phytocenotically balanced combinations of zonally typical dominants of the natural flora which are the most ecologically suitable for arid conditions of the region. Pasture suffrutex-grassy agro-ecosystems for spring-summer period have high and stable productivity and may be used as pastures for sheep, meat cattle, and camels. Fall-winter agro-ecosystems consist of shrubs, semi-shrubs, suffrutices and grasses, occupy various ecological niches in the aboveground and underground (edaphic) spheres, complement each other by important ecological, biological and economic characteristics, and effectively use environmental resources. It allows us to restore biodiversity, to increase yielding on low-productive territories and badlands, to optimize arid biocenoses of the Central-Asian region. All these will ensure sustainability of the pasture livestock sector.

REFERENCES

1. Odum Yu. *Ekologiya* [Ecology]. Moscow, 1986 (in Russ.).
2. Pianka E. *Evolyutsionnaya ekologiya* [Evolutionary ecology]. Moscow, 1981 (in Russ.).
3. Gilyarov A.M. *Uspekhi sovremennoi biologii*, 1978, 85(3): 431-446 (in Russ.).
4. Elton CH. *Ekologiya nashestviya zhivotnykh i rastenii* [Ecology of animal and plant invasion]. Moscow, 1960 (in Russ.).
5. Gauze G.F. *Zoologicheskii zhurnal*, 1935, 14(2): 243-270 (in Russ).
6. Hutchinson G.E. Soncludingn remarks. *Cold Spring Harbor Symposia on Quantitative Biology*, 1957, 22: 415-427.
7. Soldbrig O., Soldbrig D. *Populyatsionnaya biologiya i evolyutsiya* [Population biology and evolution]. Moscow, 1982 (in Russ).
8. McGill B.J. A renaissance in the study of abundance. *Science*, 2006, 314: 770-772 (doi: 10.1126/science.1134920).
9. McGill B.J., Etienne R.S., Gray J.S., Alonso D., Anderson M.J., Benecha H.K., Dornelas M., Enquist B.J., Green J.L., He F., Hurlbert A.H., Magurran A.E., Marquet P.A., Maurer B.A., Ostling A., Soykan C.U., Ugland K.I., White E.P. Species abundance distributions: moving be-

yond single prediction theories to integration within an ecological framework. *Ecol. Lett.*, 2007, 10: 995-1015 (doi: 10.1111/j.1461-0248.2007.01094.x).
10. Hubbell S.P. *The unified neutral theory of biodiversity and biogeography*. Princeton and Oxford, Princeton University Press, 2001.
11. Hubbell S.P. Neutral theory in community ecology and the hypothesis of functional equivalence. *Funct. Ecol.*, 2005, 19: 166-172 (doi: 10.1111/j.0269-8463.2005.00965.x).
12. Hubbell S.P., Foster R.B. Biology, chance, and history and the structure of tropical rain forest tree communities. In: *Community ecology*. J.M. Diamond, T.J Case (eds.). Harper and Row, NY, 1986: 314-329.
13. Bell G. Neutral macroecology. *Science*, 2001, 293: 2413-2418 (doi: 10.1126/science.293.5539.2413).
14. Whitfield J. Ecology: neutrality versus the niche. *Nature*, 2002, 417: 480-481 (doi: 10.1038/417480a).
15. Ricklefs R.E. Naturalists, natural history, and the nature of biological diversity. *The American Naturalist*, 2012, 179(4): 423-435 (doi: 10.1086/664622).
16. Shipley B., Paine C.E.T., Baroloto C. Quantifying the importance of local niche-based and stochastic processes to tropical tree community assembly. *Ecology*, 2012, 93(4): 760-769 (doi: 10.1890/11-0944.1).
17. Hille Ris Lambers J.H., Clark J.S., Beckage B. Density-dependent mortality and the latitudinal gradient in species diversity. *Nature*, 2002, 417, 6890: 732-735 (doi: 10.1038/nature00809).
18. ter Steege H., Zagt R. Density and diversity. *Nature*, 2002, 417, 6890: 698-699 (doi: 10.1038/417698a).
19. Volkov I., Banavar J.R., Hubbell S.P., Maritan A. Neutral theory and relative species abundance in ecology. *Nature*, 2003, 424: 1035-1037 (doi: 10.1038/nature01883).
20. Adler P.B. Neutral models fail to reproduce observed species-area and species-time relationships in Kansas grasslands. *Ecology*, 2004, 85: 1265-1272 (doi: 10.1890/03-0602).
21. Harpole W.S., Tilman D. Non-neutral patterns of species abundance in grassland communities. *Ecol. Lett.*, 2006, 9: 15-23 (doi: 10.1111/j.1461-0248.2005.00836.x).
22. Clark J.S. Beyond neutral science. *Trends Ecol. Evol.*, 2009, 24: 8-15 (doi: 10.1016/j.tree.2008.09.004).
23. Gravel D., Canham D., Beaudet M., Messier C. Reconciling niche and neutrality: the continuum hypothesis. *Ecol. Lett.*, 2006, 9: 399-409 (doi: 10.1111/j.1461-0248.2006.00884.x).
24. Adler P.B., Hille Ris Lambers J., Levine J.M. A niche for neutrality. *Ecol. Lett.*, 2007, 10: 95-104 (doi: 10.1111/j.1461-0248.2006.00996.x).
25. Chesson P. Mechanisms of maintenance of species diversity. *Annu. Rev. Ecol. Syst.*, 2000, 31: 343-366 (doi: 10.1146/annurev.ecolsys.31.1.343).
26. Pavlov D.S., Striganova B.R., Bukvareva E.N., Dgebuadze Yu.Yu. *Vestnik RAN*, 2010, 80(2): 131-140 (in Russ).
27. Bukvareva E.N., Aleshchenko G.M. *Uspekhi sovremennoi biologii*, 2012, 132(4): 337-353 (in Russ).
28. Komarov A.S., Zubkova E.V. *Matematicheskaya biologiya i bioinformatika*, 2012, 7(1): 152-161 (in Russ).
29. Zubkova E.V. *Dinamika raspredelenii ekologicheskikh nish rastenii pri suktsessiyakh lesnykh soobshchestv. Avtorefat kandidatskoi. dissertatsii* [Dynamics of ecological niche distribution under succession in forest plant communities. PhD Thesis]. Kazan', 2013 (in Russ.).
30. Seledets V.P. *Botanicheskii zhurnal*, 2013, 98(1): 25-40 (in Russ.).
31. Shamsutdinov Z.Sh., Ubaidullaev Sh.R., Blagorazumova M.V., Shamsutdinova E.Z., Nasiev B.N. *Aridnye ekosistemy*, 2013, 19(4/57): 5-13 (in Russ.).
32. Dorokhina L.N., Geguchadze E.S. *Uspekhi sovremennogo estestvoznaniya*, 2006, 4: 36-37 (in Russ.).
33. Shamsutdinov Z.Sh., Shamsutdinova E.Z. L.G. Ramenskii theory about types of vital strategies and its importance for development of arid forage resources. *Agricultural Biology*, 2011, 2: 32-40.
34. Mack R.N. Biotic invasions: causes, epidemiology, global consequences and control. *Ecol. Appl.*, 2000, 10: 689-710 (doi: 10.1890/1051-0761(2000)010%5B0689:BICEGC%5D2.0.CO;2).
35. Jakobs G., Weber E., Edwards P.J. Introduced plants of the invasive *Solidago gigantea* (*Asteraceae*) are larger and grow denser than conspecifics in the native range. *Diversity and Distributions*, 2004, 10: 11-19 (doi: 10.1111/j.1472-4642.2004.00052.x).
36. Callaway P.M., Aschchoug E.T. Invasive plant versus their new and old neighbors: a mechanism for exotic invasion. *Science*, 2000, 290: 521-523 (doi: 10.1126/science.290.5491.521).
37. Chumanova N.N., Grebennikova V.V., Kondaurova I.G. *Vestnik Kemerovskogo gosudarstvennogo universitet*a, 2015, 3(4/64): 116-120 (in Russ.).
38. Puzachenko Yu.G., Zheltukhin A.S., Sandlerskii R.B. *Zhurnal obshchei biologii*, 2010, 71(6): 467-487 (in Russ.).
39. Korablev M.P., Korablev N.P., Korablev P.N., Tumanov I.L. *Vestnik okhotovedeniya*, 2014, 11(2): 110-115 (in Russ.).
40. Gilyarov A.I. *Zhurnal obshchei biologii*, 2010, 71(5): 386-401 (in Russ.).
41. Shamsutdinov Z.Sh., Shamsutdinov N.Z. *Aridnye ekosistemy*, 2012, 18(3/52): 5-21 (in Russ.).

42. Ramenskii A.G. *Vvedenie v kompleksnoe pochvenno-geobotanicheskoe obsledovanie zemel'* [Introduction to the complex soil-geobotanical survey of lands]. Moscow, 1938 (in Russ.).
43. Kurkin K.A. *Byul. MOIP. Otd. biol.*, 1983, 8(4): 3-14 (in Russ.).
44. Nechaeva N.T., Shamsutdinov Z.Sh. V sbornike: *Problemy antropogennoi dinamiki biogeotsenozov (Chteniya pamyati akademika V.N. Sukacheva)* [In: Anthropogenic dynamics of biocoenoses — V.N. Sukachev Memorial Readings]. Moscow, 1990: 31-53 (in Russ.).
45. Zaletaev V.S. *Zhizn' v pustyne. Geografo-botanicheskie i ekologicheskie problem* [Life in the desert. Geographical and botanical and ecological aspects]. Moscow, 1976 (in Russ.).
46. Shamsutdinov Z.Sh., Shamsutdinov N.Z. Biogeocenotic principles and methods of degraded pastures phytomelioration in Central Asia and Russia. In: *Prospects for saline agriculture*. Netherlands, 2002: 29-35.
47. Shamsutdinov Z.Sh., Shamsutdinov N.Z. Halophytes utilization for biodiversity and productivity of degraded pastures restoration in arid region of Central Asia and Russia. In: *Biosaline agriculture and high salinity tolerance*. C. Abdelly, M. Öztürk, M. Ashraf, C. Grignon (eds.). Switzerland, Birkhauser Verlag, 2008: 293-240 (doi: 10.1007/978-3-7643-8554-5_21).
48. Rodin L.E. Produktivnost' pustynnykh soobshchestv. V knige: *Resursy biosfery* [In: Biosphere resources. Iss. 1]. Leningrad, 1975, vypusk 1: 128-166 (in Russ.).
49. Rabotnov T.A. *Fitotsenologiya* [Phytocenology]. Moscow, 1983 (in Russ.).

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GENETIC DIFFERENTIATION OF UKRANIAN CHICKEN BREEDS USING VARIOUS TYPES OF MOLECULAR GENETIC MARKERS

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Abstract

Modern poultry breeding is aimed towards maximizing productive performance and genetic potential of chicken breeds and lines used for different purposes in order to obtain the greatest profit. Prevalence of foreign highly productive commercial chicken lines and crosses is determined by several factors, the most important of which are the high productivity of chicken lines, as well as the lack of support and ineffective implementation of programs targeted to genetic conservation of native breeds. Preferences given to highly productive chicken breeds in breeding and poultry farming also have negative effects which manifest in a reduced genetic diversity due to narrow specialization of selected breeds and lead to the reduction of national genetic resources. The study of genetically determined features of different chicken breeds is one of the priority tasks of the gene pool conservation problem. In this study, we used two types of molecular genetic markers, PCR-RFLP and Indel, to investigate the genetic differentiation of Ukrainian chicken breeds in comparative aspect based on polymorphism of different functional genes whose allelic variants are associated with productive traits. The Ukrainian chicken breeds for different primary use, i.e. Borkovskaya Barvistaya line A, Plymouth Rock White line G-2, Poltava clay line 14 and Rhode Island Red line 38, were compared. Genetic differentiation of the chicken populations was performed by analyzing frequencies of alleles in polymorphic loci of prolactin gene (*PRL*), growth hormone gene (*GH*), insulin-like growth factor I gene (*IGF-I*), gene family of transforming growth factors β (*TGF-β1*, *TGF-β2* and *TGF-β3*), pituitary transcription factor-1 gene (*PIT-1*) and Mx gene (*Mx*). For generalized estimation of breed diversity, the genetic distances were calculated based on the studied polymorphic loci for both PCR-RFLP and Indel markers. The most genetically distant breeds were Borkovskaya Barvistaya and Rhode Island Red (24.9 % of the differences). In general, the largest differences can be noted between the egg-lying and dual-purpose chicken breeds. In this, the allelic differences with the lines used for both eggs and meat were most pronounced (23-25 %). Differences between the breeds of dual use, i.e. primary for meat and eggs or for eggs and meat, were not expressed enough. Maximum differences were between populations of Poltava clay and Plymouth Rock White chicken (11.2 %), while minimum differences were between Rhode Island Red and White Plymouth Rock chicken (4.2 %). In turn, the genetic distance between the two egg-meat breeds studied was intermediate compared to the above-mentioned (7.1 % difference). The pattern of phylogenetic tree corresponds to the previously described regularities and reflects differentiation of the chicken lines by their primary use. As follows from the dendrogram, the chickens of egg-meat primary use form a separate cluster. At the same time, meat-egg and egg-lying chickens form separate branches, while the egg-lying breed shows the greatest genetic differences compared to the other lines.

Keywords: polymorphism, allele, population, chicken, genetic distances, egg chicken breeds, dual-purpose chicken breeds

Modern poultry breeding is aimed towards maximizing the potential use of chicken breeds and lines used for different purpose in order to obtain the greatest profit from sale of poultry. Worldwide spread of foreign highly productive commercial chicken crosses and lines depend on the several factors, the most important of which is high productivity values in poultry, as well as lack of

support and effective realization of the programs for conservation of genetic sources of the domestic breeds in general. Endless hurry for profit may often literally result in extermination of the breeds in creation of which decades of work were spent by the national geneticists and animal breeders. The key concept of modern poultry breeding industry is effectiveness that is expressed in the growing values of poultry yield [1]. However breeding of highly productive poultry may also have negative effects manifested in reduced genetic diversity because of narrow specialization of breeds and lines, leading to a decrease in national genetic resources [2, 3]. Lower interest in various genetic resources endangers their existence in general and may lead to loss of the unique genetic properties, which are alien to modern industrial poultry and which are characterizing local breeding groups in particular [4, 5].

At beginning of 1990s, the Poultry Breeding Institute at the Ukrainian Academy of Agrarian Sciences had tenths of agricultural poultry breeds and lines used for different purpose, including rare breeds — Yurlovo Crower, Italian partridge, bare-necked chicken breeds, mini chickens, etc. [6]. Today, the State Trial Poultry Breeding Station (successor of the above-named poultry breeding institute) has the limited number of the Ukrainian chicken breeds represented by only several lines. The most spread representatives of such "genetic core" are breeds of egg laying chickens — Borkovskaya Barvistaya (line A), meat-egg chickens — Plymouth Rock White (line G-2), dual-purpose chickens — Poltava clay (line 14), and Rhode Island Red (line 38 and line 02). Recently, the above-listed chicken breeds lack the importance for the industrial poultry breeding and are sold only for the needs of small farming units, which is to the most extent defined by good adaptive properties of the Ukrainian chicken upon keeping them at the courtyard. Lack of the expressed state support and interest of the large poultry producers in Ukraine endanger the existence of the genetic breeds in general, which, in case of their extermination, would result in permanent loss of the unique genetic material adapted to keeping conditions in relevant geographic zone. Use of genetic properties of various poultry breeds refers to priority genetic conservation issues [7]. Therefore, analysis of specific properties of the genetic structure of the Ukrainian chicken populations (along with genetic conservation in general) becomes the paramount task for the Ukrainian poultry breeding industry.

We have already studied genetic and population parameters of trial chicken lines. In this publication we for the first time put an emphasis on the genetic differentiation of selected Ukrainian populations in the comparable aspect based on data on polymorphism of various functional genes, allele variants of which are associated with emergence of the economically useful traits.

Purpose of the present study is molecular and genetic differentiation of the Ukrainian chicken breeds.

Technique. The studies of Ukrainian trial chicken populations including Borkovskaya Barvistaya (line A) egg-laying chickens, Plymouth Rock White (line G-2) meat-egg chickens, and Poltava clay (line 14) and Rhode Island Red (line 38) dual purpose chickens were carried out from 2011 to 2015.

Polymorphism of target genes by PCR-RFLP and Indel markers was studied. These were 57 bps insertion in intron 2 of *PIT-1* (gene of pituitary transcription factor-1); 24 bps insertion in promoter area and transition of cytosine to thiamin in position –2402 of *PRL* (prolactin gene); MspI polymorphism in intron 1 and intron 4, and SacI and AluI polymorphisms in intron 4 of *GH* (growth hormone gene); HinfI polymorphism in promoter area and PstI polymorphism in 5′UTR region of *IGF-I* (gene of insulin-like growth factor I); MboII polymorphism of *TGF-β1* (gene of transformation growth factor β1) exon

area; RsaI polymorphism of *TGF-β2* (gene of transformation growth factor β2) promoter area; BslI polymorphism in intron 4 of *TGF-β3* (gene of transformation growth factor β3); RsaI polymorphism in exon 13 of *Mx* (gene Mx).

The primers, protocols, and restriction enzymes were as described [8-15].

Amplification was done with the use of DreamTaq PCR Master Mix reagents (Thermo Scientific, USA) and a programmed thermal cycler TherCyc (DNA Technology, Russia) as per the protocol: denaturation for 5 min at 94 °C (1 cycle); denaturation for 1 min at 94 °C, annealing for 1 min at the temperature specific for each locus, elongation for 1 min at 72 °C (35 cycles); final elongation for 10 min at 72 °C (1 cycle). The final mixture volume was 20 μl, and concentration of primers was 0.2 μM. Genotyping was based on electrophoretic analysis.

Polymorphic allele frequency was determined by maximum likelihood formulas [16]. Based on the obtained data, the Nei genetic distances and Wright F-statistics were calculated by common methods with the use of Popgen32 software (https://sites.ualberta.ca/~fyeh/popgene_do-wnload.html). Divergence degree between the populations was determined based on F_{st}, with F_{st} of 0.00-0.05 for poor divergence, of 0.06-0.15 for medium divergence, of 0.16-0.25 for high divergence, and of > 0.25 for ultrahigh divergence [17]. Philogenetic tree was plotted using PHILIP 3.69 (http://evolution.gs.washington.edu/phylip/getmenew1.html) and MEGA 7 (https://www.megasoftware.net/download_form) softwares. Validity of allele frequency values and confidence limits of their diversity were determined by statistical error and t-test [16]. The differences were statistically significant at $p < 0.05$.

Results. Use of PCR method and restriction analysis enabled us to determine polymorphous variants of the selected genes in the Ukrainian chicken breeds. Structure of the primers, relevant restriction enzymes, as well as relative sizes of the amplification and restriction products are provided in table 1 below.

1. Nucleotide sequences of primers, relevant restriction enzymes, and relative sizes of the amplification/restriction products

Locus	Nucleotide sequences of primers (references)	Annealing	Restriction endonuclease	Amplification/restriction products, bps
PIT-1 (intron 2)	gtcaaggcaaatattctgtacc; tgcatgttaatttggctctg [8]	58 °C		I — 387; D — 330
PRL (promoter)	tttaatattgtgggtgaagagaca; atgccactgatcctcgaaaactc [9]	54 °C		I — 154; D — 130
PRL (C-2402T)	agaggcagcccaggcattttac; cctgggtctggtttggaaattg [9]	62 °C	AluI	C — 160/144/81/54; T — 304/81/54
GH (intron 1)	atccccaggcaaacatcctc; cctcgacatccagctcacat [10]	55 °C	MspI	A — 539/237; B — 392/237/147; C — 267/237/147/125
GH (intron 4)	ctaaaggacctggaagaaggg; aacttgtcgtaggtgggtctg [10]	61 °C	MspI	A — 1200; B — 600/600; C — 500/700
GH (intron 4)	ctaaaggacctggaagaaggg; aacttgtcgtaggtgggtctg [10]	61 °C	SacI	A —584/440/144; B — 1024/144
GH (intron 4)	ctgagggacgtggttatgggcac; gacctcaaggattgcagggct [11]	63 °C	AluI	C — 167/293; T — 108/185/167
IGF-1 (promoter)	cattgcgcaggctctatctg; tcaagagaagcccttca [12]	55 °C	HinfI	C —622/191; A — 378/244/191
IGF-1 (5′UTR)	gactatacagaaagaaccac; tatcactcaagtggctcaagt [13]	53 °C	PstI	C_1 — 621; C_2 — 257/364
TGF-β1 (exon)	ggggtcttcaagctgagcgt; ttggcaatgctctgcatgtc [14]	65 °C	MboII	B — 173/67; F — 240
TGF-β2 (promoter)	gccataggttcagtgcaag; tgacagaagctctcaagcc [14]	52 °C	RsaI	B — 100/184; L — 284
TGF-β3 (intron 4)	tcagggcaggtagagggtgt; gccactggcaggattctcac [14]	64 °C	BslI	B — 125/75/74/20; L — 145/75/74
Mx (exon 13)	ccttcagcctgtttttctcctttaggaa; cagaggaatctgattgctcaggcgtgta [15]	60 °C	RsaI	A — 100; G — 73/27

Table 2 shows allele frequencyies of the studied loci in the lines.

2. Allele frequencies of the studied loci in the populations of Ukrainian chicken breeds

Locus, restriction endonuclease	Breeds			
	Plymouth Rock White	Borkovskaya Barvistaya	Poltava clay	Rhode Island Red
PRL	0,135 (I)^{a};	0,710 (I)^{b};	0 (I)^{c};	0,060 (I)^{d};
24 Indel	0,865 (D)^{a}	0,290 (D)^{b}	1 (D)^{c}	0,940 (D)^{d}
PRL	0,155 (C)^{a};	0,710 (C)^{b};	0,372 (C)^{c};	0,140 (C)^{ad};
C-2402T	0,845 (T)^{a}	0,290 (T)^{b}	0,628 (T)^{c}	0,860 (T)^{ad}
GH	0,435 (A)^{a};	0,650 (A)^{b};	0,908 (A)^{c};	0,390 (A)^{ad};
intron 1	0,395 (B)^{a};	0,270 (B)^{b};	0,020 (B)^{c};	0,130 (B)^{d};
MspI	0,170 (C)^{a}	0,080 (C)^{b}	0,072 (C)^{bc}	0,480 (C)^{d}
GH	0,560 (A)^{a};	0,750 (A)^{b};	0,100 (A)^{c};	0,270 (A)^{d};
intron 4	0,160 (B)^{a};	0,080 (B)^{b};	0,070 (B)^{bc};	0,310 (B)^{d};
MspI	0,280 (C)^{a}	0,170 (C)^{b}	0,830 (C)^{c}	0,420 (C)^{d}
GH	0,030 (A)^{a};	0,550 (A)^{b};	0,036 (A)ac;	0,110 (A)^{d};
intron 4	0,970 (B)^{a}	0,450 (B)^{b}	0,964 (B)^{ac}	0,890 (B)^{d}
SacI				
GH	0,140 (C)^{a};	0,080 (C)^{ab};	0,040 (C)^{bc};	0,300 (C)^{d};
intron 4	0,860 (T)^{a}	0,920 (T)^{ab}	0,960 (T)^{bc}	0,700 (T)^{d}
AluI				
IGF-I	0,180 (C_1)^{a};	0,270 (C_1)^{b};	0,380 (C_1)^{c};	0,350 (C_1)^{bcd};
PstI	0,820 (C_2)^{a}	0,730 (C_2)^{b}	0,620 (C_2)^{c}	0,650 (C_2)^{bcd}
IGF-I	0,680 (A)^{a};	0,270 (A)^{b};	0,290 (A)^{bc};	0,420 (A)^{d};
HinfI	0,320 (C)^{a}	0,730 (C)^{b}	0,710 (C)^{bc}	0,580 (C)^{d}
TGF-β1	0,210 (B)^{a};	0,540 (B)^{b};	0,310 (B)^{c};	0,150 (B)^{ad};
	0,790 (F)^{a}	0,460 (F)^{b}	0,690 (F)^{c}	0,850 (F)^{ad}
TGF-β2	0,460 (B)^{a};	0,600 (B)^{b};	0,790 (B)^{c};	0,610 (B)^{bd};
	0,540 (L)^{a}	0,400 (L)^{b}	0,210 (L)^{c}	0,390 (L)^{bd}
TGF-β3	0,240 (B)^{a};	0,170 (B)^{ab};	0,520 (B)^{c};	0,330 (B)^{d};
	0,760 (L)^{a}	0,830 (L)^{ab}	0,480 (L)^{c}	0,670 (L)^{d}
Mx	0,210 (A)^{a};	0,375 (A)^{b};	0,140 (A)^{ac};	0,125 (A)^{cd};
	0,790 (G)^{a}	0,625 (G)^{b}	0,860 (G)^{ac}	0,875 (G)^{cd}
PIT-1	0,520 (I)^{a};	0,360 (I)^{b};	0,630 (I)^{c};	0,650 (I)^{cd};
	0,480 (D)^{a}	0,640 (D)^{b}	0,370 (D)^{c}	0,350 (D)^{cd}

Note. Sizes of amplification/restriction products for each studied locus (A, B, C, C_1, C_2, D, F, G, I, L, T) are provided in table 1; different letters in the upper index (a, b, c, d) signify the statistically significant differences ($p < 0.05$) within the locus limit.

Insertion in the promoter of prolactin gene indicates the expressed prevailing of allele I in the population of egg-laying chickens. The dual-purpose chickens (both egg and meat, and meat-egg) show prevailing of allele D, whereas in Poltava chickens this locus is monomorphic, i.e. the population entirely consists of individuals with DD genotype. Distribution of alleles for C-2402T mutation in *PRL* locus was somewhat similar. Thus, frequencies of C and T alleles in the population of egg-laying chickens were similar to those for I and D alleles. This interesting phenomenon is due to practically absolute prevalence of IC haplotype over IT haplotype, and DT haplotype over DC haplotype in the said population. Herewith, such trend was not observed in other populations. We assume that prevalence of IC haplotypes in egg-laying chickens directly reflects the effect of performed selection. Such assumption is confirmed by many authors who report on the relationship of I and C alleles with egg yield in chickens of various breeds that, in its turn, correlates with results of our studies [9, 18, 19]. Note, for this mutation, unlike the above-described, prolactin locus was polymorphic in the Poltava chicken population. Herewith, C allele frequency in line 14 was the highest for the dual-purpose chicken populations. In its turn, lines G-2 and 38 were practically identical by proportion of C and T allele frequencies.

Chicken lines for different primary use significantly differ in MspI polymorphism in intron 1 of growth hormone gene. Thus, Rhode Island Red line was characterized by prevalence of C allele (0.480), while its frequency was small in other populations. It should be noted that the smallest C allele frequency was in Poltava clay line (0.072), which just like Rhode Island Red line refers to dual-

purpose type. Possibly, the interbreed differences were more important than productivity types. At the same time, Poltava chickens were characterized by the highest A allele frequency (0.908) and the smallest B allele frequency (0.020) as compared to other studied lines. At that, only A allele was found homozygous in the said population. According to results of foreign authors, C allele is completely absent in the commercial lines of egg-laying chickens (Hy-Line), whereas its frequency in the native populations expressly varies [20]. Chicken lines of different primary use also significantly differ in allele frequencies on MspI polymorphism of intron 4 of growth hormone gene. Thus, no BB homozygotes were found in the Poltava clay and Borkovskaya Barvistaya populations. This results in low frequency of B allele. Herewith, Poltava clay line has the highest frequency of CC genotypes and, accordingly, the highest frequency of C allele. The highest frequency of allele A was in egg-laying chickens, and the smallest one was in dual-purpose chickens (line 14). Line 38 has the highest frequency of allele B. Egg-laying chickens expressly differed from other birds by SacI polymorphism of intron 4 of growth hormone gene due to prevalence of allele A (0.550). In other populations allele A was significantly less frequent, from 0.030 to 0.110, and found only in heterozygotes. Differences between lines for AluI polymorphism in intron 4 of growth hormone gene are sufficiently smoothed, except for the Rhode Island Red population with the highest allele C frequency. Allele T is expressly dominating in other populations.

Allele C_2 prevails for insulin-like growth factor I locus in all populations studied. This is mostly expressed in line G-2 of Plymouth Rock White (only one bird homozygous for allele C_1 was found). The closest allele frequencies were in chickens used for egg and meat. Intermediate position is characteristic of egg-laying chickens. Foreign researchers report that commercial meat chicken crosses have a significant prevalence of allele C_1 (for Cobb 500, C_1 frequency is 0.84) or sufficiently close ratio of allele frequencies (for Hubbard, C_1 frequency is 0.42) [21]. Other studies show high egg yield of C_2C_2 individuals compared to C_1C_1 ones in populations of the native Korean and Chinese breeds [22, 23]. The dual-purpose chicken lines have the highest differences from other studied populations by HinfI polymorphism of insulin-like growth factor I gene promoter area. This population was characterized by prevalence of AA homozygotes, which results in prevalence of relevant allele. The other populations did not practically differ from each other. These data correlate with foreign study results showing association of allele A in poultry with meat properties [12, 24]. In fact, dual-purpose chicken lines have higher meat properties (i.e. live weight, carcass weight, etc.) as compared to other breeds. Therefore, the observed distribution of allele frequencies in the studied populations is quite reasonable, but further studies are necessary to found out the links of *IGF-I* allele variants with productive properties of Plymouth Rock White line, given breed specificity of molecular markers.

The studied population of egg-laying chickens is leading on MboII polymorphism of *TGF-β1* exon area with the prevalence of allele B and maximum number of BB homozygotes. Allele F significantly prevails among chicken for different primary use with maximum frequency in the Rhode Island Red population which lacks BB homozygotes. It should be noted that during studies of the productive properties of chickens depending on allele variants *TGF-β1*, positive association of allele F with meat yield values was in Poltava clay breed that, in its turn, allows us to explain the observed allele frequencies [25]. As to RsaI polymorphism of *TGF-β2* promoter, we have not found specially expressed differences between the lines. In general, allele B prevails, except for Plymouth Rock White breed, with maximum frequency for Poltava chicken line which also has the

lowest heterozygosity. The proportion of *TGF-β3* alleles in populations of dual-purpose and egg-lying chickens was practically the same (no valid differences were found) with significant prevalence of allele L. Line 38 was denoted by practically double prevalence of allele L regarding B. Differences in allele frequencies in line 38 compared to other populations were valid. Population of Poltava clay chickens in which frequencies of B and L alleles have practically coincided (0.520 vs. 0.480) validly differed from other studied lines.

Similar RsaI polymorphism for gene *Mx* was denoted in all populations. Allele G prevailed over allele A that was mainly expressed in egg-and-meat chicken and the least expressed in egg-laying chickens. Moreover, it is egg-laying chicken line where we have found the greatest number of AA homozygotes. By *PIT-1* allele ratio, egg-lying and egg-and-meat chickens significantly differed while dual-purpose breeds are the intermediates (see Table 2).

For generalized assessment of the genetic differentiation in the populations of chickens for different primary use, we have estimated genetic distances based on the studied polymorphic loci (both PCR-RFLP and Indel markers were analyzed). The values and the opposite genetic likelihood indices are provided in the Table 3.

3. Genetic distances and genetic likelihood between the the populations of the Ukrainian chicken breeds

Breed	Plymouth Rock White	Borkovskaya Barvistaya	Poltava clay	Rhode Island Red
Plymouth Rock White		0.1951	0.1124	0.0424
Borkovskaya Barvistaya	0.8228		0.2319	0.2488
Poltava clay	0.8937	0.7930		0.0712
Rhode Island Red	0.9585	0.7797	0.9313	

N o t e. Genetic distance values are above the diagonal, genetic likelihood values are under the diagonal.

The most genetically apart breeds were Borkovskaya Barvistaya and Rhode Island Red (24.9 % of differences). In general, the least likelihood was found in egg-laying and dual-purpose breeds, whereas the least likelihood was noted in egg-meat chickens (23-25 % of differences in allele variants of loci) (see Table 3). Dual-purpose and egg-and-meat chickens differ slightly, Poltava clay and Plymouth Rock White lines are the most differentiated (11.2 %), Rhode Island Red and Plymouth Rock White are the least differentiated (4.2 %). In turn, intermediate genetic distances were between the two breeds of egg-and-meat types (7.1 % differences).

Having analyzed the genetic differentiation in chicken populations, we could note specific distribution of allele frequencies of the studied polymorphic loci depending on the productivity type. In this, the extent of differences by different markers was not the same. In this regard, it is reasonable to assess the degree of dissimilarities between the populations by separate loci. In addition to estimation of the genetic distances by Nei, coefficient F_{st} may serve as a good instrument as it directly reflects subdivision of the populations and may be calculated for individual loci. F_{st} values for each locus within all studied populations may be used to estimate dissimilarities (genetic subdivision) in the chickens as per the selected genes.

Both studied mutations in prolactin locus clearly display the dissimilarities between the egg-laying and dual-purpose chickens as it is evidenced by F_{st} values from 0.34 to 0.55 for 24 Indel and from 0.11 to 0.33 for C-2402T, respectively. Maximum dissimilarities for the insertion in the prolactin locus were in line 14 due to monomorphism of this locus in the studied population. F_{st} deviations for dual-purpose lines were insignificant. Wright's genetic subdivision values (F_{st}) correlate with Nei's genetic distance (Dn) calculated separately for prolactin locus. Dn values for populations of egg-laying and dual-purpose chickens are from 0.66 to 0.97 for 24 Indel and from 0.22 to 0.65 for C-2402T.

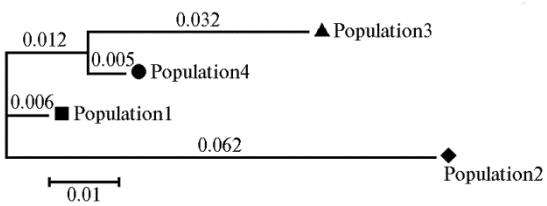
The allele frequencies of growth hormone locus are directly determined by mutation type. By intron 1 polymorphism (MspI-polymorphism), the Poltava clay population stands apart and clearly differ from the meet-and-egg lines (Fst = 0.19) and the egg-meet lines (Fst = 0.23) but not from the egg-laying lines (0.09). Dissimilarities between other lines were insignificant. Only the Rhode Island Red chickens are somewhat dissimilar from other studied populations by AluI polymorphism in intron 4, with F_{st} from 0.04 to 0.12 vs. F_{st} of 0.03-0.08. By SacI polymorphism in *GH* intron 4 compared to other mutations in this locus, the egg-laying and combines lines are the most dissimilar, with F_{st} of 0.23 to 0.33. Combined breeds have practically minimum F_{st} values of 0.02-0.08. The highest similarity by MspI polymorphism in *GH* intron 4 was between Plymouth Rock White and Borkovskaya Barvistaya breeds (F_{st} = 0.03), as well as Rhode Island Red breed (F_{st} = 0.05), while the last two breeds, in turn, differed from each other (F_{st} = 0.14). The Poltava clay and egg-laying chickens (F_{st} = 0.39), as well as Plymouth Rock White chickens (F_{st} = 0.23) are the most diverse. These trends completely correspond to the Nei's genetic distance.

Study of transforming growth factor β gene family polymorphism revealed that by MboII polymorphism of *TGF-β1* exon the egg-laying chicken population is the least diverse from line 14 (F_{st} = 0.11) and the most diverse from line 38 (F_{st} = 0.24) as compared to meet-egg chickens (F_{st} = 0.18). The populations for combined use do not differ. No expressed dissimilarities between the lines were found for RsaI polymorphism of *TGF-β2* promoter fragment. A comparison of Plymouth Rock White and Poltava clay populations results in the highest F_{st} value. F_{st} for locus *TGF-β3* in all populations also did not reflect significant dissimilarities.

We did not found any significant F_{st} distance between all populations for PstI polymorphism of insulin-like growth factor I locus. The meat-egg chicken line shows the highest HinfI polymorphism of insulin-like growth factor I gene promoter, except for comparisons with Rhode Island Red chickens. For all other lines dissimilarities are insignificant.

Also, we have not found any significant differences of F_{st} for *PIT-1* locus. RsaI polymorphism of *Mx* gene is similar for all populations, with F_{st} from 0.01 to 0.08.

In general, allele frequencies in chicken populations may depend on both selection purpose (towards egg or meat yield, etc.), and characteristic features of breeds. Moreover, in phenotypic-based selection of individuals for nest formation the breeder often uses several characteristics of which each is due to effects of several genes (alleles). Quantitative traits are determined by the aggregate activity of the significant number of genes and, thus, the selected individuals with desired performance parameters may also have nonproductive alleles especially manifested in heterozygotes. All these factors together result in the distribution we observed.



Dendrogram of interpopulation relationships by Nei's genetic distance (Neighbor Joining method): Population 1 — Plymouth Rock White, line G-2; Population 2 — Borkovskaya Barvistaya, line A; Population 3 — Poltava clay, line 14; Population 4 — Rhode Island Red, line 38.

Dendrogram of genetic diversity of the chicken populations (Fig.) was plotted by Neighbor Joining method with the use of genetic distances for all studied loci. The obtained phylogenetic tree generally corresponds to earlier described regularities and reflects the dissimilarities between the chicken lines de-

pending on the chicken types. Populations of egg-meat chickens are clustered together. At the same time, meet-and-egg and egg-laying chickens form separate branches, provided that the egg-laying breed demonstrates the highest genetic dissimilarities as compared to other lines.

Our results testify that, traditional phenotype-based selection in fact does not allow for the desired genotypes and elimination of individuals possessing nonproductive alleles of the wide range of loci. Traditional selection practice shows that Ukrainian breeds are inferior to the imported chicken lines, mainly because of limited use of marker associated selection (MAS) in poultry breeding. MAS is a quit routine tool enabling an increase in poultry productivity to the level of foreign lines with a number of monomorphic candidate genes, as it is shown by foreign researches.

Thus, our data validly prove that genetic differentiation of the chicken populations by a set of polymorphic loci mainly depends on the poultry primary use. However, the effect of this factor may vary. Genetic variability revealed in each of the studied breeds allows targeted selection with the use of molecular and genetic methods, including individual QTL genotyping, to produce the lines with a certain set of desired genotypes and their combinations. It will enable breeders to maximum use of the productive potential of the Ukrainian chicken breeds.

REFERENCES

1. Fisinin V.I., Cherepanov S.V. *Materialy XVII Mezhdunarodnoi konferentsii «Innovatsionnye razrabotki i ikh osvoenie v promyshlennom ptitsevodstve»* [Proc. XVII Int. Conf. WSAP «Innovative developments in poultry and their practical use»]. Sergiev Posad, 2012: 3-7 (in Russ.).
2. Stolpovskii Yu.A. *Vavilovskii zhurnal genetiki i selektsii*, 2013, 17(4/2): 900-915 (in Russ.).
3. Semik E., Krawczyk J. The state of poultry genetic resources and genetic diversity of hen populations. *Ann. Anim. Sci.*, 2011, 11(2): 181-191.
4. Paronyan I.A. *Genetika i razvedenie zhivotnykh*, 2014, 3: 43-48 (in Russ.).
5. Roiter Ya.S. *Ptitsa i ptitseprodukty*, 2016, 3: 45-47 (in Russ.).
6. *Katalog pleminnikh resursiv sil's'kogospodars'koï ptitsi* /Pod redaktsiei Yu.O. Ryabokonya [Catalogue of poultry breed gene pool. Yu.O. Ryabokon' (ed.)]. Kïïv, 2006 (in Russ.).
7. Gal'pern I.L., Segal E.L., Fedorov I.V. *Materialy XVIII Mezhdunarodnoi konferentsii VNAP «Innovatsionnoe obespechenie yaichnogo i myasnogo ptitsevodstva Rossii»* [Proc. XVIII Int. Conf. WSAP «Innovative provision of egg and meat poultry in Russia»]. Sergiev Posad, 2015: 45-48 (in Russ.).
8. Nie Q., Fang M., Xie L., Zhou M., Liang Z., Luo Z., Wang G., Bi W., Liang C., Zhang W., Zhang X. The PIT1 gene polymorphisms were associated with chicken growth traits. *BMC Genet.*, 2008, 9: 20-24 (doi: 10.1186/1471-2156-9-20).
9. Cui J.-X., Du H.-L., Liang Y., Deng X.-M., Li N., Zhang X.-Q. Association of polymorphisms in the promoter region of chicken prolactin with egg production. *Poultry Sci.*, 2006, 85: 26-31 (doi: 10.1093/ps/85.1.26).
10. Feng X.P., Kuhnlein U., Aggrey S.E., Gavora J.S., Zadworny D. Trait association of genetic markers in the growth hormone and the growth hormone receptor gene in a White Leghorn strain. *Poultry Sci.*, 1997, 76: 1770-1775 (doi: 10.1093/ps/76.12.1770).
11. Kulibaba R.A., Liashenko Y.V., Yurko P.S. Novel AluI-polymorphism in the fourth intron of chicken growth hormone gene. *Cytol. Genet.*, 2017, 51(1): 54-59 (doi: 10.3103/S0095452717010091).
12. Zhou H., Mitchell A.D., McMurtry J.P., Ashwell C.M., Lamont S.J. Insulin-like growth factor-i gene polymorphism associations with growth, body composition, skeleton integrity, and metabolic traits in chickens. *Poultry Sci.*, 2005, 84: 212-219 (doi: 10.1093/ps/84.2.212).
13. Nagaraja S.C., Aggrey S.E., Yao J., Zadworny D., Fairfull R.W., Kuhnlein U. Trait association of a genetic marker near the IGF-I gene in egg-laying chickens. *J. Hered.*, 2000, 91: 150-156.
14. Li H., Deeb N., Zhou H., Mitchell A.D., Ashwell C.M., Lamont S.J. Chicken quantitative trait loci for growth and body composition associated with transforming growth factor-β genes. *Poultry Sci.*, 2003, 82: 347-356 (doi: 10.1093/ps/82.3.347).
15. Luan D.Q., Chang G.B., Sheng Z.W., Liu Y., Chen G.H. Analysis on the polymorphism and the genetic effects on some economic traits of mx gene S631N mutation site in chicken. *Thai J. Vet. Med.*, 2010, 40(3): 303-310.
16. Merkur'eva E.K. *Geneticheskie osnovy selektsii v skotovodstve* [Genetic basis of breeding in animal husbandry]. Moscow, 1977 (in Russ.).
17. Wright S. *Evolution and the genetics of populations. V. 4: Variability within and among natural*

populations. Chicago, 1978.
18. Jiang R.-S., Xu G.-Y., Zhang X.-Q., Yang N. Association of polymorphisms for prolactin and prolactin receptor genes with broody traits in chickens. *Poultry Sci.*, 2005, 84: 839-845 (doi: 10.1093/ps/84.6.839).
19. Bagheri Sarvestani A.S., Niazi A., Zamiri M.J., Dadpasand Taromsari M. Polymorphisms of prolactin gene in a native chicken population and its association with egg production. *Iranian Journal of Veterinary Research*, 2013, 14(2): 113-119.
20. Ip S.C.Y., Zhang X., Leung F.C. Genomic growth hormone gene polymorphisms in native Chinese chickens. *Experimental Biology and Medicine (Maywood, N.J.)*, 2001, 226(5): 458-462.
21. Al-Hassani A.S., Al-Hassani D.H., Abdul-Hassan I.A. Association of insulin-like growth factor-1 gene polymorphism at 279 position of the 5′UTR region with body weight traits in broiler chicken. *Asian Journal of Poultry Science*, 2015, 9(4): 213-222 (doi: 10.3923/ajpsaj.2015.213.222).
22. Kim M.H., Seo D.S., Ko Y. Relationship between egg productivity and insulin-like growth factor-I genotypes in Korean native Ogol chickens. *Poultry Sci.*, 2004, 83: 1203-1208 (doi: 10.1093/ps/83.7.1203).
23. Li H.F., Zhu W.Q., Chen K.W. Polymorphism in NPY and IGF-I genes associates with reproductive traits in Wenchang chicken. *African Journal of Biotechnology*, 2009, 8(19): 4744-4748.
24. Moe H.H., Shimogiri T., Kawabe K., Nishibori M., Okamoto S., Hashiguchi T., Maeda Y. Genotypic frequency in Asian native chicken populations and gene expression using insulin-like growth factor I (IGFI) gene promoter polymorphism. *Japan Poultry Science*, 2009, 46: 1-5 (doi: 10.2141/jpsa.46.1).
25. Kulibaba R.A., Tereshchenko A.V. Transforming growth factor β1, pituitary-specific transcriptional factor 1 and insulin-like growth factor I gene polymorphisms in the population of the Poltava clay chicken breed: association with productive traits. *Agricultural Science and Practice*, 2015, 2(1): 67-72.

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AUTOIMMUNITY AND THE ENDOGENOUS HORMONE PROFILES OF BULL SIRES

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Abstract

Morpho-functional changes in reproductive organs of bulls due to the influence of continental climate with cold winters and hot summers can repress reproductive function (I.M. Donnik et al., 2005). It requires a deep knowledge of immunobiological, hormonal and biochemical mechanisms regulating the reproductive function in animals, with regard to their adaptability to various environmental and farming conditions (I.M. Donnik et al., 2015). Here, we compared for the first time the autoimmune state of domestic- and foreign-bred Holstein bull sires aged 3 to 9 years (n = 101) which were housed in two ecologically different regions, the Moscow Province (Head Center for Reproduction of Farm Animals) and the Sverdlov Province (Uralplemcenter, Regional Information and Selection Center). Autoimmunity detected by sperm immobilization test was unidentified in 69 % and 83 % of bull sires from the Sverdlov Region and the Moscow Province, respectively. The percentages of bulls with a high titer (> 1:8) of sperm immobilization comprised 3 % and 8 % in the Sverdlov Region and the Moscow Region, respectively. At the titers of approximately 1:4, the autoimmune anti-sperm state of the animals was reversible and easily restored following recommended technologies during two cycles of spermatogenesis. A comparatively high titer was recorded during more intense exploitation of bulls. The Holstein bulls of different origin also differed in susceptibility to developing the autoimmune anti-sperm response. The sires of the Canadian selection were more susceptible, while the lowest-level autoimmunity was characteristic of the Holland bulls. Endogenous hormone levels correlated with the autoimmunity: in case of the titer elevation, the endogenous hormone concentrations decreased, but unequally, i.e. by 38 % and 46 % for testosterone and estradiol, respectively. The variations in correlation between testosterone and estradiol could reach 49 %. The percentage of animals with the titers of 1:4 to 1:8 was higher among the young bull sires aged 28 months, while the anti-sperm antibodies were not revealed or autoimmunity was weakly expressed in the mature bulls aged 35 months, with the titers of 0 to 1:2. Therefore, the young bulls at the start of their intensive use are more susceptible to various impacts as compared to the pubertal sires.

Keywords: autoimmunity, bull sires, testosterone, estradiol, thyroxin

Reproductive function is closely related to animal metabolism, resistance, and adaptiveness [1]. Deviations in morphofunctional state of the reproductive organs in bovine animals located in different natural zones may result in sexual disorders and deterioration of reproductive ability [2]. Thus, in the context of extreme continental climate the ovarian function in females is reduced with noted lack of primary and secondary follicles in atresia phase. In the diary breeding context, up to 95 % of the population genetic progress is attributed to the influence of servicing bulls. To ensure qualitative production of sperm it

is required to adhere to feeding, maintenance and sperm collection technologies with simultaneous systematic multifactor analysis of the head count, including biochemical, hormonal, spermatologic, histological, and immunological methods (identification of circulating autoimmune antibodies to cell structure antigens of testicular apparatus) [3, 4].

The unfavorable properties such as increased sensitivity to stresses, and pathologic response to unfavorable environmental effects are identified in high productive animals of pure-bred lines. They are susceptible even to insignificant deviations from the diet and maintenance regime, which is expressed by metabolic disorder affecting their immune status. It also results in reduction of the productive, reproductive properties, advance cull, and, consequently, in large economic damage [5].

Pathogenesis of autoimmune diseases is very complicated and relates to interoperation of many endogenous and exogenous factors [6]. Anti-sperm antibodies are able to disturb spermatogenesis and may cause pathospermia, may prevent penetration of spermatozoids through the cervical mucus and ovum fertilation [7-10]. Autoimmune response against spermatozoids may be caused by mechanical trauma, overheating, infections of the reproductive tract, cryptorchidism, prostatitis [7, 11-14], and genetic predisposition [15]. Production of anti-sperm antibodies is deemed to be one of the male infertility reasons [13, 16-18], but may be usually found even in fertile males [18, 19].

Real reasons for deterioration of the qualitative sperm characteristics could not be always foreseen. Any unbalance in the diet by nutritional value (deficit or excess) or by any component has negative effect on all spermatogenesis stages: division of seminiferous epithelium, formation and maturing of spermatozoids, biochemical content of secretion of accessory sex organs, and integrity of blood-testis barrier [20]. Physiological state and resistance ability of the livestock in the industrial intensive animal breeding are under effect of a number of technological stresses [21]. Unfavorable ecologic factors (technogenic environmental pollution, natural and climate conditions of highlands) also change the biochemical state in animals. Unfavorable ecologic zones are considered to be the regions close to industrial enterprises, atomic, aluminum, and metallurgical plants-polluting water and fed for animals by toxic elements [22]. Natural resistance, humoral and cell immunity are weakened under the effect of negative factors, accompanied by pollution by heavy metals, secondary immunodeficits, and deterioration of reproductive ability [23].

Autoimmunity, as a consequence of secondary immunedeficit, may serve one of the main reasons of reproductive malfunction in agricultural animals. Depending on the duration of factor and its nature, autoimmune state may have temporary, and reversible or long-lasting and non-reversible effects which significantly affect the reproductive ability in males. Negative correlation was established between the presence of autoimmune antibodies to sperm in blood of servicing bulls and fertilization effectiveness. Sperm concentration in ejaculates and quantity of semen suitable for cryo-conservation is validly reduced in bulls with autoimmune anti-sperm antibodies. Systematic and timely identification of the sires with autoimmune antibodies to sperm is practically important in breeding enterprises. Normative value of immune status accounting for the main ecologic factors should be developed at studying of the populations of practically healthy animals [24-26].

For a long time, reproductive and immune systems in males were studied independently. However, the interest in their interoperation, especially in autoimmunity effect on infertility or reduction of the male fertility, constantly grows [13, 27]. Autoimmune process in testicular apparatus in males is considered to

be an etiologic factor of testicular failure, resulting in reduction of the hormonal function of gonad [28]. Admittedly, valid ($P < 0.001$) reduction in volume and mass of both testis were found in animals suffering from autoimmune orchitis, and testosterone content was significantly reduced in animals suffering from autoimmune hypogonadism. Relationship between the immune and hormonal systems (disorders in one of them leads to failure of the other one) is a known fact. As exemplified by highly productive cows, it was testified that simultaneous correction of immune, hormonal, and biochemical state in animals has more appreciable effect [29].

Information on effect of anti-sperm autoimmune antibodies on fertilization is contradictory. There is no common understanding of the role of humoral immunity upon reproduction and anti-sperm antibodies in development of infertility [7, 8, 30]. Practically, no clinical studies of the autoimmune process in interstitial Leydig's testicular cells producing the main androgene – testosterone were conducted [28]. Since there is no clear diagnostics algorithm of autoimmune hypogonadism in males and females, it challenges the real assessment of its spread and correction ability. Presence of autoimmune antibodies to steroid producing cells of other endocrine organs and nonorganospecific antibodies may serve as a supplementary diagnostic criterion of such pathology. Development of the methods for assessment of the influence of anti-sperm antibodies on fertility of spermatozoids and male infertility has an undisputable clinical importance [31].

Here, we have for the first time conducted a comprehensive immunobiological and hormonal monitoring of producing bulls in the conditions of Moscow and Sverdlovsk regions, accounting for the country of origin, location, and use mode, age, interbreed differences (animal paints), hormonal status in terms of testosterone and estradiol, as well as concentration in blood serum of cholesterine, the precursor of such hormones. Relation of autoimmune titer with age, origin, and geographical location of the animals, as well as the number of endogenous hormones that grew up with decreasing of the autoimmunity titer has been established. Share of the aforesaid autoimmune animals is found among young bulls, whereas autoimmune state is not found or is poorly manifested in majority of mature species.

Purpose of this research was assessment of autoimmune antibody titer and hormonal profile in servicing bulls at adaptation to different conditions of care and use.

Techniques. The studies in November 2016 involved superior servicing bull sires of the dmestic and foreign selection aged 3-9 years ($n = 101$) in two regions, Moscow (JSC Head Center for Reproduction of Farm Animals, $n = 64$) and Sverdlovsk (OJSC Uralplemcentr, $n = 37$). Blood was collected from the jugular vein in sterile vials. Blood serum was separated, and samples were inactivated at temperature of 56 °C in thermostat during 30 minutes and were kept at temperature of below –18 °C prior to their use. Sperm was individually collected on artificial vagina and was immediately used during studies after assessment.

Titer of autoimmune antibodies to spermatozoids was determined by sperm immobilization test (SIT) in the presence of complement (I.I. Sokolovskaya et al., 1990) of Guinea pig. The semen used in SIR has spermatozoid mobility scoring ≥ 0.7 with concentration of > 0.8 billion/ml preliminary diluted with 0.9 % NaCl solution up to concentration of 300 million/ml. For SIR, we used 32-well plates (MiniMed LLC, Russia) with 0.1 ml of 1 % NaCl solution added to each well. The first upper well served as the control. A 0.1 ml aliquot of undiluted blood serum of the studied male was added to the next well in vertical row, and 0.1 ml of 1:2, 1:4, 1:8, 1:16 and 1:32 dilutions of titrated serum were added to other wells of the vertical row. Then, 0.1 ml of 1:10 diluted Guinea pig

blood serum and 0.1 ml of diluted semen aliquots were added to each well. The plates were shaken delicately, covered with glass cup and half-dumped in water-bath (37 °C). Results were recorded 2 hours after formation of precipitates. The last blood serum dilution providing sperm immobilization was considered the sperm-immobilizing antibody titer of the bull (the autoimmunity titer). Reaction was conducted in three analytical replicates.

Endogenous blood testosterone and estradiol concentration was assayed by ELISA test with the use of a Uniplan AFG-01 device (CJSC Pikon, Russia), testosterone reagent kit Immuno-FA-TS, and estradiol reagent kit Immuni-FA-E (Russia). Analysis kit (Spinreac, Spain) and a ChemWell® 2902 automated analyzer (Awareness Technology, Inc., USA) were used to measure blood cholesterol concentration.

Mean (*M*) and standard error of the mean (±SEM) was calculated using Microsoft Office (MS Excel) software. Validity of differences were assessed by Student *t*-test, differences were statistically significant at $P < 0.05$.

Results. When comparing bulls' immune status, we accounted for animal location, climate zone, and ecologic factors, age, breed, and the mode of exploitation. Sperm immobilization test we used is based on binding autoimmune anti-sperm antibodies circulating in the blood with spermatozoids via their surface antigens in the presence of Guinea pig complement (Table 1).

1. Blood anti-sperm antibody titers in servicing bulls of different origin at two breeding enterprises (November 2016)

Enterprise, region	Bull number (%)	Total (share, %)	SIT titer$^+$ (total and share)							
			0	1:2	1:4	1:8	1:16	*M*±SEM		
								0-1:2	1:4-1:8	1:16
OJSC Uralplemcentr (Sverdlov Region)	37 (100)	35 (94.59)	17 (48.57)	7 (20.00)	10 (28.57)	1 (2.86)	0 0	24 (68.57±7.85)	11 (31.43±7.85)	0 0
AO GCV (Moscow)	64 (100)	61 (95.31)	34 (53.12)	19 (29.68)	3 (4.69)	4 (6.25)	1 (1.56)	53 (82.81±4.72)	7 (10.94±3.90)	1 (1.56±1.55)
Differences compared to the values for OJSC Uralplemcentr								+14.24	–20.49*	±1.56

N o t e. AO GCV — OJSC Head Center for Reproduction of Farm Animals. SIT — sperm immobilization test.
* Differences between animals of OJSC Uralplemcentr and OJSC Head Center for Reproduction of Farm Animals are statistically significant at $P < 0.05$.

2. Blood anti-sperm antibody titers in Holstein Black and White bulls depending on their age ($n = 35$, OJSC Uralplemcentr, Sverdlov Region, November 2016)

Bull age, months	*n*	SIT titer$^+$						
		total					portion of the total amount, % (*M*±SEM)	
		0	1:2	1:4	1:8	1:16	0-1:2	1:4-1:8
16-24	9	4	2	3	0	0	66.67±15.71	33.33±15.71
25-36	11	3	2	6	0	0	45.45±15.01	54.55±15.01
37-48	11	8	0	2	1	0	72.73±13.43	27.27±13.43
Over 48	4	2	2	0	0	0	100	0

N o t e s. SIT — sperm immobilization test.

The obtained results testify that the animals of OJSC Head Center for Reproduction of Farm Animals are superior, as 82.8 % of livestock (53 out of 61 bulls) lack autoimmune antibodies or have the lowest titers (0-1:2). That is, the autoimmune status of the bull sires is normal. In OJSC Uralplemcentr this parameter is 14 % lower and the number of bulls with relatively high SIT titers (1:4-1:8) is significantly higher compared to the animals of OJSC Head Center for Reproduction of Farm Animals ($P < 0.05$). At that, 10 of 11 bulls from OJSC Uralplemcentr had relatively low titers of anti-sperm antibodies — 1:4. Such titers may result from physiological disorders of technological origin, e.g. at sperm collection, and/or because of improper diet, mainly at lack of carotene. According to our previous studies, such autoimmune state can be easily corrected

during one or two cycles of spermatogenesis.

The age of bulls from OJSC Uralplemcentr significantly affected their autoimmunity (Table 2). Similar results were also obtained in bulls from JSC Head Center for Reproduction of Farm Animals (n = 15, data not shown).

Comparison of autoimmunity titers in Holstein bulls of different selection at two breeding enterprises (Tables 3) revealed the influence of the origin and place of use of the animals on their immune status.

3. Blood anti-sperm antibody titers in Holstein bulls of different origin at two breeding enterprises (November 2016)

Country of origin	n	SIT titer $^{+}$							
		total					portion of the total amount, % (M±SEM)		
		0	1:2	1:4	1:8	1:16	0-1:2	1:4-1:8	1:16
OJSC Uralplemcentr (Sverdlov Region)									
Netherlands	11	5	3	3	0	0	72.73±13.43	27.27±13.43	0
Denmark	10	8	–	1	1	0	80.00±12.65	20.00±12.65	0
Russia	5	1	2	2	0	0	60.00±21.91	40.00±21.91	0
JSC Head Center for Reproduction of Farm Animals (Moscow Region)									
Canada	30	5	11	5	7	2	53,33	40,00	6.67±4.03

N o t e. SIT — sperm immobilization test. Dash means absence of data.

SIT titers of 0-1:2 were characteristic of most tested Danish bulls. About half of Canadian bulls also had such titers, and autoimmune shifts were typical of a significant part of the population, i.e. in 40 % that approximately is 2 times higher than in Danish bulls. A total of 6.7 % of the Canadian bulls showed the highest SIT titer (1:16) indicating serious immune disorders. These bulls were 4-5 year-old animals which have been exploited more intensively. Apparently, their autoimmune disorder is caused by improper diets during active use leading to a decline in carotene and vitamin A blood level below the norm. Improper semen collection may also cause small traumas of sexual apparatus that is one of the main etiological factors of bulls' autoimmunity.

To study the dependence of immune status on the intrabreed differences, we compared SIT titers in the Black Pied and Red Pied Canadian Holsteins (Table 4). A total of 42.86 % Black Pied bulls had rather high titers, whereas among the Red Pied bulls this parameter was approximately 2 times lower. Nevertheless, final conclusions about the discovered phenomenon are not possible until more animals will be involved in the studying.

4. Blood anti-sperm antibody titers in Black Pied and Red Pied Holstein bulls (JSC Head Center for Reproduction of Farm Animals, Moscow Region, November 2016)

Suit	n	SIT titer^{+}							
		total					portion of the total amount, (M±SEM)		
		0	1:2	1:4	1:8	1:16	0-1:2	1:4-1:8	1:16
Black Pied	19	1	8	3	6	1	42.86±0.80	42.86±10.80	4.76±4.65
Red Pied	11	4	3	2	1	1	53.85±15.03	23.08±11.69	7.62±7.34

N o t e. SIT — sperm immobilization test.

5. Blood endogenous hormones (μmol/l) **in Holstein bulls depending on anti-sperm antibody titers** (M±SEM, JSC Head Center for Reproduction of Farm Animals, Moscow Region, November 2016)

SIT titer^{+}	n	Thyroxine	Estradiol	Testosterone
0-1:2	22	66.50±9.64	0.087±0.050	16.81±5.33
1:4-1:16	18	66.22±10.16	0.087±0.080	15.13±5.55

N o t e. SIT — sperm immobilization test.

In studying endogenous hormone production in a relationship with autoimmunity, we conditionally divided the Canadian Holstein 24-40 month-old bulls from JSC Head Center for Reproduction of Farm Animals into two groups,

with SIT titer of 0-1:2 (conventional lack of autoimmunity) and more than 1:4 (different degree of autoimmunity) (Table 5). The results show trend towards 10 % reduction in testosterone concentration with an increase in anti-sperm antibody titers. The thyroxine content shows no reliable differences. Among the animals of JSC Uralplemcentr a decrease in blood testosterone concentration with an increase in SIT titers is even more pronounced (by 38 %) (Table 6). Cholesterol content also increased (by 14 %) within the physiological limits (see Table 6).

6. Blood endogenous hormones and cholesterol, their precursor, in Holstein bulls depending on anti-sperm antibody titers (*M*±SEM, JSC Uralplemcentr, Sverdlov Region, November 2016)

SIT titer+	*n*	Testosterone, μmol/l	Estradiol, μmol/l	Cholesterol, μmol/l	Testosterone/estradiol	Age, months
Total	34	17.5±3.1	0.44±0.21	3.50±0.10	5.2±1.6	35.1±3.1
of which:						
0-1:2	23	20.0±3.8	0.45±0.24	3.27±0.20	6.3±2.3	38.1±4.1
1:4-1:8	11	12.4±4.9	0.32±0.44	3.72±0.20	3.2±1.1	28.0±4.6
difference		–37.9%	–46 %	+13.7 %	–48.9 %	–10.0

N o t e. SIT — sperm immobilization test.

The data we obtained in different regions correlate (see Tables 5, 6) that is indicative of the relationship between the autoimmune status and androgenous hormones.

Thus, alteration of anti-sperm antibody titers depends on the bulls' age. High titers (from 1:4 to 1:8) are characteristic of younger animals aged 28 months, while low titers (0-1:2) are typical for bulls aged 35 months. Accordingly, young bulls at the beginning of intensive use are more subjected to different affecting factors, whereas mature animals are more sustainable. Testosterone/estradiol ratio of the bulls aged 38 months is 6.3 at higher autoimmune titers. In these bulls testosterone/estradiol ratio is approximately 2 times higher compared to that of the animals aged 28 months. We did not detect any significant differences in the content of cholesterol, the precursor of most sexual hormones, between the bulls of different age. More animals with relatively high autoimmunity are among the bulls of Russian and Canadian selection. The studied Red Pied Holstein bulls are more subjected to different changes or damages of testicular apparatus than Black Pied sires (7.62 and 4.76 %, respectively, the titer of 1:16). Anti-sperm titers are significantly higher in Holstein bulls from JSC Uralplemcentr. In our view, this is caused by adverse technogenic agents in the regions. However, the increase of titer (1:4) is reversible and can be easily normalized for 1-2 cycles of spermatogenesis in the absence of the etiologic factor. Sperm immobilization test, together with measurement of blood concentration of endogenous hormones may be helpful tools for diagnostic of autoimmune disorders in male reproductive system.

REFERENCES

1. Wade G.N., Schneider J.E., Li H.Y. Control of fertility by metabolic cues. *Am. J. Physiol.*, 1996, 270(1 Pt 1): E1-19 (doi: 10.1152/ajpendo.1996.270.1.E1).
2. Donnik I.M., Bol'shakov V.N. V sbornike: *Nauchnye osnovy profilaktiki i lecheniya boleznei zhivotnykh* [In: Fundamentals of preventing diseases and animal therapy]. Moscow, 2005: 433-443 (in Russ.).
3. Shulman S., Hu C.Y. A study of the detection of sperm antibody in cervical mucus with a modified immunobead method. *Fertil. Steril.*, 1992, 58(2): 387-391 (doi: 10.1016/S0015-0282(16)55214-5).
4. Francavilla F., Romano R., Santucci R. Effect of sperm-antibodies on acrosome reaction of human sperm used for the hamster egg penetration assay. *Am. J. Reprod. Immunol.*, 1991, 25(2): 77-80 (doi: 10.1111/j.1600-0897.1991.tb01067.x).

5. Seliverstov V.V., Shakhov F.G. *Korrektsiya ekologo-adaptatsionnoi teorii vozniknoveniya, razvitiya massovoi patologii i zashchity zdorov'ya zhivotnykh v sel'skokhozyaistvennom proizvodstve* [Revision of eco-adaptation theory of origin and development ща mass pathologies, and animal health protection in commercial farming]. Moscow, 2000 (in Russ.).
6. Davidson A., Diamond B. Autoimmune diseases. *N. Engl. J. Med.*, 2001, 345(5): 340-350 (doi: 10.1056/NEJM200108023450506).
7. Bozhedomov V.A., Loran O.B., Sukhikh G.T. *Andrologiya i genital'naya khirurgiya*, 2001, 1: 72-77 (in Russ.).
8. Bohring C., Krause E., Habermann B., Krause W. Insolation and identification of sperm membrane antigens recognized by antisperm antibodies, and their possible role in immunological infertility disease. *Mol. Hum. Reprod.*, 2001, 7(2): 113-118.
9. Lombardo F., Gandini L., Lenzi A., Dondero F. Antisperm immunity in assisted reproduction. *J. Reprod. Immunol.*, 2004, 62(1-2): 101-109 (doi: 10.1016/j.jri.2003.08.005).
10. Clarke G. Etiology of sperm immunity in women. *Fertil. Steril.*, 2009, 91(2): 639-643 (doi: 10.1016/j.fertnstert.2007.11.045).
11. Dimitrov D.G., Urbanek V., Zverina J., Madar J., Nouza K., Kinsky R. Correlation of asthenozoospermia with increased antisperm cell-mediated immunity in men from infertile couples. *J. Reprod. Immunol.*, 1994, 27: 3-12 (doi: 10.1016/0165-0378(94)90011-6).
12. Dimitrov D.G., Petrovska M. Effects of products of activated immune cells and recombinant cytokines on spontaneous and ionophore-induced acro-some reaction. *Am. J. Reprod. Immunol.*, 1996, 36(3): 150-156 (doi: 10.1111/j.1600-0897.1996.tb00156.x).
13. Walsh T.J., Turek P.J. Immunologic infertility. In: *Infertility in the male. 4th ed.* L.I. Lipshuitz, S.S. Howards, C.S. Niederberger (eds.). Cambridge University Press, Cambridge, 2009: 277-294 (doi: 10.1017/CBO9780511635656.017).
14. Kurpisz M., Havryluk A., Nakonechnyj A., Chopyak V., Kamieniczna M. Cryptorchidism and long-term consequences. *Reprod. Biol.*, 2010, 10(1): 19-35 (doi: 10.1016/S1642-431X(12)60035-7).
15. Omu A.E., al-Qattan F., Mohammed A. Expression of human leukocyte antigens in patients with autogenic and allogenic circulating antisperm antibodies. *Archives of Andrology*, 1996, 37(3): 155-162.
16. Check J.H. The infertile male — diagnosis. *Clinical and Experimental Obstetrics & Gynecology*, 2006, 33(3): 133-139.
17. Lee R., Goldstein M., Ullery B., Ehrlich J., Soares M., Razzano R., Herman M., Callahan M., Li P., Schlegel P., Witkin S. Value of serum antisperm antibodies in diagnosing obstructive azoospermia. *J. Urology*, 2009, 181(1): 264-269 (doi: 10.1016/j.juro.2008.09.004).
18. Naz R.K. Modalities for treatment of antisperm antibody mediated infertility: novel perspectives. *Am. J. Reprod. Immunol.*, 2004, 51(5): 390-397 (doi: 10.1111/j.1600-0897.2004.00174.x).
19. Bohring C., Krause W. The role of antisperm antibodies during fertilization and for immunological infertility. *Chem. Immunol. Allergy*, 2005, 88: 15-26 (doi: 10.1159/000087818).
20. Kombarova N.A., Abilov A.I. *Molochnoe i myasnoe skotovodstvo*, 2009, 3: 30-32 (in Russ.).
21. Argunov M.N., Mel'nikova N.V. *Materialy I S'ezda veterinarnykh farmakologov Rossii* [Proc. I Congress of Russia Veterinary Pharmacologists]. Voronezh, 2008: 88-90 (in Russ.).
22. Shakhov A.G. *Ekologicheskie problemy patologii, farmakologii i terapii zhivotnykh* [Ecological aspects of animal pathology, pharmacology and therapy]. Voronezh, 1997 (in Russ.).
23. Topuriya G.M., Topuriya L.Yu. *Veterinariya Kubani*, 2011, 1: 22-23 (in Russ.).
24. Kochetkov A.A., Abilov A.I., Tag T.A. Specificity of methods for revealed of sensitization against different nature antigens of ejaculates. *J. Reprod. Immunol.*, 1989, 15(Suppl. 1): 133 (doi: 10.1016/0165-0378(89)90275-1).
25. Abilov A.I., Tag T.A. Bulls autoimmunity: Relationship with protein-vitamin A deficiency and its removed. *J. Reprod. Immunol.*, 1989, 15(Suppl. 1): 51 (doi: 10.1016/0165-0378(89)90122-8).
26. Sokolovskaya I., Radchenkov V., Bronskaya A., Oyvadis R., Abilov A., Tag T., Solovyov N., Subbotin A., Oshadchuk V. The significance of female's immune reactions on foetus at pregnancy. I. The prenatal livability at experimental blockading of pregnant female's immune. *J. Reprod. Immunol.*, 1983, 5(Suppl. 1): 72 (doi: 10.1016/0165-0378(83)90161-4).
27. Madar J., Urbanek V., Chaloupkova A., Nouza K., Kinsky R. Role of sperm antibodies and cellular of male to sperm in the pathologenesis of male infertility. *Ceska Gynekologie*, 2002, 67(1): 3-7.
28. Yarnykh A.L., Vorokhobina N.V., Khokhlov P.P., Gzgzyan A.M. *Meditsinskaya immunologiya*, 2002, 4(2): 217-218 (in Russ.).
29. Abilov A.I., Amerkhanov Kh.A., Eskin G.V., Fedorova E.V., Zhavoronkova N., Kombarova N.A., Varennikov M.V. *Zootekhniya*, 2013, 9: 25-28 (in Russ.).
30. Shiraishi Y., Shibahara H., Koriyama J., Hirano Y., Okazaki H., Minota S., Suzuki M. Incidence of anti-sperm antibodies in males with systemic autoimmune diseases. *Am. J. Reprod. Immunol.*, 2009, 61(3): 183-189 (doi: 10.1111/j.1600-0897.2008.00676.x).
31. Nikolaeva M.A. *Rossiiskii immunologicheskii zhurnal*, 2005, 9(2): 56-60 (in Russ.).

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CYTOGENETIC STATUS OF MARES (*Equus caballus*) OF UKRAINIAN RIDING BREED INFLUENCES THEIR FERTILITY

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Abstract

Cytogenetic studies of mares are widely used in practice (in case of embryonic death this is a mandatory test) in countries with developed horse breeding. Genetic evaluation of *Equus caballus* is also widely performed. Nevertheless, in the available literature, we could not find publications on the relationship between cytogenetic disorders in mares and the effectiveness of artificial insemination with frozen and thawed sperm. This paper is the first report on the impact of the cytogenetic status of mares *E. caballus* of Ukrainian horse breed on their sexual cycle and the efficacy of the artificial insemination by Kharkov technology. It has been shown that in case of ovary hypofunction caused by an increased chromosomal variability, as estimated by the per cent of aberrant metaphases with no genome mutations and the transmitted cytogenetic disturbances found, it is necessary to divide the mares into three groups: up to 5 %, from 5 to 10 % and over 10 % overall chromosomal instability. This allows better characterization of mares' physiological condition to optimized treatment and the artificial insemination procedure by Kharkov technology. When cooled semen used, the fertility of the mares having more than 10 % aberrant metaphases was the lowest, by 29.81 and 31.86 % less ($p < 0.01$) compared to mares from the groups with lower chromosomal instability. When thawed semen was used, the fertility was the highest in the mares with the chromosomal instability up to 5 %, that is, on average 14.93 % higher ($p < 0.05$) compared to the mares with more than 5 % of metaphases with aberrations. The influence of cytogenetic status on the fertility in the insemination was clearly seen when the cryopreserved semen was used as compared to cooled semen. The fertility of the mares inseminated with cryopreserved sperm averaged was 71.60 % in group 1 (up to 5 % aberrant metaphases), 56.67 % in group 2 (5-10 % aberrant metaphases), and only 37.04 % in group 3 (> 10 % aberrant metaphases). So, estimation of cytogenetic status ensures optimization of artificial insemination and an increase in mares' fertility when used cooled and frozen-thawed semen.

Keywords: *Equus caballus*, cytogenetic status of mares, fertility, aberrations

Genetic evaluation of *Equus caballus* is performed in many countries [1-3]. Nevertheless, we could not find in the available literature any publications on the relationship between cytogenetic disorders in mares and the effectiveness of artificial insemination with frozen and thawed sperm. Cytogenetic studies are more practically used in countries with developed horse breeding than in Ukraine, and in mandatory manner in case of embryonic death [4-6]. It should be noted that intensive selection in the Ukrainian horse breeds without sufficient fundamental studies could result in spread of cytogenetic anomalies which are able to reduce reproductive function [7-9]. Probably, for this particular reason foal crop across the Ukrainian horse breeding industry does not exceed 50 % lacking the required minimum of breeding reproductive stock for majority of the raised breeds. Only 3 out of the 12 *E. caballus* breeds officially registered in the Ukraine have sufficient quantity of breed animals; herewith the total number of horse stock at beginning of 2015 had decreased to 320-330 thousands [10]. Thus,

the fundamental objective of the horse breeding development program in Ukraine is to preserve and to increase the number of stock by 2020. Although use of modern reproduction biotechnology methods has its role to play [11, 12], they are ineffective upon diagnosis of anovulatory sex cycle in mares caused by hypovarianism or cytogenetic disorders.

It should be noted that over the last years we have witnessed decrease in fertility in all animal species (just like in humans) and growth of a number of obstetric-gynecologic diseases that, possibly, is partially due to deterioration of the ecologic situation [13-16]. Cytogenetic studies are wider used in the context of zoological gardens to preserve the appropriate gene pool and to ensure the effective animal reproduction [17-18].

These findings point out to the need for use of cytogenetic studies to find the reasons of anovulatory sex cycles and to increase fertilization rates in mares both in the natural horsing and after artificial insemination. First of all, it is necessary to perform cytogenetic tests on mares with rectally confirmed hypovarianism as the most spread pathology. However, only few cytogenetic tests were performed on *E. caballus* in Ukraine as regards to the reproductive function, without studying the link with effectiveness of the artificial insemination [19, 20].

In this paper we have for the first time shown that fertilization of horses of the Ukrainian roadster breed depends on the instability degree of their chromosomes apparatus (provided lack of genome mutations and balanced genetically transmitted cytogenetic disorders), provided that this effect is manifested stronger if mares are inseminated by frozen-thawed, rather than by frozen sperm.

Purpose of this study was establishment of the impact of the cytogenetic status in mares *E. caballus* of the Ukrainian horse breed on their sexual cycle and the efficacy of the artificial insemination by Kharkov technology.

Techniques. Tests on 143 mares of the Ukrainian roadster breed were conducted during 3 years (2012-2015) at one and the same farming units (private stud farms and breed reproducing farms in Kharkov, Poltava, Dnipropetrovsk, Zaporozhian, and Kiev regions of the Ukraine).

Blood for cytogenetic tests was collected weekly in sterile vials with heparin from the jugular vein before insemination by commonly accepted methods with adherence to the aseptic and antiseptic regulations. Lymphocytes were cultivated during 48-56 hours in sterile conditions in Eagle medium or 199 medium (Sigma, USA) with addition of the inactivated calf serum, Phytohaemagglutinin (Sigma, USA) and antibiotics (penicillin and streptomycin, 100 mg/cm^3) at temperature of 37 °C, following which preparations based on metaphase lymphocyte plates were produced ([21, 22]. Medicines were examined by optical microscope Jenaval (Carl Zeiss, Germany) under oil immersion at magnification of ×1000.

Based on cytogenetic data, mares were divided into groups prior to the horsing and insemination period depending on the percentage of metaphases with aberrations (up to 5 %, 5-10 % and over 10 %). Afterwards, a set of zootechnical and veterinary actions were taken for improvement of fertility (better feeding and raising, longer motion, use of developed complex for individual care) in mares with established hypovarianism. Animals with genome mutations and balanced genetically transmitted cytogenetic anomalies were excluded from the studies.

Optimal insemination time was comprehensively diagnosed by ultrasound scanner Aquila Pro (Esaote, Spain) for veterinary purpose with rectal linear probe (6-8 mHz).

Artificial insemination was performed in the first full-value ovulatory sex cycle by Kharkov technology [10] with the use of our instrument [23]. Cooled and frozen-thawed sperm was used. Prior to insemination, cooled stud sperm

was stored for no more than 48 hours in the household refrigerator at temperature of 2-4 °C. Sperm was frozen by Kharkov technology [10], spermatozoids were packed up in tube syringes of 5 cm^3 with sperm concentration of 150-200 million/cm^3. One sperm dose was used for one insemination. Spermatozoids were unfrozen (tube syringes) in water bath at temperature of 38-40 °C during 2-3 minutes. After defrosting, only such spermatozoids were used for insemination purposes, in which sperm mobility rate scored over 3 (at least 30 % of spermatozoon with rectilinear motion). Donor studs had acceptable chromosomal variability (at least 5 % of metaphases with aberrations) and no genome mutations and balanced cytogenetic disorders.

Study results were statistically processed by commonly accepted methods [24], as well as with the use of specialized program SPSS (IBM, USA). Mean (*M*) values and standard errors of the mean (±SEM) are provided in table below. Deviations were assessed by Student's *t*-test and were considered statistically significant at $p < 0.05$.

Results. The paramount problem occurring at preservation of the Ukrainian gene pool of *E. caballus* breed is in decrease of the number of mares with anovulatory sex cycles as fertilization becomes impossible in such animals [25]. At complex diagnostics of the state of reproductive range, deviations from the physiological norm were noted in animals with chromosomal variability exceeding 5 %. Physiological pre-ovulation mare follicle at acceptable number of metaphases with aberrations and hypo-ovaria in species with high percentage is presented in Fig. 1 below.

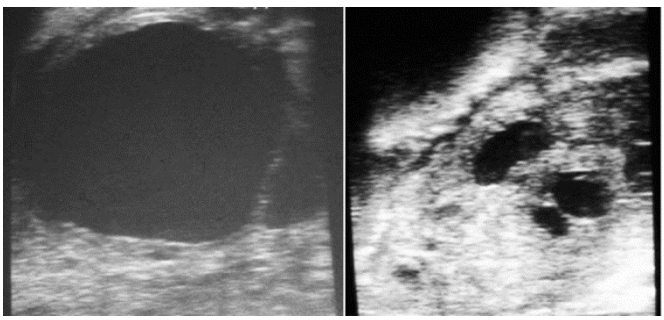

Fig. 1. Physiological follicle of mare (*Equus caballus*) of Ukrainian roadster breed with acceptable cytogenetic status (on the left) **and hypo-ovaria at high percentage of metaphases with aberrations** (on the right)**.**

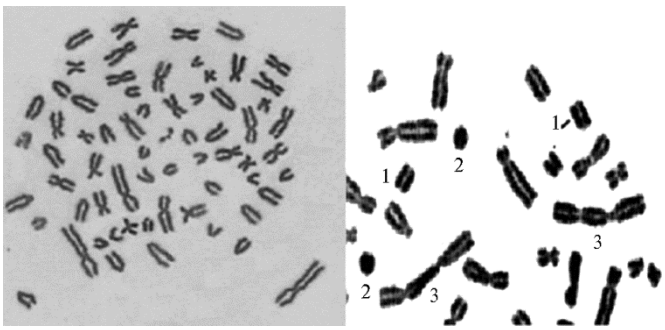

Fig. 2. Metaphase plates in mares (*Equus caballus*) of Ukrainian roadster breed without aberrations (on the left) **and high chromosomal instability** (on the right)**:** 1 — paired fragments, 2 — circular bodies, 3 — dicentrics.

Normal metaphases and observed cytogenetic disorders are illustrated in Fig. 2.

Data of the table 1 show that the mares with total chromosomal instability of up to 5 % and physiological sex cycle had metaphases with aberrations by 5.31 % less ($p < 0.001$) than animals from the group with 5-10 % aberrations, and by 17.09 % less ($p < 0.001$) metaphases with aberrations as compared to mares among which over 10 % of aberration metaphases are registered.

Upon use of defrost sperm, effect of cytogenetic status in mares on their fertilization by way of insemination was more displayed than in case of use of the cooled sperm, which correlates to other study results of use of frozen-thawed

stud sperm [26, 27]. Insemination was performed in the first full-value ovulation sex cycle. Fertilization rate upon use of the cooled sperm (by percentage of newborn studs) in mares with cytogenetic status of over 10 % metaphases with aberrations was the least as follows: in average by 29.81 and 31.86 % lower ($p < 0.01$) than in animals of the groups with 5-10 % and < 5 % metaphases with aberrations, respectively. Upon insemination by cooled sperm, fertilization rates in mares from the first (up to 5 % of metaphases with aberrations) and the second (5-10 % of metaphases with aberrations) groups lacked valid deviations.

1. Cytogenetic status of mares (*Equus caballus*) of the Ukrainian roadster breed and their fertilization at artificial insemination by Kharkov technology (*M*±SEM, private horse breeding plants and breeding reproducing centers of the Ukraine, years 2012-2015)

Indicator	Metaphases with aberrations		
	< 5 %	5-10 %	> 10 %
Number of studied metaphases	2418	2486	2930
Number of mares in a group	44	45	54
Metaphases with aberrations	1.48±0.08	4.40±0.11***	10.67±0.22***
Relative number of metaphases with aberrations, %	2.70±0.14	8.01±0.22***	19.79±0.46***
Number of studs born from the cooled sperm, %	92.00±1.15	90.00±1.11	60.19±0.93**
Number of studs born from the thawed sperm, %	71.60±1.14	56.67±1.11*	37.04±1.85**

*, **, *** Deviations are statistically significant as compared to the first group of mares (up to 5 % of metaphases with aberrations) at $p < 0.05$; $p < 0.01$ and $p < 0.001$, respectively.

Fertilization rates by insemination by thawed sperm was the highest in the group with total chromosomal instability of < 5 %, having in average exceeded by 14.93 % ($p < 0.05$) the rates in mares from the second group and by 34.56 % ($p < 0.01$) in mares with the highest percentage of metaphases with aberrations (> 10 %). Other researchers also note that cytogenetic status in mares is especially important upon use of the unfrozen sperm [28, 29].

2. Structure of chromosomal aberrations in mares (*Equus caballus*) of the Ukrainian roadster breed with different degree of chromosomal instability (*M*±SEM, private horse breeding plants and breeding reproducing centers of the Ukraine, years 2012-2015)

Indicator	Metaphases with aberrations		
	< 5 %	5-10 %	> 10 %
Total aberrations	1.95±0.10	6.69±0.27***	14.41±0.33***
Structure of aberrations, %:			
single fragments of chromosomes	70.08±3.78	47.57±2.10***	43.02±1.85***
paired fragments of chromosomes	23.11±5.10	27.75±1.65	23.11±0.77
circular chromosomes	0.00±0.00	10.36±1.70	16.84±1.20**
gaps and breaks in chromosomes	6.81±5.29	13.74±1.95	16.27±1.03
dicentrics	0.00±0.00	0.58±0.66	0.76±0.87

*, **, *** Deviations are statistically significant as compared to the first group of mares (up to 5 % of metaphases with aberrations) at $p < 0.05$; $p < 0.01$ and $p < 0.001$, respectively.

Studies of the structure of chromosomal aberrations (Table 2) had shown that their total number in mares from the first group (with acceptable total chromosomal instability) was in average by 4.74 less ($p < 0.001$) than in the second group, and by 12.46 less ($p < 0.001$) than in the third group. Single fragments of chromosomes had prevailed amongst the aberrations, whereas in the first group such indicator was by 22.51 % higher ($p < 0.001$) than in the second group and by 27.06 % higher ($p < 0.001$) than in the third group, where number of anovulation cycles was the highest. Percentage of paired chromosome fragments was in average practically similar in mares with total chromosomal instability of up to 5 % and over 10 %, and in the second group such indicator had invalidly exceeded thereof in the first and third groups by 4.64 %. Circular chromosomes were not found in animals from the first group and in the third group it was by 6.48 % more ($p < 0.01$) than in the second one. Number of gaps

and breaks in the studied metaphase plates was the least in mares from the first group that in average was by 6.93 % less than in the second group and by 9.46 % less than in the third group. No dicentrics were found in the studied mares with the acceptable degree of chromosomal instability (up to 5 %), their highest percentage was established in animals from the third group with over 10 % metaphases with aberrations that was by 0.18 % more than in the second group with total chromosomal instability 5-10 %.

Therefore, we have studied the impact of cytogenetic status of mares *Equus caballus* of the Ukrainian roadster breed on full-value of their sex cycle and fertilization by Kharkov technology. It is established that in order to increase fertilization rates in mares it is reasonable to separate them depending on their cytogenetic status (with number of metaphases with aberrations of up to 5 %, 5-10 % and over 10 %). Such division allows the breeders to increase the fertilization rates both naturally and at artificial insemination. Herewith, fertilization of mares by unfrozen sperm with number of aberration metaphases of up to 5 % in average comprises 71.60 %, with 5-10 % of such metaphases — 56.67 %, over 10 % — only 37.04 %. Accordingly, it is very important to account for the cytogenetic status of mares upon use of the unfrozen sperm.

REFERENCES

1. Aldridge L.I., Kelleher D.L., Reilly M., Brophy P.O. Estimation of the genetic correlation between performances at different levels of show jumping competition in Ireland. *J. Anim. Breed. Genet.*, 2000, 117: 65-72 (doi: 10.1046/j.1439-0388.2000.00232.x).
2. Shubertova Z., Candrak J., Rolinec M. Genetic evaluation of show jumping horses in the Slovak Republic. *Ann. Anim. Sci.*, 2016, 16(2): 387-398 (doi: 10.1515/aoas-2015-0072).
3. Stefaniuk-Szmukier M., Ropka-Molik K., Zagrajczuk A., Piorkowska K., Szmatola T., Luszczynski J., Bugno-Poniewierska M. Genetic variability in equine GDF9 and BMP15 genes in Arabian and Thoroughbred mares. *Ann. Anim. Sci.*, 2018, 18(1): 39-52 (doi: 10.1515/aoas-2017-0035).
4. Zabek T., Semik E., Fornal A., Bungo-Poniewierska M. Genetic variation of two horse breeds in Cpg islands of Oas1 locus. *Ann. Anim. Sci.*, 2014, 14(4): 841-850 (doi: 10.2478/aoas-2014-0069).
5. Curik I., Zechner P., Sölkner J., Achmann R., Bodo I., Dovc P., Kavar T., Marti E., Brem G. Inbreeding, microsatellite heterozygosity, and morphological traits in Lipizzan horses. *J. Hered.*, 2003, 94: 125-132 (doi: 10.1093/jhered/esg029).
6. Khanshour A., Juras R., Blackburn R., Cothran E.G. The legend of the Canadian Horse: genetic diversity and breed origin. *J. Hered.*, 2015, 106(1): 37-44 (doi: 10.1093/jhered/esu074).
7. Ducro B.J., Koenen E.P.C., Tartwijk J.M.F.M., Bovenhuis H. Genetic relations of movement and free-jumping traits with dressage and show-jumping performance in competition of Dutch Warmblood horses. *Livest. Sci.*, 2007, 107: 227-234 (doi: 10.1016/j.livsci.2006.09.018).
8. Druml T., Grilz-Seger G., Neuditschko M., Neuhauser B., Brem G. Phenotypic and genetic analysis of the leopard complex spotting in Noriker horses. *J. Hered.*, 2017, 108(5): 505-514 (doi: 10.1093/jhered/esx039).
9. Collins C.W., Songsasen N.S., Vick M.M., Wolfe B.A., Weiss R.B. Abnormal reproductive patterns in Przewalski's mares are associated with a loss in gene diversity. *Biol. Reprod.*, 2012, 86(2): 1-10 (doi: 10.1095/biolreprod.111.092676).
10. Tkachev A.V., Sheremeta V.I., Tkacheva O.L., Rossokha V.I. Physiological relationship of erythrocyne antigens with indicators of horse spermogram. *Fiziol. Zh.*, 2017, 63(1): 84-90 (doi: 10.15407/fz63.01.084).
11. Choi Y.H., Love C.C., Chung Y.G., Varner D.D., Westhusin M.E., Burghardt R.C., Hinrichs K. Production of nuclear transfer horse embryos by Piezo-driven injection of somatic cell nuclei and activation with stallion sperm cytosolic extract. *Biol. Reprod.*, 2002, 67(2): 561-567 (doi: 10.1095/biolreprod67.2.561).
12. McPartlin L.A., Suarez S.S., Czaya C.A., Hinrichs K., Bedford-Guaus S.J. Hyperactivation of stallion sperm is required for successful in vitro fertilization of equine oocytes. *Biol. Reprod.*, 2009, 81(1): 199-206 (doi: 10.1095/biolreprod.108.074880).
13. Moskalets V.V., Moskalets T.Z., Vasylkivskyi S.P., Grynyk I.V., Vovkohon A.H., Tarasyuk S.I., Rybalchenko V.K. Adaptability and stability mechanisms of Triticeae tribe to epiphytoparasites in anthropical ecosystem. *Ukrainian Journal of Ecology*, 2017, 7(2): 230-238 (doi: 10.15421/2017_41).
14. Taguchi T., Kubota S., Mezaki T., Tagami E., Sekida S., Nakachi S., Okuda K., Tominaga A. Identification of homogeneously staining regions by G-banding and chromosome mi-

crodissection, and FISH marker selection using human Alu sequence primers in a scleractinian coral *Coelastrea aspera* Verrill, 1866 (*Cnidaria*). *Comp. Cytogenet.*, 2016, 10(1): 61-75 (doi: 10.3897/CompCytogen.v10i1.5699).
15. Dean W., Santos F., Reik W. Epigenetic reprogramming in early mammalian development and following somatic nuclear transfer. *Seminars in Cell and Developmental Biology*, 2003, 14: 93-100 (doi: 10.1016/s1084-9521(02)00141-6).
16. Savina N.V., Smal' M.P., Kuzhir T.D. *Molekulyarnaya i prikladnaya genetika*, 2008, 10: 108-114 (in Russ.).
17. Kosowska B., Strzała T., Moska M. *Vestnik zoologii*, 2015, 49(6): 529-536 (doi: 10.1515/vzoo-2015-0063) (in Russ.).
18. Perevozkin V.P., Bondarchuk S.S., Manich A.S. *Genetika*, 2015, 51(8): 924-933 (in Russ.).
19. Pawlina K., Bugno-Poniewierska M. The application of zoo-fish technique for analysis of chromosomal rearrangements in the *Equidae* family. *Ann. Anim. Sci.*, 2012, 12(1): 5-13 (doi: 10.2478/v10220-012-0001-y).
20. Danielak-Czech B., Rejduch B., Kozubska-Sobocińska A. Identification of telomeric sequences in pigs with rearranged karyotype using prins technique /Identyfikacja sekwencji telomerowych u wi z rearan acj kariotypu przy wykorzystaniu techniki PRINS. *Ann. Anim. Sci.*, 2013, 13(3): 495-502 (doi: 10.2478/aoas-2013-0022).
21. Warchałowska-Śliwa E., Grzywacz B., Heller K.G., Chobanov D.P. Comparative analysis of chromosomes in the Palaearctic bush-crickets of tribe *Pholidopterini* (*Orthoptera*, *Tettigoniinae*). *Comp. Cytogenet.*, 2017, 11(2): 309-324 (doi: 10.3897/CompCytogen.v11i2.12070).
22. Sadílek D., Angus R.B., Táhlavský F., Vilímová J. Comparison of different cytogenetic methods and tissue suitability for the study of chromosomes in *Cimex lectularius* (*Heteroptera*, *Cimicidae*). *Comp. Cytogenet.*, 2016, 10(4): 731-752 (doi: 10.3897/CompCytogen.v10i4.10681).
23. Tkachov O.V. *Pristrii atravmatichnii dlya shtuchnogo osimeninnya kobil. Patent Ukraïni na korisnu model' № 105004, MPK A61D 19/02. Zayavl. 05.10.2015. Opubl. 25.02.2016. Byul. № 4* [Devise for non-traumatic artificial insemination of mares. Patent of Ukraine № 105004, MPK A61D 19/02. Appl. 05.10.2015. Publ. 25.02.2016. Bul. № 4] (in Russ.).
24. Plokhinskii N.A. *Rukovodstvo po biometrii dlya zootekhnikov* [Guide to biometrics for livestock specialists]. Moscow, 1969 (in Russ.).
25. Wejer J., Lewczuk D. Effect of the age on the evaluation of horse conformation and movement. *Ann. Anim. Sci.*, 2016, 16(3): 863-870 (doi: 10.1515/aoas-2015-0092).
26. Melo C.M., Papa F.O., Fioratti E.G., Magalhaes L.C.O., Alvarenga M.A. Application of equine epididymal sperm. *Biol. Reprod.*, 2010, 83(Suppl_1): 672-680 (doi: 10.1093/biolreprod/83.s1.672).
27. Robeck T.R., Steinman K.J., Gearhart S., Reidarson T.R., McBain J.F., Monfort S.L. Reproductive physiology and development of artificial insemination technology in killer whales (*Orcinus orca*). *Biol. Reprod.*, 2004, 71(2): 650-660 (doi: 10.1095/biolreprod.104.027961).
28. Lindsey A.C., Morris L.H., Allen W.R., Schenk J.L., Squires E.L., Bruemmer J.E. Hysteroscopic insemination of mares with low numbers of nonsorted or flow sorted spermatozoa. *Equine Vet. J.*, 2002, 34: 128-132 (doi: 10.2746/042516402776767178).
29. Lindsey A.C., Schenk J.L., Graham J.K., Bruemmer J.E., Squires E.L. Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa. *Equine Vet. J.*, 2002, 34: 121-127 (doi: 10.2746/042516402776767321).

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THE ASSOCIATION BETWEEN BLOOD GROUP AND REPRODUCTIVE PERFORMANCE IN CATTLE

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Abstract

Modern development of animal breeding is impossible without the implementation of molecular genetic methods, the use of which allows searching for reliable biomarkers of the desired development of economically useful traits in farm animals based on the analysis of hereditary information. Thus, immunogenetic methods allowed significant reduction of errors in the control of pedigree of breeders. Studies of the interrelationships of blood groups with economically useful signs of animals are in progress. Infertility and barrenness in cattle lead to huge economic losses. The purpose of this work was to identify the relationship between antigenic similarity of blood groups in parents and reproduction indicators in cattle. The study was carried out from 2009 to 2014 in Sverdlovsk Region in the livestock farms Mezenskoe LLC (Beloyarsky Region), Pervouralsky Integrated Agricultural Production Center (Pervouralsk Town District) and Artemovsky Farming Company LLC (Artemovsky Region) using 1300 pairs of Ural-type black-and-white cattle. The mated animals were authentic in origin and certified for blood groups. For their certification, 54 monospecific sera to 11 blood group systems were used. In mated animals the index of antigenic similarity of blood groups, expressed as the ratio of similar antigens in bulls and cows to their total number, and homozygosity on EAA, EAF, and EAZ blood group systems were calculated. These blood group systems were represented by several antigens which makes it possible to trace immune conflict in mated animals. By biometrical processing, it was identified that the pairs which were heterozygous in EAA and EAF antigens showed higher efficiency of conception and better survival of the offspring, though the twinning frequency decreased. The parents homozygous in EAA system produced the progeny in which the amount of aborted embryos was 2.5 % higher than that in the heterozygous pairs. The number of calves born dead from the EAF-homozygous pairs was twice as much as that of heterozygous pairs. Apparently, the immune conflict, which leads to the extended period from delivery to productive insemination and higher offspring death, decreases in animals homozygous in EAZ system. The number of calves born dead was 3.2 % for homozygous pairs and 4.7 % for heterozygous pairs. The index of antigenic similarity of parents varied from 0.1 to 0.8. If the antigenic similarity of the mated animals was 10 %, significant losses of calves were observed. If the antigenic similarity was 20 %, such losses increased but not significantly. The similar patterns were observed for the twinning. If the index of antigenic similarity of the mated animals was from 0 to 0.1, the maximum twins were born, and if the index of similarity was 0.2 or more, the number of twins increased, but gradually. Thus, the antigenic similarity of mated animals influences the number and survival of born calves.

Keywords: cattle, antigens, blood groups, index of antigenic similarity, homozygosity, multiple fetus, survival of calves

The most acute problem in modern cattle breeding industry is improvement of reproduction indicators in cattle at preservation of high milk yields. One of ways to improve reproduction indicators is search of relationships between the antigen alleles and blood groups and economically useful traits. Determination of blood groups (protein antigens of erythrocyte plasma membrane) in cattle is widely used for validity check of origin, studying of the gene pool of cattle populations, studying of relations between the blood groups and economically useful traits, and selection of pairs by antigen similarity [1, 2]. Identification of genes polymorphism of which is associated with variability in performance of economically useful traits became possible due to progress of molecular genetics and

molecular biology [3, 4]. Studies and comparative analysis of blood groups are important for studying the evolution of agricultural animal breeds [5]. These studies enable revising the breed formation processes and forecasting of heterotic effects in crossbred animals [6].

One of reproduction indicators is fertility as an evolutional ability of each kind to give birth in natural conditions, thus, compensating death rates. Multiple pregnancies are an important reproduction indicator in animals which depends on fertility [7]. The bigger the animal, the longer its life span is, and the longer is the embryonic development, the lesser is multiple pregnancies' rates. Cattle refers to oligocarpous category, whereupon the effectiveness of reproduction in cattle acquires an important economic value [8]. Growth of milk yields in cows is often accompanied by decrease of its adaptive potential and decrease of reproduction effectiveness [9]. Moreover, animal genotypes have a great effect on reproduction indicators [10]. Spontaneous mutations occurring in the seed bulls may be widely spread across the breed in cattle of highly-specialized milk breeds, such as Holstein, which are under the intensive pressure of artificial selection [11-13]. In particular, such mutations include BLAD (bovine leukocyte adhesion deficiency associated with death in calves aged under 1 year) and CVM (complex vertebral malformation) [14]. Reproduction indicators in cattle are not only influenced by physiological but also anthropogenic factors [15]. Thus, infertility and dry stat of cows are negative consequences of improperly performed insemination [16].

Infertility in both sexes is a loss of animal ability to reproduce because of a dysfunction in mature organism. Temporary (reversal) and persistent (irreversal) infertility is distinguished in cows [17]. At that, genetic infertility happens quiet rarely since from the biological point of view there are no animals with genetically limited reproduction indicators [18, 19]. Annually, each infertile cow produces less milk products (nearly 3 kg of milk per each day of infertility). Signs of infertility in cows and young cows are manifested in longstanding lack of estrum, availability of infertile inseminations [20]. Usually, 17-23 % of animals are involved in estrum during the month 1, 48-55 % during month 2, and 18-22 % during month 3 after calving. Interval from calving to the first estrum is 2 months. Therefore, each cow failing to be fertilized during 60 days after calving should be deemed infertile.

Effective prevention of infertility and dryness are important to increase cattle livestock, milking capacity, and to optimize reproduction [20, 21]. Dryness is an economic parameter applied to cattle females only [22, 23]. Dryness is mainly caused by innate infertility, false identification of estrum and untimely insemination, lack of motion, low quality of feed, infringement of insemination technology, and use of pure quality sperm. Infertility and dryness may be also caused by infection diseases [24, 25] and genetically determined disorders of reproductive health [26]. High yieldness in stud stock results in production of less milk, calves, and causes significant economic damage. In breeding farms allowing for 10 % infertility in cows at milk yield of 2500-3000 kg, milk yield is reduced by 5 % and consumption of feed per unit of produced milk increases. Increased duration of infertility in cows by 1 day decreases the annual milk in cows by 6 kg. For preservation of the high calving it is required to optimize the insemination terms and animal selection for mating [27], to use vitamin and mineral additives improving the reproduction indicators [28].

At embryo formation in humans, antigens of mother and father blood groups affect the immunologic balance [29, 30]. Similar tests were conducted in animals. It was noted that several loci and blood group antigens in cattle also affect fertilization and gestation courses [31]. S.L. Gridina et al. [32] had estab-

lished that fertilization in cows depends on antigen combination. Fertility is increased at absence of J antigen, homozygosity of parents by antigens Y_2 and G_2, and heterozygosity by antigens A_2 and S_2. Presence of A_1, B_2, G_3, I_2, O_2, A^I_2, G^I, R_2, W, X_2, and S antigens in blood of bulls and absence thereof in cows promotes growth of fruitful insemination frequency. S.L. Gridina et al. [33] and V.F. Gridin et al. [34] had found alleles (cohesively inherited antigens) of cattle blood groups controlling the economically useful traits. Studies of I.M. Starodumov et al. [35] and L.V. Kerro [36] had shown that blood groups may be used for selection and breeding, as well as for improvement of economically useful traits in cattle.

The present paper for the first time shows correlation between the antigen similarities in cattle pairs of Ural breed and reproduction properties, and also indicates that the animal blood groups are unequally associated with different traits.

Purpose of our paper is studying the relationship between the inheritance of blood antigens and reproductive indicators in groups of Ural Holstein Black Pied cattle.

Techniques. Study was conducted from 2009 to 2014 in Sverdlov Region in Mezenskoye LLC (Beloyarskiy Region), Agricultural Breeding Cooperative Pervouralsky (Pervouralsk urban district), and Agrofirm Artyemovsky LLC (Artyemovsky Region) in 1300 pairs of Black Pied Holsteins of Ural type. Tie-up housing of cattle with milking through milk line is used by the above enterprises. Cattle were fed according to standard diets. Cattle groups included cows from the 1st to 2nd calving and servicing bulls. These were 200 cows and 10 bulls in Mezenskoye LLC, 400 cows and 11 bulls in Agricultural Breeding Cooperative Pervouralsky, and 900 cows and 15 bulls in Agrofirm Artyemovsky LLC. Pairs for artificial insemination were formed out of these animal groups.

Blood groups were assayed as per P.F. Sorokov [37]. Material for analysis was collected once from the tail vein by single-use vacuum blood collection system in animals aged over 6 months. Blood antigens were found by hemolysis test (dissolution of erythrocytes under the effect of specific antiserum at presence of complement). Antiserums were produced at immunization of recipient animals by donor blood; complement was collected from the rabbit blood serum. Total 54 monospecific serums were used for identification of 11 blood groups systems.

Index of antigen similarity of cows with mated bulls was the main cow grouping criteria based on the aggregate of blood antigens. Index of antigen similarity C_{as} was calculated by formula of S.I. Shadmanov [38]:

$$C_{as} = (A_{bi} + A_{ci})/(A_b + A_c),$$

where A_{bi} and A_{ci} are the amounts of similar mother and father antigens, A_b is the number of identified father antigens, A_c is the number of identified mother antigens.

Homozygosity in parent pairs was accounted by blood group systems EAA, EAF, and EAZ.

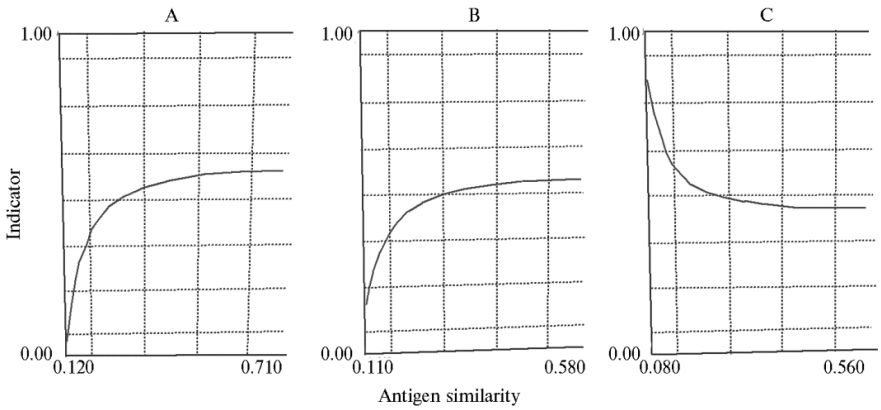
The following values were accounted for at assessment of the reproductive properties in cows: period from calving to successful insemination, fertilization index, number of abortions, survivability of calves, and multiple pregnancies.

Software packages Snedecor V3.5 and Microsoft Excel were used for biometrical processing. Mean values of reproduction indicators by samples (*M*), standard errors of the mean (±SEM) were used, statistical significance was assessed at $p \le 0.05$; $p \le 0.01$, and $p \le 0.001$, and correlation coefficients between the studied reproduction indicators and antigen similarity of pairs were calculated.

Results. Fertility is genetically transmitted, whilst propensity for abortions and dead-born calves is also conditioned upon the genetic and immunologic fac-

tors [10, 17, 18, 29]. Total 3 % of dead-born calves and 2 % of abortions were registered in population of Agricultural Breeding Cooperative Pervouralsky amongst the calves, 5 and 2 % were registered in Mezenskoye LLC, and 2 % and 0.2 % were registered in Agrofirm Artyemovsky LLC. Abortions and dead-born calves, even in such insignificant quantities, decrease the calf yield due to which breeding enterprises suffer economic losses. Simultaneous birth of two and more female calves results in growth of the number of calves by 100 cows and is a desired sign.

We have revealed weak positive relations between the antigen similarity in pairs and abortions ($r = 0.2$, $p \leq 0.05$), as well as dead-born calves ($r = 0.1$, $p \leq 0.05$) and negative correlation between antigen similarity in pairs and birth of twins ($r = -0.14$, $p \leq 0.05$). Loss of calves may be slightly reduced and birth of twins may be slightly increased at mating of bulls and cows with high antigen similarity [35]. An observed effect of the antigen similarity on survivability of calves is 1-4 %.



Reproductive indicators in of Black Pied Holstein Ural cattle depending on antigen similarity of parent pairs: A — abortions, B — dead-born calves, C — birth of twins (Sverdlov Region, 2009-2014).

Figure (A) displays the dynamic changes in survivability of calves depending on antigenic similarity of mated animals. Change of function from 0.00 to 1.00 shows the growth in abortion probability. Number of abortions had sharply increased at index of antigen similarity in pairs of up to 10 %. With increase of the antigen similarity indicator (> 20 %), survivability of calves was growing in stepwise manner. At the same time, probability of dead-born calve had increased with increasing of the antigen similarity (see Fig., B). Significant growth in dead birth frequencies is established at antigen similarity of up to 10 %, whereas at further growth of C_{as} dead birth frequency is slightly growing. Calf loss trend at close antigen similarity is, apparently, due to the fact that recessive parent alleles with unfavorable phenotype effects merge into homozygotous state as it occurs, for instance, in case of BLAD and CVM [14]. Increase of antigen similarity in bull and cow pairs had also resulted in decrease of twin birth frequency (see Fig., C) (0.00 at graph means absence of multiple pregnancies, 1.00 means birth of twins). At similarity index of < 0.1, number of twins was maximum. At increase of antigen similarity in parents to values of over 10 %, probability of twin birth slightly reduces.

Index of antigen similarity in the mated animals not exceeding 0.10 is optimal to decrease the calf loss and to promote twin birth. Regardless of weak correlation, the entire sample shows trend towards the better survivability of calves at antigen similarity index in bull and cow of 10 %. The use of cattle pairs with 11-20 % similar antigens gives a noticeable loss of calves.

An increase of propensity for spontaneous abortions and dead-burn calves is characteristic of some cows [20, 39]. It is necessary to identify and withdraw them from the population to increase the calf yield by 100 cows. Conversely, animals propensive for birth twins are selected for breeding. Daughters of such cows are also more propensive for birth twins [22]. This approach promotes health care and growth of cattle population at exception of free martinisme cases (different sex twins). Besides, it is required to select cows with significant milk yields for production of breeds with the highest milk yield. Such indicator is affected by genetic and feeding factors. It should be noted that sensitivity in cows to environment factors growth with increase of the genetic productivity potential in cows [40].

Antigen similarity, surely, affects the reproduction and production indicators in animals. At that, separate antigens or blood group systems often control certain traits significantly more than others [31]. Similarity in parents in one system may result in improvement of indicators, whereas may result in deterioration in the other one. We have analyzed the relationships between homo- and heterozygosity by A, F/V, Z blood group systems and duration of the period from calving to successful insemination by fertilization index and calf survivability index. These systems consist of one-two antigens. This enables us to track presence or absence of immunological conflict between the mother cow and calf by certain antigens.

Among the studied animals we revealed A_1 and A_2 antigens of EAA blood group. The following mother and father antigen combinations are found: homozygotes A_1/A_1, A_2/A_2, A_1A_2/A_1A_2, no antigens (–/–); heterozygotes A_1/A_2, A_1A_2/A_2, A_1A_2/A_1, $A_1A_2/–$, $A_1/–$, $A_2/–$. Period from calving to successful insemination for EAA homozygous animals from Agricultural Breeding Cooperative Pervouralskiy and Argofirm Artyemovskiy LLC is longer compared to that for heterozygous ones (see Table). Abortions and dead-born calves amongst homozygous pairs are more frequent (by 2.5 and 0.3 %, respectively) than amongst heterozygotes, that is due to a greater genetic similarity of EAA system parent antigens. At the same time, number of twins from homozygous pairs is significantly higher (3.3 %).

EAF system in the sample consists of F and V antigens. Antigen F is practically found in all animals of the sample, while antigen V is present only in few animals. Both blood antigens are rarely found. The sample contains homozygotes F–/F–, V–/V–, FV/FV, –/– and heterozygotes F–/V–, FV/F, FV/V, FV/–. As to EAF system, the homozygous pairs in all studied enterprises are more numerous than heterozygous pairs (see Table). Survivability of calves from homozygous pairs was less than from heterozygous. Frequencies of abortions, dead-born calves and multiple pregnancies in homozygous pairs are 1.9; 4.8, and 2.9 %, respectively. That is, selection of EAF heterozygous pairs leads to better survivability of calves. However, frequency of twin births was 0.7 % lower. Most healthy calf was produced in combination of F or F/V antigens from bull and V antigen from cow. Insufficient heterogeneity of blood groups results in calf loss. High frequency of twin birth was at mating bulls and cows with the same EAF system antigens.

EAZ blood system antigen groups among the studied animal populations were heterozygotes Z/– and homozygotes Z/Z, –/–. Total number of pairs homozygous for EAZ blood system antigens in all populations was 442, that is 102 more compared to heterozygous pairs (see Table). Period from calving to successful insemination among homozygous pairs from Argofirm Artyemovskiy LLC is 90 days that is 3 days shorter compared to heterozygous ones. In ABC Pervouralskiy, this period in heterozygous pairs was also longer than in homozy-

gous pairs by 6 days. That is, we have revealed the trend towards a decrease in the interval from calving to successful insemination in mating animals homogenous for EAZ system. According to V.K. Milovanov at al. [39], immunologic conflict occurring due to lack of Z antigen in one parent and presence in the other parent results in insemination problem. There are cases when at insemination embryo is lost in the first months of pregnancy. Among the studied populations, abortions and dead births are more often from EAZ-heterozygous pairs.

Reproductive indicators depending on combinations of parents by blood group systems in Black Pied Holsteins of Ural type (*M*±SEM, Sverdlov Region, 2009-2014)

Indicator	Mezenskoye LLC	ABC Pervouralskiy"	Agrofirm Artyemovskiy LLC	Total, mean value
EAA system				
Homozygous pairs				
Number of pairs	26	40	113	179
Period from calving to successful insemination, days	87.0±2.0	106,0±3,0*	93,0±2,0	95,0±1,0
Fertilization index	1.30±0.10	1,17±0,30	1,20±0,20**	1,22±0,30
Number (%) of abortions		6 (3.3 %)		
Number (%) of dead-born calves		7 (3.9 %)		
Multiple pregnancy (twins), number (%)		6 (3.3 %)		
Heterozygous pairs				
Number of pairs	95	88	421	604
Period from calving to successful insemination, days	90.6±2.0	82,9±3,0	90,9±1,0	88,1±3,0
Fertilization index	1.32±0.10	1,13±0,20	1,14±0,10	1,19±0,10
Number (%) of abortions		5 (0.8 %)		
Number (%) of dead-born calves		22 (3.6 %)		
Multiple pregnancy (twins), number (%)		17 (2.8 %)		
EAF system				
Homozygotous pairs				
Number of pairs	67	81	269	417
Period from calving to successful insemination, days	83.0±1.0	95,0±3,0**	92,0±2,0	90,0±2,0
Fertilization index	1.32±0.20	1,13±0,20	1,14±0,20	1,19±0,10
Number (%) of abortions		8 (1.90 %)		
Number (%) of dead-born calves		21 (4.80 %)		
Multiple pregnancy (twins), number (%)		12 (2.90 %)		
Heterozygous pairs				
Number of pairs	54	47	262	363
Period from calving to successful insemination, days	94.0±3.0	70,0±2,0	92,0±3,0	85,5±2,0
Fertilization index	1.43±0.10***	1,12±0,10	1,12±0,20	1,22±0,30
Number (%) of abortions		2 (0.55 %)		
Number (%) of dead-born calves		8 (2.20 %)		
Multiple pregnancy (twins), number (%)		8 (2.20 %)		
EAZ system				
Homozygotous pairs				
Number of pairs	67	72	303	442
Period from calving to successful insemination, days	88.0±2.0	80,0±1,0	90,0±3,0	86,0±3,0
Fertilization index	1.34±0.30	1,22±0,10	1,10±0,20	1,22±0,20
Number (%) of abortions		6 (1.4 %)		
Number (%) of dead-born calves		14 (3.2 %)		
Multiple pregnancy (twins), number (%)		6 (1.4 %)		
Heterozygous pairs				
Number of pairs	54	56	230	340
Period from calving to successful insemination, days	87.0±3.0	84,0±2,0	93,0±2,0	88,0±3,0
Fertilization index	1.34±0.10	1,19±0,10	1,18±0,30	1,37±0,20
Number (%) of abortions		5 (1.5 %)		
Number (%) of dead-born calves		16 (4.7 %)		
Multiple pregnancy (twins), number (%)		6 (1.8 %)		

, **, * Differences between the heterozygous and homozygous pairs are statistically significant at $p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$, respectively.

Thus, reproductive properties of Holstein Black Pied cattle depend on blood group similarities of the parents. Association of blood group system antigens with a certain trait can be more pronounced compared to other traits. At similarity of the parents, one blood antigen system can be associated with improved reproduction, whereas the other one have negative effects. There is a trend towards better survivability of calves from heterozygous pairs for EAZ system (i.e. a 2.5-2.7 % reduced number of dead-born calves and 0.3-1.4 % less abor-

tions). Apparently, it is an immunologic conflict that results in death of calves. We also have discovered trend towards improved survivability of the calves and an increase in animal populations at 10 % antigenic similarity of parents.

REFERENCES

1. Palaniappan S. Blood grouping in animals. *The Hindu Online edition of India's National Newspaper*, 2004, 9: 61.
2. Shukyurova E.B. *Open Scientific Bulletin*, 2014, 1: 1-5 (in Russ.).
3. Foster M. Genomanaliyse. In: *Tierzucht und allgemeine Landwirtschaftslehre fur Tiermediziner*. H. Kräusslich, G. Brem (eds.). Enke, 1997: 77-109.
4. Buvaeva N.V. *Ispol'zovanie grupp krovi v selektsii krupnogo rogatogo skota kalmytskoi porody. Avtoreferat kandidatskoi dissertatsii*. [Use of blood groups in the selection of cattle of Kalmyk breed. PhD Thesis]. Stavropol', 2012 (in Russ.).
5. Charoensook R., Knorr C., Brenig B., Gatphayak K. Thai pigs and cattle production, genetic diversity of livestock and strategies for preserving animal genetic resources. *Maejo Int. J. Sci. Tech.*, 2013, 1: 113-132.
6. Serdyuk G.N., Katalupov A.G. *Zootekhniya*, 2008, 8: 8-10 (in Russ.).
7. Neves J.P., Miranda K.L., Tortorella R.D. Scientific progress in reproduction research during the first decade of XXI century. *Revista brasileira de zootecnia*, 2010, 1: 414-421 (in Russ.).
8. Van Eetvelde M., Heras S., Leroy J.L.M.R., Van Soom A., Opsomer G. The importance of the periconception period: immediate effects in cattle breeding and in assisted reproduction such as artificial insemination and embryo transfer. In: *Periconception in physiology and medicine. Advances in experimental medicine and biology*. A. Fazeli, W. Holt (eds.). Springer, Cham, 2017, V. 1014: 41-68 (doi: 10.1007/978-3-319-62414-3_3).
9. Shkuratova I.A., Donnik I.M., Nevinnyi V.K., Shusharin A.D., Vereshchak N.A., Ryaposova M.V., Belyaev I.P., Sbitnev E.V. *Veterinariya*, 2007, 7: 14-15 (in Russ.).
10. Purfield D.C., Bradley D.G., Berry D.P., Evans R.D., Kearney F.J. Genome-wide association study for calving performance using high-density genotypes in dairy and beef cattle. *Genet. Sel. Evol.*, 2015, 1: 47 (doi: 10.1186/s12711-015-0126-4).
11. Oleshko V.P., Babenko E.I. *Tekhnologiya virobnitstva i pererobki produktsiї tvarinnitstva*, 2014, 1: 66-69 (in Russ.).
12. Fisher M., Small B., Roth H., Mallon M., Jerebine B. What do individuals in different science groups within a life sciences organization think about genetic modification? *Public Underst. Sci.*, 2005, 3: 317-326 (doi: 10.1177/0963662505048594).
13. Rodríguez F.M., Salvetti N.R., Panzani C.G., Ortega H.H., Rey F., Barbeito C.G. Influence of insulin-like growth factor-binding proteins-2 and -3 in the pathogenesis of cystic ovarian disease in cattle. *Anim. Reprod. Sci.*, 2011, 1-4: 1-10 (doi: 10.1016/j.anireprosci.2011.08.007).
14. Kumar V., Chakravarty A.K. Genetic disorders in dairy cattle: an Indian perspective. *Indian J. Anim. Sci.*, 2015, 8: 819-827.
15. Shendakov A. Improvement system of biological factors management in the breeding of farm animals. *Russian Journal of Agricultural and Socio-Economic Sciences*, 2012, 12: 3-18.
16. Masalov V.N., Enin Yu.M., Sinitsyn A.N., Kozlov A.S. *Vestnik Orlovskogo gosudarstvennogo agrarnogo universiteta*, 2007, 1: 23 (in Russ.).
17. Gukezhev V.M., Gabaev M.S., Batyrova O.A. *Agrarnyi vestnik Urala*, 2012, 7: 42-44 (in Russ.).
18. Flisikowski K., Schwarzenbacher H., Fries R., Venhoranta H., Flyckt A., Taponen J., Andersson M., Nowacka-Woszuk J., Szczerbal I. Switonski M., Mckay S.D., Schnabel R., Taylor J.F., Lohi H. A novel mutation in the maternally imprinted *PEG3* domain results in a loss of *MIMT1* expression and causes abortions and stillbirths in cattle (*Bos taurus*). *PLoS ONE*, 2010, 11: e15116 (doi: 10.1371/journal.pone.0015116).
19. Wolf E., Bauersachs S. Functional genome research in reproductive biology and biotechnology — a minireview. *Animal Sci. P.*, 2010, 2: 123-132.
20. Gabaev M.S., Gukezhev V.M. Plodovitost' i plemennaya tsennost' korov. *Agrarnyi vestnik Urala*, 2011, 7: 33-34.
21. Musallyamova M.F., Antonova N.V. Calculation of product costs of dairy cattle breeding in Russia. *Mediterranean Journal of Social Sciences*, 2014, 24: 403-406 (doi: 10.5901/mjss.2014.v5n24p403).
22. Pronina M.Yu. *Uchetnye zapisi Kazanskoi gosudarstvennoi akademii veterinarnoi meditsiny imeni N.E. Baumana*, 2012, 12: 358 (in Russ.).
23. Ilshatovna S.A., Vildanovna N.R. Application of fuzzy-set and multiple approaches in evaluation of effectiveness of agricultural industry enterprises activities (as an example of animal breeding of the Republic of Tatarstan). *Biosciences Biotechnology Research Asia*, 2014, 11: 37-42.
24. Smith R.L., Groen Y.T., Strawderman R.L., Schukken Y.H., Pradhan A.K., Wells S.J., Espejo L.A., Whitkock R.H., Van Kessel J.S., Smith J.M., Wolfgang D.R. Effect of Johnes disease status on reproduction and culling in dairy cattle. *J. Dairy Sci.*, 2010, 8: 3513-3524 (doi: 10.3168/jds.2009-2742).

25. Bozymov K.K., Nassambayev E., Bayakhov A.N., Upievich B.Y., Sultanova A.K. Experience of using ultrasonography in the diagnosis of cattle reproductive track diseases in the West Kazakhstan region. *Biomedical and Pharmacology Journal*, 2015, 1: 21-25 (doi 10.13005/bpj/577).
26. Wickramasinghe S., Rincon G., Medrano J.F. Variants in the pregnancy-associated plasma protein-a2 gene on *Bos taurus* autosome 16 are associated with daughter calving ease and productive life in holstein cattle. *J. Dairy Sci.*, 2011, 3: 1552-1558 (doi: 10.3168/jds.2010-3237).
27. Goncharenko I.V., Vinnichuck D.T. *Haukovii visnik NUBiP Ukraïni. Seriya: Tekhnologiya virobnitstva i pererobki produktsiï tvarinnitstva*, 2015, 205: 264-273 (in Russ.).
28. Bushueva İ.V., Knysh E.G., Panasenko O.İ. Doslidzhennya likuval'noï efektivnosti morfolinii 2-[5-(piridin-4-il)-1,2,4-triazol-3-iltio] atsetatu pri khvorobakh deyakikh vidiv tvarin. *Sciencerise*, 2014, 1: 100-104.
29. Aleshkin V.A., Lozhkina A.N., Zagorodnyaya E.D. *Immunologiya reproduktsii* [Immunology of reproduction]. Chita, 2004 (in Russ.).
30. Pfeffer P.L., Pearton D.J. Trophoblast development. *Reproduction*, 2012, 3: 231-246 (doi: 10.1530/REP-11-0374).
31. Politkin D.Yu. *Zootekhniya*, 2011, 5: 6-7 (in Russ.).
32. Gridina S.L., Shatalina O.S. *Dostizheniya nauki i tekhniki APK*, 2011, 6: 69-70 (in Russ.).
33. Gridina S.L., Romanenko G.A. *Vestnik Kurganskoi GSKHA*, 2013, 4: 29-31 (in Russ.).
34. Gridin V.F., Tkachenko I.V. Gridina S.L. *Vestnik Kurganskoi GSKHA*, 2013, 1: 140-42 (in Russ.).
35. Starodumov I.M., Panina S.V. *Estestvennye i tekhnicheskie nauki*, 2008, 4: 96-97 (in Russ.).
36. Kerro L.V. *Ispol'zovanie antigenov grupp krovi v plemennoi rabote s cherno-pestrym skotom Zaural'ya. Avtoreferat kandidatskoi dissertatsii* [Use of blood group antigens in breeding black-motley cattle of the Trans-Urals. PhD Thesis]. Kurgan, 2006 (in Russ.).
37. Sorokovoi P.F. *Metodicheskie ukazaniya po issledovaniyu i ispol'zovaniyu grupp krovi v selektsii krupnogo rogatogo skota* [Methodological guidelines for the study and use of blood groups in cattle breeding]. Dubrovitsy, 1974 (in Russ.).
38. Shadmanov S.I., Stolbov V.M., Pepina G.D. V sbornike: *Novoe v razvedenii i genetike sel'skokhozyaistvennykh zhivotnykh* [New facets in the breeding and genetics of farm animals]. St. Petersburg, 1973 (in Russ.).
39. Milovanov V.K., Sokolovskaya I.I. *Zhivotnovodstvo*, 1964, 6: 75-83 (in Russ.).
40. Voroshilova E.D. *Sibirskii vestnik sel'skokhozyaistvennoi nauki*, 2007, 1: 120-121 (in Russ.).

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COMPARISON OF SEMEN CHARACTERISTICS IN ROMANOV AND Lori Bakhtiari RAMS

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Abstract

One of the important aims in animal breeding is using the advantages of bio diversity. Romanov sheep is Russian native breed and has prominent features, such as high percent of twinning. Lori-Bakhtiari breed is dual-purpose (meat and wool) in Iran. Regarding the governmental policies, confluence between these two breeds is on the agenda. The aim of this research is to study comparatively some of the sperm features of rams in both breeds in terms of concentration, motility, morphometry. Semen samples of Lori and Romanov rams were collected in husbandry research station known as Shuli in Chaharmahal Bakhtiari Province in Iran. In this experiment, sperm parameters in Romanov ram including large diameter, small diameter, perimeter and area of head, tail length, mid piece and end piece length were estimated as 9.16±0.37 μ, 4.77±0.41 μ, 24.47±0.97 μ, 24.89±1.94 μ^2, 57.59±1.50 μ, 15.40±1.20 μ and 42.19±2.10 μ, respectively. A significant difference was observed between the large diameter and head area of sperm, while the small diameter and perimeter of sperm were similar. Motility of type A in Romanov rams was significantly higher. The tail and mid piece of Romanov sperms was significantly taller than these parts in Lori's rams. It seems that taller length of the mid piece and more mitochondria concentration are the causes of higher motility in Romanov spermatozoa.

Keywords: ram, Romanov breed, Lori Bakhtiari breed, sperm, motility, morphology

Iran also has numerous comparative advantages in terms of animal husbandry and products in such a way that every domestic animal nearly can be reared and maintained. This advantage has led to place Iran as one of the most lucrative and important countries. Of the goals of the animal breeding institutions is to apply cross breeding in order to use the genetic potentials and increase livestock resistance. Advancements could be seen considerably in the first generation. Different goals are determined due to the environmental conditions for sheep management. Those areas in where wool production is most important, crossbreeding program tends to select wool breeds. In areas where meat is more important economically, those breeds are used which have high growth rate, feed efficiency and meat quality [1]. Exploiting the most production potential of sheep is the only way which is profitably suitable and explainable [2].

Romanov sheep is Russian native breed and imported to France for research purposes by IRNA (French National Institute for Agricultural Research) research center in 1963 for the first time. This breed is premature and has outstanding characteristics such as a high percentage of twinning, high maternal instinct and high physical ability of the newborn lambs. This breed has rather tall legs. The other important aspect of Romanov is high resistant of ewes and lambs and their vigor. Lambing is easy. The mean weight of Romanov ram and

ewe are 55-80 kg and 40-50 kg, respectively. Rams have aggressive behavior during breeding, and they have sexual activity in all of seasons [3].

Lori Bakhtiari sheep is a heavy and outstanding breed in terms of the production features in Iran. They are reared in the south-west of Iran, especially in Chaharmahal Bakhtiari province. The main place of its rearing is Chaharmaahal Bakhtiari. For its top features, it is reared in the neighboring provinces such as Isfahan, Lorestan, Kohgiloye va Boyerahamd and Khuzistan. It's ewe known as Haftlang. Its fat tail is big and deep. Fat tail is a groove cause to divide and suspense. It's divided into two completely separate sections (Fig. 1). Sometimes the end of the fat tail is placed in the lower hocks [3].

Fig. 1. Rams of the local Iranian breed Lori Bakhtiari (left) **and a crossbred animal F_1** (right). A very long fat tail characteristic of the breed is considerably shortened in the offspring from crossing.

Exposing Romanov ram to the sun decreased sperm volume, total motility, individual motility, concentration, viability and normal sperms. Testis length and its diameter in Romanov ram were higher than the native Finns [4]. The color of semen is dependent upon the sperm concentration and varied from cream to white. If the sperm concentration is high in the semen, the semen color tends to be cream and if the sperm concentration in semen is low, its color tends to be white and watery [5]. Studies show that there is a direct relationship between the number of live sperms and pregnancy [6].

It is planned in order to produce the hybrid of Romanov breed and reduce some features of Lori Bakhtiari breed such as the large size of fat tail and low twining [7]. There is a project for the possibility of crossbreeding between Romanov ram and Lori Bakhtiari ewes (see Fig. 1). There is not any study about morphologic parameters in Lori Bakhtiari semen and Romanov ram in Iran.

The aim of this research was the comparison of the semen parameters of both breeds in terms of volume, concentration, motility and sperm morphometry.

Techniques. Rams were managed in the research station of Jahad Keshavarzi Organization in Chaharmahal Bakhtiari province — Iran at the same feeding and maintenance conditions. A total of 24 rams aged 2-3 years, were kept in the husbandry research station known as Shuli station which 12 of them were Lori Bakhtiari and the other 12 were Romanov rams. Shuli belongs to Jahad Keshavarzi Organization in Chaharmahal Bakhtiari province with longitude 50°, latitude 32° and the height of 2017 meters above the sea level. With the help of artificial vagina semen samples were collected. Semen was stored at 36 °C to prevent the cold shock. Samples were transferred in the laboratory for semen analysis.

Fresh semen was diluted with Hepes TCM 199 (Tissue Culture Medium, Sigma-Aldrich, Inc., USA) in proportion 1:250. From diluted sample 5μl was placed on Makler Counting Chamber (New York Microscope Company, Inc., USA) and evaluated under the microscope using CASA (Computer-aided sperm

analysis) software. To study the sperm morphology, smear was prepared. For smears staining, Rapid Sperm Staining (RSS) Differentiation quick kit was used. This kit contains three solutions of A, B and C. At first, solution A was poured on the smear in such a way that the smear surface is completely covered. After 75 seconds, it is evacuated and the surface of smear was covered with B solution for 60 seconds and evacuated. Finally, C solution was poured on the smear for 15 sec and it was rinsed with a very slow water flow. Smears were dried completely at room temperature. After staining stage, about 100 sperms in the field of microscope equipped with Isc capture program were examined in ×40 lenses. Different parameters, including area, perimeter, large and small diameter of sperm head, different parts of the sperm body and tail were determined.

Mean (M), standard errors of mean (±SE) and statistical significance (P) of the differences between the studied parameters in the two rocks were calculated.

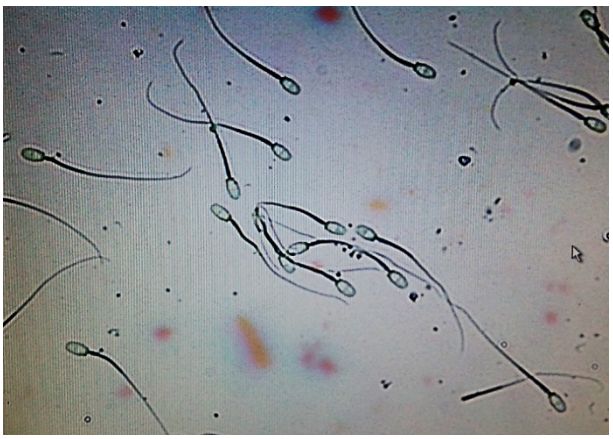

Fig. 2. Sperm of Lori Bakhtiari ram (magnification lenses ×20).

Results. Figure 2 shows the sperm samples under the microscope with ×20 magnifications in Lori Bakhtiari ram.

Different characteristics of semen samples of Lori Bakhtiari and Romanov rams are shown in Table 1. Morphological parameters and motility features of sperm between two breeds of Romanov and Lori Bakhtiari were compared.

Morphological parameters of experiment, including large diameter, small diameter, perimeter (μ) and area (μ^2) related to the sperm head are shown in Table 2. The length of tail (μ), length of the mid piece and length of end piece are related in the body of sperm. The results of comparing of sperm morphology in Lori and Romanov rams showed that there was a significant difference between large diameter and the head area of sperm ($P < 0.01$). There was not any significant difference between the small diameter and the perimeter of the head sperm between these two breeds. There was a significant difference between the sperm tail and the length of mid piece while there was not any significant difference between two breeds in the end piece (see Table 2).

Sperm motility class A, B, C, D, actual sperm velocity, straight movement, mean of the straight movement (μ/sec), mean of rotation angle, maximum range of lateral motility (μ), frequency of lateral motility (Hz), percent of linear movement, percent of rotation angle and standard percentage of straight motility are among sperm motility. The results are shown in Table 3.

Comparing the mean of sperm parameters in Lori Bakhtiari and Romanov rams indicated that sperm concentration, percent of sperm motility, percent of proceeding sperm, percent of sperm motility class A, sperm motility class D, real velocity of sperm, sperm velocity in the straight line, mean of velocity in the straight line, mean rotation angle and frequency of lateral motility had a significant difference in both breeds ($P < 0.05$). There is a significant relationship between the percent of sperm motility Class B, percent of sperm motility Class D, percent of linear of sperm motility, mean rotation angle and percent standard

straight sperm motility in both breeds (see Table 3).

1. Some parameters of ram and semen analysis in Romanov and Lori Bakhtiari rams (Shuli, Chaharmahal Bakhtiari, Iran)

Breed	Age, years	Ram color	Ram No	Replication of semen sample	Semen color	Sperm concentration, $\times 10^{12}$/ml
Lori Bakhtiari	3.0	White	885954	1	Beige	2.2
Lori Bakhtiari	2.5	White	917163	1	Beige	1.6
Lori Bakhtiari	2.5	White	865821	1	Beige	1.0
Lori Bakhtiari	2.5	White	917163	2	Beige	1.5
Lori Bakhtiari	2.5	White	865821	2	Beige	1.8
Lori Bakhtiari	3.0	White	885954	2	Beige	2.0
Lori Bakhtiari	3.0	White	885954	3	Beige	1.9
Lori Bakhtiari	2.5	White	917163	3	Beige	1.6
Lori Bakhtiari	2.5	White	865821	3	Beige	1.6
Lori Bakhtiari	2.5	White	865586	1	Beige	1.6
Lori Bakhtiari	2.5	White	865586	2	Beige	1.5
Lori Bakhtiari	2.5	White	865586	3	Beige	1.6
Romanov	2.0	Black	50202	1	Beige to white	1.8
Romanov	2.0	Black	50202	2	Beige to white	1.5
Romanov	2.0	Black	50202	3	Beige to white	1.6
Romanov	2.0	Black	52028	1	Beige to white	1.7
Romanov	2.0	Black	52028	2	Beige to white	1.6.
Romanov	2.0	Black	52028	3	Beige to white	1.7
Romanov	2.0	Black	52017	1	Beige to white	1.6
Romanov	2.0	Black	52017	2	Beige to white	1.4
Romanov	2.0	Black	52017	3	Beige to white	1.6
Romanov	2.0	Black	50141	1	Beige to white	1.8
Romanov	2.0	Black	50141	2	Beige to white	1.5
Romanov	2.0	Black	50141	3	Beige to white	1.6

2. Comparison of morphological parameters in semen sample of Lori Bakhtiari and Romanov rams (M±SE, Shuli, Chaharmahal Bakhtiari, Iran)

Parameter	Lori Bakhtiari	Romanov	P
Large diameter, μ	8.10±0.37	9.16±0.37	0.000
Small diameter, μ	4.63±0.33	4.77±0.41	0.130
Perimeter, μ	24.29±1.50	24.47±0.97	0.570
Area, μ^2	26.97±1.18	24.89±1.94	0.000
Tail length, мк	56.11±1.90	57.59±1.50	0.007
Middle piece, μ	14.11±1.10	15.40±1.20	0.000
End piece, μ	42.01±2.60	42.19±2.10	0.750

3. Comparison of sperm characteristics in Lori Bakhtiari and Romanov rams (M±SE, Shuli, Chaharmahal Bakhtiari, Iran)

Parameter	Lori Bakhtiari	Romanov	P
Concentration, $\times 10^6$/мл	5308.8±0.15	6764.4±1.2	0.028
Sperm motility, %	39.24±0.80	59.47±1.60	0.048
Percent of proceeding sperm	33.55±0.42	52.37±2.10	0.042
Sperm motility class A, %	21.47±1.10	38.26±1.20	0.320
Sperm motility class B, %	12.07±1.90	14.11±0.60	0.490
Sperm motility class C, %	5.68±1.90	7.09±1.60	0.290
Sperm motility class D, %	60.76±0.34	40.53±0.72	0.048
Actual sperm velocity, μ/sec	38.87±0.42	58.95±1.70	0.019
Straight movement, μ/sec	24.12±1.40	41.08±0.30	0.027
Mean of the straight movement, μ/sec	27.87±1.60	45.82±0.36	0.025
Mean of rotation angle, °	9.27±1.60	15.17±1.50	0.047
Maximum range of lateral motility, μ	1.72±1.40	2.23±1.90	0.030
Frequency of lateral motility, Hz	0.41±0.22	0.74±0.36	0.022
Linear movement, %	36.63±0.65	48.26±2.00	0.086
Rotation angle, %	50.15±0.72	60.49±1.50	0.089
Standard of straight motility, %	52.79±1.20	63.29±1.60	0.069

According to Table 3, the highest number of sperm was obtained in Romanov ram (6764.4 million/ml vs. 5308.8 million/ml). The highest percentage of motile sperm was in Romanov (59.47 % vs. 39.24 %). The highest percentage of proceeding of sperm was related to Romanov (52.37 % vs. 33.55 %). The highest percentage of sperm motility in Class A was seen in Romanov ram (38.26 % vs.

21.47 %). The highest real velocity of sperm was in Romanov (58.95 μ/sec vs. 38.87 μ/sec). Sperm velocity in straight line (μ/sec) was higher in Romanov (45.82 μ/sec vs. 27.87 μ/sec). The highest rotation angle of sperm was observed in Romanov (15.13° vs. 9.27°). Sperm lateral motility was higher in Romanov ram (2.23 μ vs. 1.73 μ) (see Table 3).

Morphology of sperm has a great influence on sperm motility and ovum fertilization. Normally, at least 30 % of sperms should have a normal shape [8]. The abnormality of sperm could have different forms. For example twin heads, two tail and microcephlic sperm. Abnormal sperm is not able to move in a balanced way and these sperms cannot fertile the ovum [9]. In this experiment, some abnormal forms such as sperm bent tail were seen in the semen sample of Romanov rams.

Semen color is one of the important indicators determining reproduction activity. Sperm color of rams and cows changes from light yellow to cream. If the concentration of sperm increases in semen, the semen color will tend to be cream and if the sperm concentration is low in semen, its color will change into watery [5]. In this experiment, the color of semen sample in Lori Bakhtiari was cream, and in Romanov was whitish. Higher concentration of sperm is in Romanov rams (6.7×1012/ml vs. 5.3×1012/ml).

Motility is the most important factor in fertility. Motility of sperm is important for its movement through the reproductive system. For this reason, there is a correlation between motility and fertility. Increasing the percent of straight motility and fertility of sperm will go up. In this experiment, there was no significant relationship between the rate of sperm motility in both breeds of Lori-Bakhtiari and Romanov in Class B and C ($P < 0.05$), while the motility in classes A and D in both breeds was significant. Sperm of Romanov breed was higher than Lori Bakhtiari breed in terms of motility A ($P > 0.05$). Using some component and time variation could change the sperm motility. In a study [10], melatonin in non-reproductive season had no significant effect on motility in Lori Bakhtiar ram. In another research [11], changes of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone hormones in Romanov and Al de France rams were studied. Results showed that FSH secretion in blood plasma had no significant difference in both breeds, while LH secretion and testosterone in Romanov were higher and its secretion started earlier. It seems that the cause of better reproduction activity performance in Romanov is the specific secretion of gonadotropins and testosterone [12]. Results from comparison of sperm motility and fresh semen of ram and the sperm motility after 24 hours in vitro showed that the motility of fresh sperms was significantly higher than the semen sample kept after 24 hours in vitro. Decreasing motility to less than 20 % will reduce fertility. The percent of motile sperms in bulls is 50-80 %. The results showed that the percent of sperm motility is high at the beginning of sample evaluation, while sperm motility reduced after a short time [13]. Some male infertile bull had high motile sperms. In fertile rams, the motility percent was 60-70 and sperms having abnormality in body and tail had poor motility [14]. In this experiment, the percent of sperm motility in Romanov ram was higher than in Lori Bakhtiari (59.47 % vs. 39.24 %). Considering a minimum motility of 60 to 70 percent for sperm, it seems that Lori Bakhtiari rams would have a low fertility rate. In this experiment, other measured factors using CASA indicate the priority of Romanov ram than Lori Bakhtiari ram. The real rate of sperm, percent of sperm proceeding, actual velocity of sperm, sperm velocity in straight line (μ/sec), mean velocity in straight line, mean rotation angle (degree), maximum range of lateral motility (μ), frequency of lateral motility (Hz), percent of

linear of sperm motility, mean percent of rotation angle and standard percent of straight line sperm motility in Romanov ram were higher than in Lori Bakhtiari ($P > 0.05$).

Morphometric study of germ cells especially sperm is very important in breed characteristics. Different methods are applied for morphometric study. J. Yániz et al. [15] investigated the morphometric features of acrosome and the nucleus of ram by staining the semen samples using florescence microscope. In the first group, some appropriate digital pictures were taken from sperm, head and nucleus using Potassium Iodide stain. The pictures were studied by a computer program of CASA. In the other group, Hoechst stain was used sperm nucleolus of ram and was investigated by CASA software. There was no significant relationship between the data from the first and second groups. Different dimensions of the sperm head were analyzed by CASA. Head length, head width, proportion of width to length, area and perimeter were estimated 8.08 r, 4.80 r, 0.59, 29.13 r^2 and 23.93 r, respectively [16]. Potassium iodine was used to compare sperm nucleuses of bull, boar and ram. Morphometric studies in ram sperm showed that acrosome of ram sperm is long and wide, while it is small in bull [17]. In this experiment, sperm parameters in Romanov ram were estimated including large diameter, small diameter, perimeter and area of head, tail length and mid piece length as 9.16±0.37, 4.77±0.41, 24.47±0.97, 24.89±±1.94, 57.59±1.5, 15.40±1.2 and 42.19±2.1 μ, respectively. Comparing data from Table 2 related to sperm morphometry of Romanov sperm and Lori Bakhtiari ram showed that head area of Romanov sperm is significantly greater than sperm head in Lori Bakhtiari ($P > 0.01$). The length of middle piece of Romanov sperm was significantly greater than Lori Bakhtiari sperm. Referring to Table 2, sperm motility of Romanov ram was higher than Lori Bakhtiari sperm. It seems more mitochondria accumulate in the mid piece of Romanov sperm (because of the longer middle part of Romanov sperm than Lori Bakhtiar). This structure causes to provide more energy for sperm motility.

Therefore, due to the comparison of motility classes of sperm in Romanov and Lori rams, it seems that increasing of sperm motility in Class A in Romanov breed is because of the longer mid piece and high concentration of mitochondria. Other features of semen analysis indicate the priority of this breed's sperm to Lori Bakhtiari breed.

REFERENCES

1. Salimi R., Asadi H. *Effective parameters on reproduction*. Danesh Parvar, 2014.
2. Kamkar K. *Sheep husbandry*. Farhang Jame Pub., 2008.
3. Soori M., Nurian Sarvar E. Sheep husbandary. Iran Razi University, Kermanshah, 2007.
4. Louda F., Doney J.M., Štolc L., Křížek J., Šmerha J. The development of sexual activity and semen production in ram lambs of two prolific breeds: Romanov and Finnish Landrace. *Anim. Prod.*, 1981, 33(2): 143-148 (doi: 10.1017/S0003356100040575).
5. Büyükleblebici S., Tuncer P.B., Bucak M.N., Taşdemir U., Eken A., Büyükleblebici O., Durmaz E., Sarıözkan S., Endirlik B.Ü. Comparing ethylene glycol with glycerol and with or without dithiothreitol and sucrose for cryopreservation of bull semen in egg-yolk containing extenders. *Cryobiology*, 2014, 69: 74-78 (doi: 10.1016/j.cryobiol.2014.05.005).
6. Mohammadi G., Mahdiun H., Gurani Nezhad S. Improvement of semen evaluation techniques. *Veterinary Journal and Laboratory*, 2011, 3: 135-145.
7. Tajik P. Tajik P., Ghasemzadeh-Nava H., Lotfollahzadeh S., Shirzad M.R. Assessment of live/dead and protoplasmic droplets in epididymal sperm cells in Iranian Zell rams. *J. Fac. of Vet. Med., University of Tehran,* 2003, 58(1): 25-28 (abs. in English).
8. Wainer R., Albert M., Dorion A., Bailly M., Bergère M., Lombroso R., Gombault M., Selva J. Influence of the number of motile spermatozoa inseminated and of their morphology on the success of intrauterine insemination. *Hum. Reprod.*, 2004, 19(9): 2060-2065 (doi: 10.1093/humrep/deh390).
9. Lindheim S.R., Barad D.H., Zinger M., Witt B., Amin H., Cohen B., Fisch H., Barg P. Abnormal sperm morphology is highly predictive of pregnancy outcome during controlled ovarian hyperstimulation and intrauterine insemination. *J. Assist. Reprod. Gen.*, 1996, 13(7):

569-572 (doi: 10.1007/BF02066610).
10. Fazli Nezhd J., Mamoeii M., Kheradmand A., Sukhteh Zari A. The effect of melatonin on testicular circumference and semen characteristics in non-breeding season in Lori-Bakhtiari ram. *Journal of Veterinary Research*, 2016, (71)1: 27-32.
11. Lafortune E., Blanc M.R., Orgeur P., Pelletier J., Perreau C., Terqui M., Hochereau-de Reviers M.T. A comparison of the changes in LH, FSH and testosterone in spring-born ram lambs of two different breeds. *Reprod. Nutr. Dev.*, 1984, 24: 947-952.
12. Druart X., Cognié J., Baril G., Clément F., Dacheux J.-L., Gatti J.-L. In vivo imaging of in situ motility of fresh and liquid stored ram spermatozoa in the ewe genital tract. *Reproduction*, 2009, 138(1): 45-53 (doi: 10.1530/REP-09-0108).
13. Noakes D., Parkinson T.J., England G.C., Arthur G.H. *Arthur's veterinary reproduction and obstetrics*. Saunders Ltd., 2001.
14. Mohammadi G., Barati F. *Artificial insemination in farm animals*. Shahid Chamran University, Ahvaz, 2009.
15. Yániz J., Capistrós S., Vicente-Fiel S., Soler C., De Murga J.N., Santolaria P. Study of nuclear and acrosomal sperm morphometry in ram using a computer-assisted sperm morphometry analysis fluorescence (CASMA-F) method. *Theriogenology*, 2014, 82(6): 921-924 (doi: 10.1016/j.theriogenology.2014.06.017).
16. Gravance C.G., Champion Z.J., Casey P.J. Computer-assisted sperm head morphometry analysis (ASMA) of cryopreserved ram spermatozoa. *Theriogenology*, 1998, 49(6): 1219-1230 (doi: 10.1016/S0093-691X(98)00069-7).
17. Yániz J.L., Capistrós, S., Vicente-Fiel S., Hidalgo C.O., Santolaria P. A comparative study of the morphometry of sperm head components in cattle, sheep, and pigs with a computer-assisted fluorescence method. *Asian J. Androl.*, 2016, 18(6): 840-843 (doi: 10.4103/1008-682X.186877).

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ORGANIC AQUACULTURE AS PROMISING TREND OF THE FISH INDUSTRY DEVELOPMENT

(review)

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Abstract

The status of organic aquaculture which presents production (cultivation) aquatic species (fish, crustaceans, molluscs, sea plants etc.) and guarantees high quality and safety of the product in accordance with specific principles is evaluated on the base of a large amount of statistical data. The main limiting factor of the development of aquaculture, in particular organic aquaculture, in Russia and in the world is deficiency of feeds (L.Yu. Lagutkina, 2017). Feed production is constrained by the deficit, expensiveness, and low ecological safety of fish meal which is a traditional raw material. The incentives of the development of aquaculture are changes in the culture of food consumption especially among young people, and willingness of the consumers to pay for the ecological safety of the products. The new formats of production (P. Edwards, 2015) based on technologies of sustainable, effective, integrated and organic production will contribute to the development of an aquaculture. Organic projects of aquaculture (S. Begleiter et al., 2015) become more attractive for investors. One of the first specialized venture capital funds, Aqua-Spark (Netherlands), has invested $7 mln in organic projects in Mozambique, Iceland and Norway since 2014. Global organic aquaculture production in 2015 is evaluated as 400 thousand tons that is about 0.54 % of the overall aquaculture production (without water plants) but twice as much as overall volume of Russian aquaculture production. Production of organic aquaculture is focused on rearing salmon (10 %), molluscs (5 %), carp (1 %), shrimp (1 %), trout (0.3 %), and sturgeons (0.3 %). Major part of organic products is produced in China (304 thousand tons, or 80 %) and in Europe (76 thousand tons, 19 %). Among manufacturers (about 1 thousand) those who are certified (200 in China, 465 in Europe) are of our particular interest. European manufacturers are certified according to the European (in fact, the international) standards, Chinese manufacturers are mainly certified according to national organic standard which is not identical to the international standards. Russian manufacturers will be certified with regard to the Russian and (or) international certification systems. Since 2011, the pilot introduction of organic aquaculture in Russia occurs in the Astrakhan region at a small innovative enterprise Modern Fishery Complex "Sharapovskii" where aquaculture is combined with crop rotation. "Vitality Leaf. Organic" of Saint-Petersburg Ecological Union (the Russian system for voluntarily ecological certification of international level) is acknowledged as congruent to European standards. To date, the development of organic aquaculture in Russia corresponds neither to natural potential of the country, nor to world average indicators of the industry. A way to solve the problem may lay in the development of feeds which will correspond to base requirements of organic standards.

Keywords: organic aquaculture, principles of organic production, organic standards, fish food products, consumer culture, certification, feeds

Global population growth necessitates rapid development of agriculture and growth of production output. This could be achieved by relying upon the intensive animal breeding, poultry breeding, and fishery forms. Aquaculture is going to play an enormous role in development of agriculture. By 2050, 9.8 billions of people shall be provided with food, i.e. growth of food production by 60 % as

compared to recent food production rates. Aquaculture, as a technological process, may warrant transparency and manageability of production that serves the basis for improvement of the safety and ecological compatibility. Contribution of the Russian aquaculture to the total volume of domestic fish products remains small – slightly over 4 % [1]. Conversely, it is aquaculture that produces more fish products than pisciculture in the global context starting from 2014 [2]. It is expected that by 2030 aquaculture would yield $^3/_4$ of fish products to be consumed by humans in the world [3]. Apparently, development of aquaculture in Russia is still not in line with natural potentials of the country and with mean global indicators of industrial development. Such low aquaculture production volumes could be, in particular, explained by the fact that until present time the national demand for fish protein was satisfied by sufficiently high pisciculture production volumes, which is in stagnation state in foreign countries.

In present paper we assess global development of organic aquaculture: production of fish, shell fish, mollusk, sea plants, and other consumable goods according to certain ("organic") principles based on statistical data provided in the available literature sources.

According to statistics, $^3/_4$ of the total aquaculture production volume is represented by hydrobionts, and $^1/_4$ — sea weed (*Algae*) [2]. In 2014, global aquaculture industry had produced 73.8 million of hydrobions (USD 160.2 billion in producer prices), of which 49.8 million tons of bony fishes (*Teleostei*) (USD 99.2 billion); 16.1 million tons of mollusks (USD 19 billion); 6.9 million tons of shell fishes *Crustacea* (USD 36.2 billion); 7.3 million tons of other sea animals, including semi-aquatic animals (*Amphibia*) (USD 3.7 billion). Volume of raised aquatic plants comprised 27.3 million tons (USD 5.6 billion) [2]. By dominant growth objects, modern global aquaculture may be defined as limnobiotic extensive, warm-water culture based on species of south-eastern faunal complex [4].

There are several stimuli for rapid development of aquaculture. First of all, it is growth of demand for aquaculture products [5] caused by growth in consumption rates of food products due to growth of the number of population and transformation of product consumption models. In 2009-2014, total consumption of fish products in humans had increased by 18 % (from 123.8 to 146.3 million tons), and consumption per capita — from 18 to 20 kg [2]. Practically all fish produced by aquaculture, as apart from fish produced by pesciculture, is used for such purpose. The second factor is inability to satisfy the growing global demand for fish products by traditional pesciculture without a threat to destruction of the natural ecosystems. Achievement of the fishing margin is indicated by the fact that during 2009-2014 global fishing volumes had increased by only 3 %, while aquaculture production volumes — by 32 % [2]. According to forecasts, by 2030 global consumption of fish caught by pesciculture would be reduced from 65 million tons (in 2006) to 58 million tons [6], and consumption volumes of fish produced by aquaculture would, conversely, grow from 47 to 94 million tons [7]. The third factor is lack of fish product safety warranties [8]. Fishing industry (first of all, pesciculture) does not always warrant ecologic compatibility of products served on the table, due to complexity of supply chains. Sometimes it is hard to validly establish the origin of fish, how it was caught and how it was shipped. Relevance of the issue of increasing the transparency and traceability of fish products had resulted in implementation of relevant international and national programs [9-12]. The fourth factor is increasing the effectiveness [13], as well as technological accessibility and feasibility of creation and development of aquaculture objects, first of all, for small and middle entrepreneurships. In 2000-2014, number of enterprises and farmers dealing with aquaculture had increased by 49 % — from 12.63 to 18.75 million [2].

Aquaculture development is also promoted by rapid spread of both new and known, and technologically realized production methods. These are technologies of stable [14, 15], resource effective [16], and integrated [17], as well as organic [18] agriculture, re-circulating aquaculture systems (RAS) [19], and aquaponics [20].

By 2050, 70 % of global population would live in the cities and, thus, in the next decade an explosive growth of demand for technologies of the urbanized agriculture allowing producing food ingredients and products in the closed controlled environment is expected [21]. These technologies allow improving the production safety by extending the access to food products by the citizens [22]. Reduction of the traditional logistics supply chain of products that recently increases the cost of food products by 40-100 % as compared to "farm gate" prices and in which 20-40 % of products are disposed to waste would become the basis for price affordability. One of such technologies is aquaponics [23-26] that is based on closed cycle aquaculture. It becomes popular around the world, especially in the cities, since people become more interested in fresh domestic food products. Aquaponics extending in closed urban premises [27] promotes rapid supply of fresh fish, vegetables, and plants to the consumers and more often requires less space and water for production purpose than pond aquaculture [28]. Aquaponics is effective even in countries with cold climate. Recently, there are 20 thousand farming units dealing with aquaponics in the world, and volume of aquaponics market comprises USD 409 million. By 2021 volume of the market would increase up to USD 907 million (http://industryarc.com).

Since 1999, area of agricultural lands certified by organic standards had increased from 11 to 51 million ha in the world; number of certified producers of organic products had increased from 200 thousand to 2.4 million; 87 countries enforced statutory regulation of the organic production and consumption [29]. Experts forecast rapid growth of global market of organic products from USD 81.6 billion in 2015 [29] to USD 238.4 billion in 2022 (http://www.marketresearchstore.com). In absolute figures (total sales of organic products), most developed markets are US (EUR 35.8 billion), Germany (EUR 8.6 billion), France (EUR 5.5 billion), China (EUR 4.7 billion), Canada (EUR 2.8 billion), and United Kingdom (EUR 2.6 billion) [29]. In relative figures, "organics" market (share of organic products in the total volume of sold products) in mostly developed in the following European countries: Denmark (8.4 %), Switzerland (7.7 %), Luxemburg (7.5 %), Sweden (7.3 %), and Austria (6.5 %). European producers of organics get significant state support [30].

Production of organics is not merely market niche or sustainable growth technology [31], but also a reaction of society, producers to the changing social and economic and social and cultural human behavior [32] as regards to consumption of food products. This trend is recently observed not only in developed, but also in developing countries [33]. People perceive "food", its quality, ecologic compatibility, and safety, and "health" as a whole concept.

Development of organics market is an innovative, but already sustainable global trend [34]. It is supported by growth of human awareness in influence of food on health, physical form, well-being; popularity of healthy life style, strive to diet individualization (functional and personalized diet) [21]; frequent incidents in many countries of the world associated with safety of food products; increase of the national wealth of developed countries and human readiness to pay for ecologic compatibility of the products. It is demands of the young generation that define the successful development of organics production industry. That is, in 2016 sales volumes in USA had exceeded USD 43.3 billion, and the most from 18 to 34 years. In average, 45 % Americans are attempting to actively

include organic products in their diet, regardless of the fact that their cost is by 20-100 % higher than the cost of regular food products.

In Russia, civil society is also concerned about safety and ecological compatibility of food products [35]. They are ready to pay more for ecologically compatible products and for increased costs, by purchasing fresh, not frozen, non-processed products free from genetically modified organisms (GMO). First certified (primarily by European systems) Russian producers of organic products face an unfair competition [36]. Over half of products marked by "eco", "bio", and "organic" are not related to organic production, thus, negatively affecting development of organic production in Russia. Fair market participants (not only producers, but also associations, certification operators) are forced to compete for positive image of organic products: mobile applications "Ecopolka" (http://ecopolka.ru) and "Navigator of Farm Products" (https://inter-start.ru/project85) are realized for information of consumers on verified organic products.

Importance of organics production in Russia and development of relevant technologies [37] was not left unnoticed by state bodies and development institutes. As it was noted in "Forecast of scientific and technological development of agro-industrial complex of the Russian Federation by 2030", this is one of the key factors securing access of the national producers to international markets [21]. Within the scope of the National Technological Initiative, organics production in Russia is considered as one of the key segments of perspective food product market (Road Map "Foonet", http://www.nti2035.ru). However the national regulatory framework for development of the organics market is not fully formed [38], first of all, due to absence of the federal law governing relations associated with production and turnover of organic products.

It terms of technology and its statutory regulation, the organics production shall comply with several principles which are recently articulated by the International Federation of Organic Agriculture Movements (IFOAM) [39]. These health principles (support and improvement of the health of ecosystems, soils, animals, humans, and planet); ecology (coexistence with essential ecologic systems and cycles; maintenance of the natural cycles and balances); fairness (protection of environment, humanity to humans and animals; assurance of conditions and opportunities for life which accord with physiology, essential behavior and health of live organism); care (preventative and responsible management of organic agriculture for protection of health of the existing and future generations and environment; use of new methods and technologies which may improve the production efficiency, and shall not jeopardize the health and well-being of humans).

Practically, it means meeting of the basic requirements of organic standards. For crop growing it is a conversion (transit) period; use of natural fertilizers; ban on use of chemical crop protection agents (except for the permitted list of agents), on treatment of seeds by chemical preparations and use of genetically modified seed material. For animal breeding it means natural feeds (the following content is permitted: 70 % organics, 30 % organics in conversion); ban on use of antibiotics, GMOs; animal packing density no more than permitted and their loose keeping. For processed products it means use of at least 95 % of organic raw materials and ban on use of artificial additives.

Different certification systems of organics production and products may impose adapted and additional requirements. Thus, Russian system for voluntarily ecological certification of international level "Vitality Leaf. Organic" (Ecologic Union of Saint Petersburg, member of IFOAM) imposes the following basic requirements for the crop raising: ban on seed treatment by chemical preparations; ban on use of GMOs; ecologically compatible raising methods (use of permitted fertilizers and crop protection means, crop rotation) [40]; laborato-

ry tests of soil for content of oil products, benzpyrene, heavy metals, persistent organic pollutants (POP); laboratory tests of products by extended list of indicators (pesticides, polyaromatic hydrocarbons, POPs, heavy metals); adherence to statutory regulations on waste disposals, discharges, and waste treatment.

Within the scope of aquaculture, organics direction has also been intensively developing during the last years [41, 42]. Organic aquaculture is defined as production (growing) of aquaculture objects (fish, shell fish, mollusk, and sea weed) and crop growing (in case of realization of mixed technology, aquaponics) according to the above-listed principles. Special requirements are also imposed in organic aquacultural production: renunciation of use of pesticides, fertilizers and GMOs, stepwise renunciation of fish flour, strict limiting the use of antibiotics and hormones [43]. Organic aquaculture is a production certified by organic standards, verified at each stage from raising (young animals, feed, and techniques) to treatment and delivery to consumer, and warranting high quality and safety of products.

Organics aquaculture is a new, extremely perspective and rapidly developing market niche satisfying the growing demand of humans for safe, ecologically compatible products in the context of stagnation in the global pesciculture [44, 45]. Organic projects within the scope of aquaculture become more appealing for investors. Thus, one of the first specialized venture funds in this domain is "Aqua-Spark" (Netherlands, founded in 2014), that invested USD 7 million in organic projects, of which USD 2 million were invested in project "Chicoa Fish Farm" (Mozambique, year 2014): ecologically compatible, vertically integrated, rapidly extendable technology of fresh-water raising of *Tilapia*; own incubator; production of planting material and feeds. It is forecasted that this production model would effectively operate in all countries of Africa to the south of Sahara desert. USD 2.5 million was invested in project "Matorka" (Island, 2015) (ecologically compatible production of *Salvelinus alpinus* from feeds to end product; recovered energy sources); USD 2.5 million was invested in project "Sogn Aqua" (Northway, 2015) (raising of *Hippoglossus*, patented water supply system with intensive aeration of purest deep water of fiords allowing renouncing of chemicals and antibiotics, low operating costs, practically complete absence of environmental impact, since 95 % of used materials are suitable for secondary processing, as well as possibility of production transfer on shore) (http://www.aqua-spark.nl).

In 2017 the Research Institute of Organic Agriculture, FiBL, Switzerland (http://www.fibl.org) and IFOAM (International Federation of Organic Agriculture Movements) (https://www.ifoam.bio) in their annual report "Global Organic Agriculture" [29] had for the first time summarized information on development of organic aquaculture in the world. Estimated global volume of organic aquaculture production in 2015 comprised 400 thousand tons (that is only 0.54 % of the total volume of aquaculture products, without sea weed), that is 2 times more than production volume of the Russian aquaculture, in general. Most part of organic products is produced in China — 304 thousand tons (80 %) and in Europe — 76 thousand tons (19 %), including Ireland — 31 thousand tons (mainly Atlantic salmon or *Salmo salar*), Northway — 17 thousand tons (salmon), Romania — 6.4 thousand tons (carp, salmon), Italy — 5.5 thousand tons (mollusks, *Dicen-trarchus labrax*, Black Sea salmon, or *Salmo trutta*, *Oncorhynchus mykiss*), Denmark — 4.1 thousand tons (mollusks), Hungary — 3.5 thousand tons, Spain — 2.7 thousand tons (mollusks, white mullet, bull trout, rainbow trout). From the other countries, the group of large producers is presented (mainly produced product is indicated) by Vietnam — 3.3 thousand tons (shrimps), Costa-Rica — 3.2 thousand tons (shrimp), Lithuania — 2.7 thousand

tons (carp), Indonesia — 1.9 thousand tons (shrimp), Ecuador — 1.8 thousand tons (shrimp), Thailand — 1.5 thousand tons. Significantly smaller production volumes of organic products are in Croatia— 1.4 thousand tons (mollusks, white mullet), in Greece — 1.1 thousand tons (white mullet), in Germany — 1.0 thousand tons (rainbow trout), as well as in Honduras — 0.6 thousand tons (shrimps) [46]. There is still no information on volumes of organic production in many countries with developed aquaculture (for instance, in Brazil). It could be expected that following such information, the adjusted global production volume of organic aquaculture would be higher.

Regardless of the fact that there is no available information on structure of the most part of organic aquaculture products, the available information reflect its specificity: production of organic aquaculture is focused on raising of salmons (10 %), mollusks (5 %), carp (1 %), shrimp (1 %), trout (0.3 %) and sturgeon (0.3 %).

Number of organic aquaculture producers constantly grows. There are 200 certified producers [47] in China, 465 — in Europe (most of all in Germany — 160) (http://ec.europa.eu/euro-stat/web/agriculture/data/database). European producers are certified according to European (in fact international) standards, Chinese − mainly according to the national organic standard which is not yet identical to the international standards. Generally, at present there are nearly 1 thousand of producers of organic aquaculture objects, and their number would grow.

Organic trend in China is deemed to be one of the key trends in development of aquaculture. About 60 % of the total volume of global aquaculture objects is produced there, of which fish products make 26 million tons, mollusks make 13.4 million tons, shell fish make 4 million tons, sea weed makes 13.3 million tons. This volume generates total 27 % (5.12 million) of the global number of producers — enterprises and farmers dealing with aquaculture [48]. Chinese aquaculture yields 540 fish, mollusk, shell fish, and other invertebrate species, several species of amphibians and aquatic reptilians, nearly 30 species of limnetic macrophytes, over 50 species of micro-seaweed and invertebrate animals. China remains the global leader in production of sturgeon roe. Experts forecast growth of Chinese aquaculture market volume by 2020 up to USD 103 billion mainly due to increase of organic aquaculture production volumes and demand for "premium" seafood.

In China, 400 thousand ha of agricultural lands (mainly in Zhejiang, Hainan, Mongol, Jiangsu, Xinjiang, Liaoning, Hunan, Anhui, Fujian, and Shandong provinces) are used for organic aquacultural production purposes. Main production technology at organic aquaculture farms in China is raised in polyculture. The most used production objects are fish, shrimp, *Pectinidae*, sea cucumber (trepan) (*Holothurioidea*), crab (*Brach-yura*), mollusks, and eel (*Monopterus albus*) [49].

Pilot region for implementation of organic aquaculture in Russia is Astrakhan Region [50, 51]. Aquaculture production in combination with crop rotation is in place here since 2011 at small innovative enterprise "Modern Fishery Complex "Sharapovskii". Production process by organic technology includes alternating growing of aquaculture objects (carp, herbivorous fish) and agricultural species (melons, grains, vegetables) without the use of substances containing synthetic materials and chemical agents that allows ensuring production safety of products. Preparation program of stagnal areas increases in 2 times the crop yields of agricultural products following predecessor (aquaculture objects), fish yields in 1.5 times [52]. Organic technology in combination with methods of adaptive agricultural production [53] and production of ecologically compatible

products [54] had shown high effectiveness in the context suitable for stagnal culture [55] that makes its large-scale application possible in fishery basins of arid area of Russia. Diagnostic audit of the food product safety management system was conducted at fishery complex “Sharapovskiy” for compliance with requirements of the state standard GOST P ISO 22000-2007 [56] and organic production by program “ECO-PRODUCT” adopted in “R-Standard” voluntary certification system [57] that confirmed development of organic aquaculture and readiness to further certification.

Effective development of organic aquaculture direction in China, as well as in Russia, suppresses the deficit of organic feeds [58] and lack of organic certification in majority of producers. Certification of organic aquaculture production and products in Russia would be done within the limits of the Russian and international certification systems and governed by relevant law and statutory regulations, including GOST P 57022-2016 [59]. Cost of certification would vary depending on the number of types of certified products, production volume, and etc. Most probably, it would be compatible with certification cost of agricultural production. Cost of certification of compliance to GOST P 56508-2015 [60] would comprise 70 thousand rubles/ year (GOST presupposes only nearly 70 % requirements of European organic standards); by European organic standards through European operators — USD 10 thousand [61]. Cost of certification for compliance to standard “Vitality Leaf. Organic” of the Ecologic Union of Saint Petersburg, recognized as compliant to the European standards, in average comprise 150 thousand rubles/year.

Therefore, aquaculture production industry is ready for radical changes, which would be associated with new formats of organic production and which would also change the food product consumption picture. Large global venture funds have been already included in investment organic aquaculture projects, and these projects are accounted for at conduction of scientific researches. Innovative developments are supported by governments of many countries, international organizations, and private investors. Perspectives of aquaculture changes are associated with practical realization of organic technologies promoted by both growth of the number of issues related to safety of food products and ecologic threats, as well as by growth of population of organic food products. For effective development of organic aquaculture direction in the world and in Russia, in particular, it is necessary to overcome a number of barriers, in particular deficit of organic feed and lack of international certification in majority of organic aquaculture operators. All these facts and factors shall be accounted for in the domestic aquaculture development strategy to enable the Russian producers to compete at the global market for a few years.

REFERENCES

1. *FAR: Ob"em proizvodstva produktsii akvakul'tury v 2016 godu uvelichilsya do 205 tys. Ton* [FAR. Aquaculture products increased to 205 thousand tons in 2016]. Available http://fish.gov.ru/press-tsentr/novosti/17053-ob-em-proizvodstva-produktsii-akvakultury-v-2016-godu-uvelichilsya-do-205-tys-tonn. Accessed October 9, 2017 (in Russ.).
2. *FAO: The state of world fisheries and aquaculture 2016*. Available http://www.fao.org/3/a-i5555e.pdf. Accessed February 2, 2017.
3. *Fish to 2030: Prospects for fisheries and aquaculture*. Available http://www.fao.org/docrep/019/i3640e/i3640e.pdf. Accessed February 2, 2017.
4. Bostock J., McAndrew B., Richards R., Jauncey K., Telfer T., Lorenzen K., Little D., Ross L., Handisyde N., Gatward I., Corneret R. Aquaculture: global status and trends. *Philos. T. Roy. Soc. B*, 2010, 365(1554): 2897-2912 (doi: 10.1098/rstb.2010.0170).
5. Ottinger M., Clauss K., Kuenzer C. Aquaculture: relevance, distribution, impacts and spatial assessments — a review. *Ocean Coast. Manage.*, 2016, 119: 244-266 (doi: 10.1016/j.ocecoaman.2015.10.015).
6. Pauly D., Alder J., Bennett E., Christensen V., Tyedmers P., Watson R. The future for fisher-

ies. *Science*, 2003, 302(5649): 1359-1361 (doi: 10.1126/science.1088667).
7. Branch T.A., Jensen O.P., Ricard D., Ye Y., Hilborn R. Contrasting global trends in marine fishery status obtained from catches and from stock assessments. *Conserv. Biol.*, 2011, 25(4), 777-786 (doi: 10.1111/j.1523-1739.2011.01687.x).
8. Leal M.C., Pimentel T., Ricardo F., Rosa R., Calado R. Seafood traceability: current needs, available tools, and biotechnological challenges for origin certification. *Trends Biotechnol.*, 2015, 33(6): 331-336 (doi: 10.1016/j.tibtech.2015.03.003).
9. Charlebois S., Sterling B., Haratifar S., Naing S.K. Comparison of global food traceability, regulations and requirements. *Comp. Rev. Food Sci. F.*, 2014, 13(5): 1104-1123 (doi: 10.1111/1541-4337.12101).
10. Borit M., Olsen P. *Seafood traceability systems: gap analysis of inconsistencies in standards and norms.* FAO, Rome, 2016.
11. *Tekhnicheskii reglament Evraziiskogo ekonomicheskogo soyuza «O bezopasnosti ryby i pishchevoi rybnoi produktsii»*. [Technical Regulations of the Eurasian Economic Union on the Safety of Fish and Food Fish Products]. Available http://docs.cntd.ru/document/420394425. Accessed October 9, 2017 (in Russ.).
12. Dvoryaninova O.P., Sokolov A.V., Alekhina A.V. *Tekhnologii pishchevoi i pererabatyvayushchei promyshlennosti APK — produkty zdorovogo pitaniya*, 2017, 1(15): 14-22 (in Russ.).
13. Edwards P. Aquaculture environment interactions: past, present and likely future trends. *Aquaculture*, 2015, 447: 2-14.
14. Pretty J. Agricultural sustainability: concepts, principles and evidence. *Philos. T. Roy. Soc. B*, 2008, 363(1491): 447-466 (doi: 10.1098/rstb.2007.2163).
15. Velten S., Lewenton J., Jager N., Nevig J. What is sustainable agriculture? A systematic review. *Sustainability*, 2015, 7(6): 7833-7865 (doi: 10.3390/su7067833).
16. Padmavathy K., Poyyamoli G. Alternative farming techniques for sustainable food production. *Sustainable Agriculture Reviews*, 2011, 7: 367-424 (doi: 10.1007/978-94-007-1521-9_13).
17. Hendrickson J.R., Hanson J.D., Tanaka D.L, Sassenrath G. Principles of integrated agricultural systems: introduction to processes and definition. *Renew. Agr. Food Syst.*, 2008, 23(4): 265-271 (doi: 10.1017/S1742170507001718).
18. Siddique S., Hamid M., Tariq A., Kazi A.G. Organic farming: the return to nature. In: *Improvement of crops in the era of climatic changes.* P. Ahmad, M. Wani, M. Azooz, LS. Phan Tran (eds.). Springer, New York, NY, 2014: 249-281 (doi: 10.1007/978-1-4614-8824-8_10).
19. Ellis R.P., Urbina M.A., Wilson R.W. Lessons from two high CO_2 worlds — future oceans and intensive aquaculture. *Glob. Change Biol.*, 2014, 23(6): 2141-2148 (doi: 10.1111/gcb.13515).
20. Goddek S., Delaide B., Mankasingh U., Ragnarsdottir K.V., Jijakli H.R. Challenges of sustainable and commercial aquaponics. *Sustainability*, 2015, 7(4): 4199-4224 (doi: 10.3390/su7044199).
21. *Prognoz nauchno-tekhnologicheskogo razvitiya agropromyshlennogo kompleksa Rossiiskoi Federatsii na period do 2030 goda* [Forecast of scientific and technological development of the agro-industrial complex of the Russian Federation up to 2030]. Moscow, 2017 (in Russ.).
22. Lyzhin D.N. *Problemy natsional'noi strategii*, 2014, 2(23): 79-94 (in Russ.).
23. König B., Junge R., Bittsanszky A., Villarroel M., Komives T. On the sustainability of aquaponics. *Ecocycles*, 2016, 2(1): 26-32 (doi: 10.19040/ecocycles.v2i1.50).
24. Kloas W., Groß R., Baganz D., Graupner J., Monsees H., Schmidt U., Staaks G., Suhl J., Tschirner M., Wittstock B., Wuertz S., Zikova A., Rennert B. A new concept for aquaponic systems to improve sustainability, increase productivity, and reduce environmental impacts. *Aquacult. Environ. Interact.*, 2015, 7: 179-192 (doi: 10.3354/aei00146).
25. Matishov G.G., Ponomareva E.N., Kazarnikova A.V., Il'ina L.P., Grigor'ev V.A., Sokolova T.A., Pol'shina T.N., Kovalenko M.V., Kuzov A.A., Korchunov A.A. *Izvestiya vysshikh uchebnykh zavedenii. Severo-Kavkazskii region. Seriya Estestvennye nauki*, 2016, 3(191): 41-48 (in Russ.).
26. Ermakova N.A., Zlotnitskaya T.S. *Rybnoe khozyaistvo*, 2016, 5: 57-62 (in Russ.).
27. Palma Lampreia Dos-Santos M.J. Smart cities and urban areas — aquaponics as innovative urban agriculture. *Urban For. Urban Gree.*, 2016, 20: 402-406 (doi: 10.1016/j.ufug.2016.10.004).
28. Shafahi M., Woolston D. Aquaponics: a sustainable food production system. *Proc. ASME 2014 International Mechanical Engineering Congress and Exposition*. Montreal, Canada, V. 3: V003T03A073 (doi: 10.1115/IMECE2014-39441).
29. *FiBL, IFOAM — Organics international: the world of organic agriculture: statistics and emerging trends 2017.* Nuremberg, BIOFACH, 2017.
30. Akimova Yu.A. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Innovatsionnoe razvitie otraslei APK: ugrozy i novye vozmozhnosti»* [Proc. Int. Conf. «Innovative development of the agribusiness sector: challenges and new opportunities»]. Moscow, 2017: 32-37 (in Russ.).
31. Rahmann G., Ardakani M.R., Bàrberi P., Boehm H., Canali S., Chander M., Wahyudi D., Dengel L., Erisman J.W., Galvis-Martinez A.C., Hamm U., Kahl J., Köpke U., Kühne S., Lee S.B., Løes A.-K., Moos J.H., Neuhof D., Nuutila J.T., Olowe V., Oppermann R., Rembiałkowska E., Riddle J., Rasmussen I.A., Shade J., Sohn S.M., Tadesse M., Tashi S., Thatcher A., Uddin N., von Fragstein und Niemsdorff P., Wibe A., Wivstad M., Wenliang W.,

Zanoli R. Organic Agriculture 3.0 is innovation with research. *Organic Agriculture*, 2017, 7(3): 169-197 (doi: 10.1007/s13165-016-0171-5).
32. Basha M.B., Mason C., Shamsudin M.F., Hussain H.I., Salem M.A. Consumers attitude towards organic food. *Proc. Econ. Financ.*, 2015, 31: 444-452 (doi: 10.1016/S2212-5671(15)01219-8).
33. Mehree I. Consumer behaviour of organic food a developing country perspective. *International Journal of Marketing and Business Communication*, 2015, 4(4): 442-452 (doi: 10.21863/ijmbc/2015.4.4.024).
34. Dorias M., Alsanius B. Advances and trends in organic fruit and vegetable farming research. *Horticultural Reviews*, 2015, 43: 185-268 (doi: 10.1002/9781119107781.ch04).
35. Belova I.N., Karslyants E.A. *Vestnik Rossiiskogo universiteta druzhby narodov. Seriya Ekonomika*, 2014, 2(40): 48 (in Russ.).
36. Gorelova I.E. *Perspektivy nauki*, 2017, 5(92): 49-53 (in Russ.).
37. Gokhberg L., Kuzminov I. Technological future of the agriculture and food sector in Russia In: *Global innovation index 2017. Innovation feeding the world. Chapter 9*. Geneva, New Delhi, Cornell University, INSEAD, and the World Intellectual Property Organization, 2017: 135-141.
38. Ryzhkova S.M., Kruchinina V.M., Gasanova Kh.N., Novoselov E.A. *Ekonomika sel'skokhozyaistvennykh i pererabatyvayushchikh predpriyatii*, 2017, 8: 57-63 (in Russ.).
39. *Printsipy organicheskogo sel'skogo khozyaistva* [Principles of organic agriculture]. Available http://www.ifoam.bio/sites/default/files/poa_russian_web.pdf. Accessed October 9, 2017 (in Russ.).
40. van Mansvelt J.D., Temirbekova S.K. General position of organic agriculture in Western Europe: concept, practical aspects and global prospects. *Agricultural Biology*, 2017, 52(3), 478-486 (doi: 10.15389/agrobiology.2017.3.478eng).
41. Jena A.K., Biswas P., Saha H. Advanced farming systems in aquaculture: strategies to enhance the production. *Innovative Farming*, 2017, 1(1): 84-89.
42. Ratheesh K.R., Sandeep K.P., Manju L.N., Sreekanth G.B. Organic aqua-farming a gateway to sustainable aquaculture. *Aqua International*, 2013, August: 25-28.
43. Bergleiter S., Meisch S. Certification standards for aquaculture products: bringing together the values of producers and consumers in globalised organic food markets. *Journal of Agricultural and Environmental Ethics*, 2015, 28(3): 553-569 (doi: 10.1007/s10806-015-9531-5).
44. Branch T.A., Jensen O.P., Ricard D.Ye.Y., Hilborn R. Contrasting global trends in marine fishery status obtained from catches and from stock assessments. *Conserv. Biol.*, 2011, 25(4): 777-786 (doi: 10.1111/j.1523-1739.2011.01687.x).
45. Mullon C., Fréon P., Cury P. The dynamics of collapse in world fisheries. *Fish Fish.*, 2005, 6(2): 111-120 (doi: 10.1111/j.1467-2979.2005.00181.x).
46. *The world of organic agriculture. Statistics and emerging trends 2013. FiBL-IFOAM Report*. FiBL-IFOAM, 2013: 340.
47. *Aquaculture market in China 2016-2020*. TechNavio — Infiniti Research Ltd., 2016.
48. *The state of world fisheries and aquaculture 2016*. FAO, 2016. Available http://www.fao.org/3/a-i5555e.pdf. Accessed March 3, 2017.
49. Xie B., Qin J., Yang H., Wang X., Wang Y.-H., Li T.-Y. Organic aquaculture in China: A review from a global perspective. *Aquaculture*, 2013, 414-415: 243-253 (doi: 10.1016/j.aquaculture.2013.08.019).
50. Lagutkina L.Yu., Ponomarev S.V. *Vestnik Astrakhanskogo gosudarstvennogo tekhnicheskogo universiteta. Seriya: Rybnoe khozyaistvo*, 2016, 4: 74-82 (in Russ.).
51. Lagutkina L.Yu., Levina O.A., Ponomarev S.V., Sheikhgasanov K.G. *Materialy III Mezhdunarodnaya nauchnaya Internet-konferentsiya «Sovremennye tendentsii v sel'skom khozyaistve»* [Proc. III Online Conf. «Current trends in agriculture»]. Kazan', 2014: 66-71 (in Russ.).
52. Lagutkina L.Yu., Sheikhgasanov K.G., Pozhidaeva M.A., Biryukova M.G. *Vestnik Astrakhanskogo gosudarstvennogo tekhnicheskogo universiteta. Seriya Rybnoe khozyaistvo*, 2016, 2: 84-93 (in Russ.).
53. Tulokhonov A.K., Namzhilova L.G., Boldanov T.A. *Materialy III Mezhdunarodnoi nauchnoi Internet-konferentsii «Sovremennye tendentsii v sel'skom khozyaistve»* [Proc. III Online Conf. «Current trends in agriculture»]. Kazan', 2014: 122-139 (in Russ.).
54. Sheikhgasanov K.G., Lagutkina L.Yu., Ponomarev S.V. *Vestnik Astrakhanskogo gosudarstvennogo tekhnicheskogo universiteta. Seriya: Rybnoe khozyaistvo*, 2014, 3: 97-103 (in Russ.).
55. Lavelina T.P. *Ratsional'noe ispol'zovanie zemel'nykh resursov Severnogo Prikaspiya pri integrirovannom proizvodstve rastitel'noi i rybnoi produktsii. Avtoreferat kandidatskoi dissertatsii* [Effective use of land resources of the Northern Caspian region in integration of plant and fish production. PhD Thesis]. Moscow, 1998 (in Russ.).
56. *GOST R ISO 22000-2007. Sistemy menedzhmenta kachestva pishchevoi produktsii. Trebovaniya k organizatsiyam, uchastvuyushchim v tsepi sozdaniya pishchevoi produktsii* [State Standard R ISO 22000-2007. Food quality management systems. Requirements for organizations involved in food production]. Available http://www.internet-law.ru/gosts/gost/529. Accessed October 9, 2017 (in Russ.).
57. *Predlagaemye OS «DEKUES» skhemy sertifikatsi* [The certification schemes proposed by the

DEKUES OS]. Available http://www.dqs-russia.ru. Accessed October 9, 2017 (in Russ.).
58. Lagutkina L.Yu. *Vestnik Astrakhanskogo gosudarstvennogo tekhnicheskogo universiteta. Seriya: Rybnoe khozyaistvo*, 2017, 1: 67-78 (doi: 10.24143/2073-5529-2017-1-67-78) (in Russ.).
59. *GOST R 57022-2016. Produktsiya organicheskogo proizvodstva. Poryadok provedeniya dobrovol'noi sertifikatsii organicheskogo proizvodstva* [State Standard R 57022-2016. Organic products. The procedure for voluntary certification of organic production]. Moscow, 2016 (in Russ.).
60. *GOST R 56508-2015. Produktsiya organicheskogo proizvodstva. Pravila proizvodstva, khraneniya, transportirovaniya* [State Standard R 56508-2015. Organic products. Rules of production, storage, transportation]. Moscow, 2015 (in Russ.).
61. *Natsional'nyi organicheskii soyuz: Tol'ko 80 proizvoditelei organicheskoi produktsii v Rossii sertifitsirovany* [National Organic Union: Only 80 manufacturers of organic products in Russia are certified]. Available http://rosorganic.ru/about/press/only-80-organic-producers-are-certi.html. Accessed March 3, 2017 (in Russ.).

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FUNCTIONAL INDICATORS OF POIKILOTHERMIC AQUATIC SPECIES FROM NATURAL AND ARTIFICIAL WATER BIOCENOSES

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Abstract

For assessment of sustainability of natural biocenoses and the physiological and immunological state of hydrobionts in aquaculture, it is necessary to know functional parameters of circulating liquids in hydrobionts of different taxonomic groups. The purpose of this study was to analyze the limits of the main indicators of homeostasis in aquatic animals and fish from natural water bodies, i.e. crayfish (*Astacus astacus* and *Pontastacus leptodactylus*), fish (carp Cyprinus caprio L., tench *Tinca tinca* L. and catfish *Silurus glanis* L.), and amphibians (frogs *Rana temporaria* and *Xenopus laevis*). Here, here is the first report on hematologic, cytochemical, biochemical indicators for these species which inhabits natural water bodies or are artificially grown in the conditions of Moscow and Pskov provinces and Chuvashiya region. Hematological investigations included differential count of blood cells of fishes and amphibians in smears stained by the Pappengeim technique; hemolymph of river crayfish was examined in Goryaev chamber. Immunological parameters were evaluated by cytochemical method as an average cytochemical coefficient (CCC) of lysosomal cationic protein in fish blood neutrophils and crayfish haemocytes in the reaction with Bromphenol blue. Biochemical parameters were assessed in blood serum using a biochemical analyzer Chem Well (Awarenes Technology, Inc., USA). The reference constants of homeostasis we found are as follows: the total number of cells in crayfish hemolymph of 700 to 800 per 1 μl; the number of red blood cells in fish of 1-2 million/μl, blood leukocytes in fish of 50-150 thousand per 1 μl. Interspecific differences in haemocyte patterns between *Astacus astacus* and *Pontastacus leptodactylus* were not revealed. Biochemical differences were as follows: glucose concentration in the *Astacus astacus* hemolymph was 64 % higher compared to that in *Pontastacus leptodactylus* whereas the alkaline phosphatase activity was almost 71 % lower. Agranular and semi-agranular haemocytes, along with juvenile forms that we called transparent cells, serve as phagocytes in crayfish. In healthy crayfish, phagocytic activity of these cells, as estimated by the average cytochemical coefficient of the lysosomal cationic protein level, was approximately the same and ranged from 1.5 to 2.0. In fish, we found gender and species-related differences of homeostatic constants. The presence of promyelocytes, the blast forms of leukocytes, in *Tinca tinca* was indicative of more intensive leukopoiesis. The percentage of neutrophils was higher in male *Tinca tinca* due to 2- to 3-fold number of band neutrophils compared to other groups. The level of non-enzyme cationic protein in the lysosomes of neutrophils of female carp, tench and catfish were higher compared to male individuals. The activity of aspartate aminotransferase (ASAT) in male tench and catfish was almost 3 times higher than that in carp. The carbohydrate metabolism in carp and catfish, in terms of lactate concentration, was more than 3 times higher compared to tench. Among the studied amphibians, we observed interspecific and gender differences. The proportion of segmented neutrophils in *Rana temporaria* was more than 4 times higher than that of *Xenopus laevis.* Gender variations in the number of segmented cells were as follows: the cell number in male *Rana temporaria* and *Xenopus laevis* were 27 and 33 % lower than that of females. The blood lymphocyte counts in *Rana temporaria* were significantly lower than that in *Xenopus laevis.* We found gender differences of *Rana temporaria* on biochemical parameters. As compared to the males, the female *Rana temporaria* showed higher activity of ALAT and ASAT (by 6 and 19 %, respectively), creatine kinase (by 29 %), and alkaline phosphatase (by 60 %). The total blood protein content in amphibians was 2-3 %, blood glucose averaged 1-4 mmol/l, triglycerides varied

from 0 to 400 mg%. It is proposed to use parameters of aquatic organisms' homeostasis for ecological monitoring of natural and artificial water biocenoses.

Keywords: natural and artificial water biocenosis, aquatic animals, lower vertebrates, crayfish, *Astacus astacus*, *Pontastacus leptodactylus*, fish, *Cyprinus caprio*, *Tinca tinca*; *Silurus glanis*, amphibians, *Rana temporaria*, *Xenopus laevis*, homeostasis

Growing demand for qualitative products promotes development of aquaculture and artificial reproduction of animal species — traditional members of the essential biocenosis [1]. At the same time, many species become extinct from water biocenosis under the pressure of antropogenous factors which destabilize hydrobiont communities. Thus, crawfish and sturgeons are very rarely found in biocenosis in Moscow Region. In the past years, number of amphibionts had sharply decreased resulting in uncontrolled reproduction of insects — mosquito, fly, and gad-fly. Recently, reduction in the number of amphibiont populations, death of separate populations or species in general becomes more global [2]. It is evident that it is impossible to secure ecologic safety around and inside big cities without artificial regulation of the population number and specific composition of animal communities. Artificial reproduction and use of shellfish, fish, amphibian species, being the biocenesis stability markers, is suppressed by the lack of detailed information on physiological norm for animals, first of all, inhabitants of water reservoirs, the hydrobionts. Since blood is a labile body system, hematological indicators to the most extent reflect physiological properties of such animals and changes of their ecosystem at pollution of water reservoirs, and serve the basis of bioindication method [3].

Invertebrate hydrobionts significantly differ from lower vertebrates not only by body constitution, but also blood system. Breathing pigment in majority crawfish species is hemocianine [4] that makes its color blue. Blood system of shellfish is open: hemolymph circulating in vessels and intercellular cavities consists of liquid part (plasma), and cell components (haemocytes). Many authors highlight three haemocytes in crawfish [5-7]. We have identified four autonomous morphofunctional types of such cells [8] well distinguished at microscoping of freshly collected hemolymph, which were called agranulocytes, semi-agranulocytes, granulocytes, and transparent cells. Agranular haemocytes (GC I) are small (3-17 μm) usually spherical cells with small number of insertions. They remain unchanged at object plate than other types. Poly-granular haemocytes (GC II) are cells of 8-40 μm in size. They are interim cells between two other cell types. They contain small number of different size granules. Their cytoplasm is destructed at object plate and in 30-40 minutes it is hard to distinguish GC II from agranular haemocytes. Granulocytes (GC III) are the biggest hemolymph cells (up to 50 μm and more) with numerous and big granules with high refringency. Outbreak of granules with further dissolution of cytoplasm commences in 15 minutes following collection of hemolymph. Size of transparent cells (GC IV) is nearly 8-35 μm, they are hardly identified and their nucleus is not visible at light microscopy of native hemolymph. Assumedly, they are non-differentiated predecessors of blood cells. Hemolymph in vitro in aerobic context rapidly changes its rheological properties, loses fluidity and transforms into a gel-like mass, haemocytes are exposed within 30-50 minutes to structural and functional changes, gradually transforming from an oval-fusiform cells into round-shaped formations. In anaerobic context, no rheological changes apparently occur in crawfish endolymph (or the process slows down) which is evidenced by continued lymphatic leakage at tamponade of traumatic injury of cuticle (endolymph does not coagulates), whereas gel-form clot is formed in a few seconds upon hemolymph contact with air [9].

Haemocyte functions in crawfish are not studied enough. However, it is

established that different haemocyte types participate in immune protection. Membranes of haemocyte-agranulocyte contain recognizing receptors. Antigen recognition (for instance, β-1,3-glucans fungi or lipopolysaccharides of bacteria Sn) happen at penetration of alien agents followed by activation of the enzyme cascade promoting discharge of phenol oxidase from semi-agranular and granular cells [10]. It is also established that phagocytosis, incapsulation by haemocyte layers, microbial killing, and agglutination of antigens are done by agranular and semi-agranular haemocytes [11]. As apart from the selective immunity in superior vertebrates, crawfish lack genetically transmitted antibodies and tolerance of immune system which provides the basis to assume that hemolymph cells exhibit phagocytosis activity in anaerobic conditions [12, 13]. Crawfish lack hematopoiesis organs, but have blood-forming tissue located at dorsal and dorsolateral surface of ventriculus. There are five types of blood-forming cells, and their number is approximately 1.4×10^6 [14].

Blood cells and immunity factors of hydrobionts are most studied in fish. Cell morphology in fish is highly diversified and is displayed in form, cell sizes, nucleus, granules, specific content of cell elements, and to a significant effect is defined by ecologic conditions of a specie ecosystem. Values of white and red blood cells depend on temperature and pollution of water, hydrochemical mode, content and quantity of consumed feed, stocking density at raising, season, age, and physiologic state [15-17]. As apart from higher vertebrates, blood of lower fishes contains sufficient number of immature cell forms. There is no unique classification of blood cells. That is, some authors [13, 18] divide normoblasts into basophils, polychromatophils, and oxilophils, and classify them as mature cells; the other authors [19] consider normoblasts as immature cells, and divide erythrocytes, by maturity extent, into basophils and polychromatophils. By maturing, cells of erythroid row pass erythroblast, normoblasts, basophilic, polychromatophilic, and mature erythrocyte stages. Subject to commonly accepted terminology, mature leucocytes are divided into granular or grainy (basophils, eosinophils, neutrophils) and agranular or non-grainy cells (lymphocytes and monocytes). Immature forms of lymphocytic row are presented by lymphoblasts, prolymphocytes, monoblasts, and promonocytes. Mieloblasts, promielocytes, mielocytes, and metamielocytes refer to precursors of cells of myeloid (granular) row [20]. As apart from mammals, fish has more hematopoiesis organs. These are gill apparatus and thymus gland stretching from it, lymphatic follicles, gastric mucosa, heart epithelium and vascular endothelium, spleen (in higher vertebrates and bone fishes it serves as blood cell destruction and phagocytosis organ], and kidneys. Hematopoiesis in bone fishes is mostly active in lymphoid organs, kidneys, and spleen, provided that principal blood circulation organ is kidneys (front part]. Formation of erythrocytes, leucocytes, trombocytes, as well as decomposition of erythrocytes occurs in kidneys and spleen. In fish (as apart from mature mammals), mature and young erythrocytes present in peripheral blood do not serve the pathology indicator.

There is scarce information on cellular content of blood in amphibians. It is known that blood erythrocytes in amphibians are bigger than in fishes, mainly nuclear ones [21-24]. Number of erythrocytes in tailed amphibians is approximately 0.07-0.08 million/μl, in untailed, according to various sources, 0.35-0.50 million/μl and 0.38-0.64 million/μl; number of leucocytes is 2.4-21.0 thousand/μl, of trombocytes — 8.5-21.6 thousand/μl [25]. Sometimes, non-nucleated erythrocytes are found (up to 5 %) [19].

For the first time we have studied hematological, cytochemical, and biological indicators in representatives of water fauna in various taxonomic (crustaceans, fish, amphibians) and gender groups in Moscow, Pskov Regions, and Chuvash Republic. The obtained results could be used at assessment of sustainability of

natural biocenosis and the adequacy of keeping conditions of such hydrobionts in aquaculture.

Purpose of present study was to determine benchmark values for principal homeostasis indicators in hydrobionts from artificial and natural water basins.

Techniques. Studies (2005-2015) were conducted on sexually mature and clinically healthy animals of different taxonomic groups: two species of crawfish: *Astacus astacus* and *Pontastacus leptodactylus*, three fish species (*Cyprinus caprio* L., *Tinca tinca* L., *Silurus glanis* L.) and two amphibian species (*Rana temporaria* and *Xenopus laevis*) from the natural and agricultural water basins of the temperate zone of the European Russia (except for *Xenopus laevis* raised in aquarium conditions). *Astacus astacus* inhabits basins of Pskov Region, *Pontastacus leptodactylus* — of Moscow Region. Carp and catfish were raised in fishing ponds of fish-breeding farm Kirya (Chuvash Republic), tench — in fish-breeding farm Osenka (Moscow Region). Animal groups were formed based on analogue principle, accounting for the generic inhering, sex, age, and live mass. Number of groups depended on availability of object and varied within the limits of small sample (from 5 to 20 imdiciduals).

Subject to our developed methodology [26], probes of circulating liquids of crawfish and fish were aseptically collected by noninvasive method. Hemolympth of crawfish was collected in vivo by puncture of ventral sinus, fish blood from the tail vein. In amphibians, blood for preparation of wipes for hematologic and cytochemical studies were collected in vivo from finger and for biochemical analysis from the heart.

Upon Pappenheim staining method which allows discrimination of nucleus and cytoplasmatic inclusions, cells were first subjected to fixation by May-Gruenwald solution for 3 minutes. After the reagent was removed by washing with distilled water, the preparations were treated with Romanovsky solution during 40 minutes, washed by tapped water, and air dried.

Total number of haemocytes (TNH) for calculation of haemocyte formula in crawfish was counted in Goryaev's chamber in native hemolymph just after collection. For determination of erythropoiesis indicators and differential leucocytes count in fish (leucocyte formula), peripheral blood wipes stained by Pappenheim were used. Hematopoiesis activity in fish was assessed as the portion of immature erythrocyte forms. Amphibian blood cells were counted in Goryaev's chamber. For erythrocyte count, blood sample was diluted 200 times with 0.9 % NaCl solution (20 μl of blood and 4 ml of the solution). Count of erythrocytes X_e in 1 μl of blood was calculated as $X_e = (a_e \times 4000 \times 200)/80$, where a_e is the number of cells in 80 small squares of Goryaev's chamber. For leucocyte count, sample was diluted 20 times with 5 % solution of acetic acid with methylene blue. Number of blood leucocytes X_l per μl was calculated as $X_l = (a_l \times 250 \times 20)/100$, where a_l is the number of leucocytes in 100 large squares of Goryaev's chamber. Digital microscope Optika DM 15 with software OPMIAS (OPTIKA Micro Image Analysis Software) (PriborUfa LLC, Russia) was used.

Immunological indicators were cytochemically assessed by mean cytochemical coefficient (CCC) of lysosomal cationic protein in blood neutrophils of fish and haemocytes of crawfish in test with bromophenol blue [27] adapted for hydrobions [28]. CCC for fish and crawfish was calculated:

$$CCC = [0 \times N_0(H_0) + 1 \times N_1(H_1) + 2 \times N_2(H_2) + 3 \times N_3(H_3)]/100,$$

where $N_0(H_0)$, $N_1(H_1)$, $N_2(H_2)$, $N_3(H_3)$ (%) are the number of neutrophils in fish (haemocytes in crawfish) with activity of 0, 1, 2, and 3 points.

During biochemical studies, hemolymph of crawfish was centrifuged for 5 minutes at 3000 rpm and 6 °C to prevent rapid coagulation. For production of serum, fish blood was placed in dry sterile vial and allowed for 1 hour at room

temperature, afterwards serum was carefully collected using syringe with thin needle, frozen at –15...–20 °C and transported in frozen state in thermal containers with ice to the laboratory. Biochemical blood indicators in fish and hemolymph indicators in crawfish were determined in a programmed automated analyzer Chem Well (Awareness Technology, Inc., USA), with the use of reagent toolkits of JSC Vital Development Corporation (Saint Petersburg) (analysis of proteins by biuret test method) subject to the producer protocol.

Mean values (M) and standard errors of the mean (±SEM) were calculated. Results were processed by variation statistical methods by Studen's t-test. Deviations were statistically significant at $P < 0.05$.

Results. We tested hematologic, cytochemical, and biochemical indicators, based on which conclusion on physiological norm for representatives of species was drawn.

Specific properties of homeostasis in crawfish. Crawfish is a group of cultivated hydrobionts, physiologic properties of which is the least studied. General clinical analysis of internal environment in crawfish provides the objective information on animal adaptiveness to ecosystem conditions and may be used for biomonitoring of environment in general and water environment in particular. Characteristics of hematologic, biochemical, and cytochemical indicators in both species of crawfish are illustrated in Table 1.

1. Hemolymph characteristics in two crawfish species from natural aquacenosis (M±SEM, Moscow and Pskov regions, 2010-2012)

Indicator	*Astacus astacus* (a) (n =10)	*Pontastacus leptodactylus* (n =10)
Haemocyte formula, %:		
agranular cells	40.0±3.9	34.9±4.8
semi-agranular cells	24.2±5.7	29.7±3.4
granular cells	27.8±2.8	32.1±2.4
transparent cells	8.0±1.9	3.3±1.6
Hematologic and biochemical indicators:		
TNH, kiloliter/μl	384±111	911±137[a]
glucose, μmol/l	2.2±0.6	< 0.5[a]
ALAT, IU/l	80.6±11.7	55.1±17.8
ASAT, IU/l	57.7±7.3	55.3±33.5
ALP, IU/l	17.1±2.1	78.0±20.2[a]
Cytochemical indicators:		
CCC, units	1.70±0.06	1.87±0.17

Note. TNH — total number of haemocytes, ALAT and ASAT — alanine and aspartate aminotransferases, respectively, ALP — alkaline phosphatase, CCC — mean cytochemical coefficient.

[a] Differences from *Astacus astacus* are statistically significant at $P < 0.05$.

In endolympth, the counts of all three cell types (agranular, semi-agranular, granular) varied in average within 32-35 %. However, in *P. leptodactylus* number of transparent cells was by 18.2 % less than in *A. astacus*. For other elements, interspecies differences were insignificant. Mean cytochemical coefficient of lysosomal cationic protein in haemocytes of crawfish *P. leptodactylus* is 9.4 % higher than in *A. astacus*.

This experiments show several trends for biochemical indicators of endolympth in crawfish. Activity of alanine aminotransferase of hemolympth (ALAT) in *A. astacus* was trice higher than in *P. leptodactylus*. Other substrate blood indicators change more notably. Mean glucose concentration in hemolympth in *A. astacus* exceeded that in *P. leptodactylus* by 64 %, while activity of alkaline phosphatase (ALP) was about 5 times lower.

Agranular and semi-agranular haemocytes are phagocytes in crawfish. Besides, so called transparent cells have phagocytosis ability (assumedly, juvenile forms) [9]. Our experiments show that phagocyte reserve in two crawfish species is approximately similar.

Homeostasis in fishes. In evolutionary hierarchy, fishes are lower than warm blooded animals and, accordingly, change limits of indicators of the internal environment in vivo are wider in them. In case of carp, tench, and catfish (Table 2), hematopoiesis occurs in approximately similar manner. Leucopoiesis is more intensive in tench (promieocytes, the blast leucocyte forms are

present). Due to rod nuclear cells, the amount of neutrophils is 2-3 times higher in female tench than in other groups. No eosinophils were found in all fish species, basophils were found in female tench, catfish, and also in female carp at insignificant level.

2. Hematologic indicators in fish species (*M*±SEM, fish breeding farms, Volgograd Region, Chuvash Republic, 2010-2012)

Indicator	carp *Cyprinus caprio* L.		Tench *Tinca tinca* L.		Catfish *Silurus glanis* L.	
	males (a) (n =23)	females (b) (n =10)	males (c) (n =7)	females (d) (n =5)	males (e) (n =12)	females (f) (n =10)
Erythropoiesis, %						
Hemocytoblasts, Erythroblasts	0.3±0 2	0.6±0.2	1.0±0.4	–	0.7±0.4	–
Normoblasts	2 9±0 4	3.4±0.3	2.9±0.1	3.0±0.1	2.7±0.4	3.0±1.4
Basophiluıc erythrocytes	8 6±0 4	9.1±1.1	6.0±3.8	12.1±4.2	11.6±4.0	7.5±0.7
Mature erythrocytes	88 2±1 5	86.9±1.4	90.1±1.1	84.9±4.3	85.0±4.4	89.5±2.1
Leucocyte formula, %						
Mieloblasts	–	–	–	–	–	–
Promielocytes	–	–	1.0±0.7	2.0±1.4	–	–
Mielocytes	0 8±0 4	1.3±0.5	–	5.7±1.7 [a,b]	0.5±0.4[d]	1.0±0.4d
Metamielocytes	4 0±0.9	4.3±0.4	–	6.2±4.2	3.0±1.4	3.5±0.7
Neutrophils:						
rod nuclear	1 4±0 3	1.0±0.4	6.0±0.2[a,b]	2.0±0.9[c]	0.7±0.5[c]	1.5±0.4[c]
microxyphil nuclear	1 6±0 4	2.4±0.5	3.2±0.4[a]	4.5±0.1[a,,b]	4.3±0.6[a,b]	4.5±0.8[a,b]
total	3 0±0 3	3.4±0.9	9.2±0.9[a,b]	6.5±0.9[a,,b]	5.0±0.8[a]	6.0±1.2[a]
Eosinophils	–	–	–	–	–	–
Basophils	0 4±0 2	0.1±0.2	2.3±0.8 [a,b]	–	0.3±0.3 [c]	–
Monocytes	3 0±0 3	2.2±0.5	2.1±1.1	5.5±3.5	3.3±2.0	2.5±0.7
Lymphocytes	88 8±1 2	88.7±1.3	85.4±4.4	74.1±5.6[a]	87.9±2.3[d]	87.0±2.8
Phagocyte activity						
CCC, units	1 81±0 07	1.94±0.05	1.68±0.01[b]	2.05±0.01[c]	1.30±0.15[a,b,c,d]	1.72±0.11[e,f]
Biochemical values						
ALAT, IU/l	40 2±10 5	41.3±12.2	39.6±8.9	32.6±5.9	45.0±4.4	75.1±12.8
ASAT, IU/l	164±13	133±39	346±18[a,b]	310±40[a,b]	402±12[a,b]	367±29[a,b]
Glucose, μmol/l	3 6±1 2	4.5±1.1	9.4±1.3[a]	6.6±0.5[a]	7.4±1.1[a]	8.1±1.3[a]
Creatine kinase, IU/l	3896±63	3877±161	3054±18 [a,b]	2990±107 [a,b]	527±93[a,b,c,d]	1185±430[a,b,c,d]
Lactate, mg/dl	66 9±7 5	68.5±5.7	19.9±4.5 [a,b]	19.1±2.7 [a,b]	116.2±5.3[a,b,c,d]	121.1±9.8[a,b,c,d]
ALP, IU/l	25 5±1 5	17.5±0.5[a]	43.6±4.7 [a,b]	56.3±11.4 [a,b]	9.9±6.3[a,c,d]	9.3±4.0[a,c,d]
Albumin, g/dl	11.5±3.4	9.1±1.7	15.2±1.7	14.8±1.4	12.2±0.3	13.7±2.7
Total protein, g/l	26.8±6.4	22.3±1.7	24.9±3.3	21.5±1.2	29.9±2.5	31.0±5.1
Triglicerides, mg/dl	124±42	105±32	76±33	94±25	271±105	178±25
Cholesterol, mg/dl	109±12	118±21	121±39	133±16	134±28	107±26

Note. ALAT and ASAT — alanine and aspartate aminotransferase, ALP — alkaline phosphatase, CCC — mean cytochemical coefficient. Dash means no available data.
[a,b,c,d,e,f] Letters in upper index indicate the variant differences with which are statistically significant at $P < 0.05$.

Quantity of non-enzyme cationic protein in neutrophil lysosomes (CCC) in females was higher than in males; in tench and catfish the differences are reliable. Differences could be explained by strengthening of non-specific cell immunity in females. Activity of aspartate aminotransferase (ASAT) in male tench and catfish was approximately 3 times higher compared to carp, with high reliability, i.e. for carp and tench $t = 13.5$. Biological role of ASAT is transamination important for energy metabolism. Any states requiring urgent mobilization of protein components to cover energy needs of a body are associated with adaptive hormonally stimulated biosynthesis of ASAT. Obtained results evidence on higher stress tolerance in tench and catfish as compared to carp. Reliable over 3-fold growth of lactate content in carp and catfish compared to tench is testified, which evidences on intensive carbohydrate metabolism. At the same time, mineral metabolism, according to ALP activity, in male tench was 2-3 times more intensive.

In general, the studied clinically healthy fishes showed differences in leukogram: tench had higher level of macrophages (neutrophils) that indicates phagocytosis potential. Phagocytic activity of these cells in studied female fishes was somewhat higher than in males.

Homeostasis in amphibians (*Rana temporaria*, *Xenopus laevis*).

In scientific literature, homeostasis indicators in amphibians are discussed unreasonably rarely. In our studies of *R. temporaria* the erythrocyte count ranges within 0.12-0.37 million/μl in males, and 0.22-0.39 million/μl in females; leucocytes range within 0.14-0.38 million/μl in males, and 0.13-0.47 million/μl in females. Frogs show both gender and interspecific differences in leucocyte formula (Table 3).

3. Hematological indicators in frog species (*M*±SEM)

Indicator	*Rana temporaria*		*Xenopus laevis*	
	males (a) (n =10)	females (b) (n =10)	males (n =5)	females (n =5)
Erythropoiesis, %:				
hemocytoblasts, erythroblasts	0.8±0.3	0.4±0.4	1.5±2.1	2.1±0.5
normoblasts	2.8±0.6	2.4±0.5	5.0±2.8	0.9±0.3
mature erythrocytes	96.4±0.7	97.2±0.9	93.5±1.3	97.0±0.6
Leucocyte formula, %:				
metamielocytes	0.2±0.3	–	–	0.8±0.4
rod nuclear neutrophils	0.2±0.3	0.6±0.4	–	1.2±0.6[a]
microxyphil nuclear neutrophils	12.2±0.9	16.8±1.5[a]	2.7±0.5 [a,b]	4.3±0.3 [a,b]
total neutrophils	12.4±0.9	17.4±1.5[a]	2.7±0.5 [a,b]	5.5±0.4 [a,b]
eosinophils	3.0±0.7	3.2±0.9	1.8±0.4	0.9±0.4 [a,b]
basophils	–	0.4±0.4	0.5±0.7	–
monocytes	2.8±0.5	2.0±0.4	2.6±0.8	2.2±0.5
lymphocytes	81.6±1.0	77.0±1.7	92.4±0.8 [a,b]	90.6±1.2 [a,b]
Bacterial activity, units	1.78±0.27	1.78±0.24	2.02±0.06	1.74±1.20

N o t e. BA — bactericide activity of blood neuthrophils. *Rana temporaria* frogs were taken from the natural aquacenosis (Moscow Region, 2014), *Xenopus laevis* grew in an aquarium. Dash means that indicators falls outside the device sensitivity limits.

[a,b] Letters in upper index indicate the variant differences with which are statistically significant at $P < 0.05$.

Blood of *Rana temporaria* males and *X. laevis* females contain metamielocytes. The number of rod nuclear neutrophils was 67 % higher in *X. laevis* females compared to males. Microxyphil nuclear neutrophils in *R. temporara* were 4-fold as much as in *X. laevis*. We revealed gender variations in microxyphil nuclear cells which counts were 27 and 33 % less in *R. temporara* and *X. laevis* males than in females. Eozinophils of white blood granulocyte row in males and females of *R. temporara* was comparable in number, and reliably lower in females of *X. laevis*. Number of blood lymphocytes in *R. temporaria* was lower than in *X. laevis*. The value of neutrophil CCC in males and females was about the same in *Rana temporara* and 22 % higher in males of *Xenopus laevis* compared to females.

4. Biochemical blood indicators in males and females of *Rana temporaria* from natural aquacenosis (*M*±SEM, Moscow Region, 2014)

Indicators	Males (a) (n =10)	Females (n =10)
ALAT, IU/l	164±36	174±33
ASAT, IU/l	88±29	109±36
Glucose, μmol/l	0.8±0.3	1.4±0.2
CK, IU/l	985±159	1388±362
Creatine, μmol/l	46±10	47±5
LDH, IU/l	3798±417	3430±220
Lactat, mg/dl	49±16	52±6
Urine acid, μmol/l	262±110	377±151
ALP, IU/l	28±16	69±45
Albumin, g/dl	21±2	25±0.5
Urea, mg/dl	48±2	56±1[a]
Total protein, g/l	24±6	34±2
Trigliceride, mg/dl	3±1	11±6
Cholesterol, mg/dl	57±11	85±18
Hemoglobin, g/l	93±15	172±6[a]

N o t e. ALAT and ASAT — alanine and aspartate aminotransferases, CK – creatinine kinase, LDH — lactate dehydrogenase, ALP — alkaline phosphatase.

[a] Letter in upper index indicates the variant differences with which are statistically significant at $P < 0.05$.

Total blood volume in amphibians is small that challenges biochemical testing. That is why we managed to determine only few indicators (Table 4). Sexual differences were characteristic of *R. temporaria*. In females as compared to males ALAT and ASAT activity is 6 and 19 % higher, creatine kinase is 29 % higher, and alkaline phosphatase is 60 % higher.

Glucose ensures metabolic processes in vivo and is important in energy metabolism in animals. In *R. temporaria* females, as compared to other studies species, this indicator exceeded that in males to a greater extent (by 43 %). Possibly, nearly 2-

fold excessive content of glucose in females is caused by higher dependence of females from physical environmental factors, especially during reproduction, which makes such indicator the most important one. Interestingly, amphibian females are also superior to males in other biochemical blood parameters (e.g. content of creatine, lactate, urine acid, albumin, urea, and total protein is 30 % higher).

Gender specificity of blood lipid metabolism in amphibians of the studied specie manifests itself in superiority of females over males (blood triglycerides and cholesterol are 68 % and 33 % higher, respectively). Other assessed biochemical blood indicators, except for activity of lactate dehydrogenase, were higher in *R. temporara* females than in males.

It should be noted that blood microxyphil nuclear cells of *R. temporara* males and females (see Table 3) exceeded in number those of *X. laevis* by 78 and 76 %, the number of neutrophils was 78 and 71 % more, and the count of eosinophils was 40 and 69 % more. Number of blood erythroblasts in *X. laevis* males and females as compared to *R. temporara* was 47 and 80 % higher. Normablasts in *R. temporara* males was 44 % lower than in *X. laevis* male. *R. temporara* females left behind *X. laevis* females as per rod nuclear and microxyphil nuclear neutrophils, their total amount and number of eosinophils (by 67, 76, 71, and 69 %, respectively). Number of rod nuclear neutrophils in *X. laevis* males exceeded the indicator of *R. temporaria* by 80 %. Portion of eosinophils in males and females of *R. temporaria* was 44 and 67 % higher compared to *X. laevis.*

Interspecific differences between studied amphibians in number of lymphocytes are not so evident. By the content of agranular haemocytes, *X. laevis* males were somewhat superior to *R. temporaria* males. The trend for lymphocytes in males and females was similar. However, average number of lymphocytes in *R. temporaria* is somewhat higher (by 15 %) compared to *X. laevis.* CCC values in *R. temporaria* and *X. laevis* differ insignificantly.

Therefore, there are specific and gender differences of the studies amphibians by homeostasis indicators that reflects adaptation in various biotopes.

It should be noted that crustaceans, fish, and amphibians have a number of common homeostatic traits, regardless of the great evolutionary remoteness. Circulating liquids contain granular, agranular, and by juvenile cell forms. In haemocyte formula of various crawfish species we identified four haemocyte types in close percentage relationship. Leucogram in the studied lower vertebral hydrobionts is similar though differs in several types of leucocytes, and intensity of erythropoiesis is similar. Biochemistry of internal environment in such different taxones is also sufficiently similar. We detected glucose, proteins, triglycerides in comparable quantities in hemolympth of crawfish, as well as blood plasma in fish and amphibians.

Thus, we suggest the criteria to estimate the adaptiveness of hydrobionts, sustainability of natural biocenoses and/or adequacy of aquaculture conditions. These parameters are as follows: total hemolymph cell number within 700-800 per μl for crawfish, the erythrocyte count of 1-2 million per μl and leucocyte count of 50-150 thousand per μl in fish and frogs. In vertebrate hydrobionts, the blood total protein concentration is 2-3%, the glucose concentration is 1-4 μmol/l and the concentration of triglycerides is 0-400 mg%. Mean cytochemical coefficient of non-enzyme cationic protein of phagocytic cell lysosomes is 1.5-2.1.

REFERENCES

1. Lavrovskii V.V. *Rybovodstvo i rybolovstvo*, 2000, 2: 18-19 (in Russ.).
2. Houlahan J.E., Findlay C.S., Schmidt B.R., Meyer A.H., Kuzmin S.L. Quantitative evidence

for global amphibian population declines. *Nature*, 2000, 404: 752-755 (doi: 10.1038/35008052).
3. Keister I.A. *Ekologiya zhivotnykh*, 2009, 3: 117-125 (in Russ.).
4. Spicer J.I., Taylor A.C. Oxygen-binding by haemocyanins from an ecological series of amphipod crustaceans. *Marine Biology*, 1994, 120(2): 231-237.
5. Martynova M.G., Bystrova O.M., Parfenov V.N. *Tsitologiya*, 2008, 50(3): 243-248 (in Russ.).
6. Söderhäll K., Johansson M.W., Smith V.J. Internal defense mechanisms. In: *Freshwater crayfish: Biology, management and exploitation*. D.M Holdich, R.S. Lowery (eds.). Croom Helm, London, 1988: 213-235.
7. Johansson M.W., Keyser P., Sritunyalucksana K., Söderhäll K. Crustacean haemocytes and haematopoiesis. *Aquaculture*, 2000, 199(1-3): 45-52 (doi: 10.1016/S0044-8486(00)00418-X).
8. Pronina G.I., Koryagina N.Yu., Revyakin A.O. *Izvestiya Orenburgckogo GAU*, 2009, 4(24): 186-189 (in Russ.).
9. Pronina G.I., Koryagina N.Yu. *Izvestiya Orenburgskogo GAU*, 2010, 3(27): 251-253 (in Russ.).
10. Chisholm J.R.S., Smith Valerie J. Comparison of antibacterial activity in the hemocytes of different crustacean species. *Comp. Biochem. Phys. A*, 1995, 110(1): 39-45.
11. *Söderhäll* K., Cerenius L. Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.*, 1998, 10(1): 23-28 (doi: 10.1016/S0952-7915(98)80026-5).
12. Johansson M.W., Soderhall K. The prophenoloxidase activating system and associated proteins in invertebrates. *Progress in Molecular and Subcellular Biology*, 1996, 15: 46-66.
13. Golovina N.A., Trombitskii I.D. *Gematologiya prudovykh ryb* [Hematology of pond fish]. Kishinev, 1989 (in Russ.).
14. Chaga O., Lignell M., *Söderhäll* K. The haemopoietic cells of the freshwater crayfish *Pacifastacus leniusculus*. *Anim. Biol.*, 1995, 4: 59-70.
15. Pickering A.D. Introduction: the concept of biological stress. In: *Stress and fish*. Acad. Press, London-NY, 1993: 1-9.
16. Van Rooij J.M., Videler J.J. Estimating oxygen uptake rate from ventilation frequency in the reef fish *Sparisoma viride*. *Mar. Ecol. Prog. Ser.*, 1996, 132(1-3): 31-41.
17. Ivanova N.T. *Atlas kletok krovi ryb (sravnitel'naya morfologiya i klassifikatsiya formennykh elementov krovi ryb)* [Atlas of fish blood cells — comparative morphology and classification]. Moscow, 1983 (in Russ.).
18. Amineva V.A., Yarzhombek A.A. *Fiziologiya ryb* [Fish physiology]. Moscow, 1984 (in Russ.).
19. Zhiteneva L.D., Makarov E.V., Rudnitskaya O.A. *Evolyutsiya krovi* [Blood evolution]. Rostov-na-Donu, 2001 (in Russ.).
20. Rey Vazquez G., Guerrero G.A. Characterization of blood cells and hematological parameters in *Cichlasoma dimerus* (*Teleostei, Perciformes*). *Tissue Cell*, 2007, 39(3): 151-160 (doi: 10.1016/j.tice.2007.02.004).
21. Hutchins M. *Grzimek's animal life encyclopedia. Vol. 6: Amphibians*. Gale Group, Farmington Hills, 2003.
22. Bagnara T.J., Larsen L.O., Elkan E., Rafferty K.A. Jr., Coopre E.L., Oksche A., Veck M., Ingle D., Capranica R.R., Dodd M.H.I., Dodd J.M. *Physiology of the Amphibia. V. 3*. B. Lofts (ed.). Academic Press, Inc., NY, 2012 (ISBN: 0-12-455403-2).
23. Wei J., Li Y.-Y., Wei L., Ding G.-H., Fan X.-L., Lin Z.H. Evolution of erythrocyte morphology in amphibians *(*Amphibia: Anura*)*. *Zoologia (Curitiba)*, 32(5): 360-370 (doi: 10.1590/S1984-46702015000500005).
24. Arikan H., Çiçek K. Haematology of amphibians and reptiles: a review. *North-West. J. Zool.*, 2014, 10(1): 190-209.
25. Lyubin N.A., Konova L.B. *Metodicheskie rekomendatsii k opredeleniyu i vyvedeniyu gemogrammy u sel'skokhozyaistvennykh i laboratornykh zhivotnykh pri patologiyakh* [Methodology of hemogram analysis of agricultural and laboratory animals under pathology]. Ul'yanovsk, 2005 (in Russ.).
26. Ivanov A.A., Pronina G.I., Koryagina N.Yu., Petrushin A.B. *Klinicheskaya laboratornaya diagnostika v akvakul'tur*e [Clinical laboratory diagnostics in aquaculture]. Moscow, 2013: 6-34 (in Russ.).
27. Shubich M.G. *Tsitologiya*, 1974, 10: 1321-1322 (in Russ.).
28. Pronina G.I. *Izvestiya Orenburgskogo GAU*, 2008, 4(20): 160-163 (in Russ.).

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GENETIC STRUCTURE OF NATURAL POPULATIONS OF STERLET (*Acipenser ruthenus* L.) IN THE CATCHMENT BASINS OF THE KAMA AND OB RIVERS BASED ON POLYMORPHIC ISSR MARKERS

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Abstract

Starlet (*Acipenser ruthenus* L.) is included in the Red Data Books of the Russian Federation, Perm Krai and Kirov Province. Inter-microsatellite DNA polymorphism analysis of sterlet populations of the Kama and Ob rivers has not been performed until now. This paper reports on genetic diversity and genetic structure of five natural sterlet populations of the Kama, Ob and Vyatka rivers based on polymorphism of ISSR-PCR markers. The study was carried out in 2015-2916. DNA was extracted from fragments of pectoral fins of fishes aged 3 to 4 years. DNA samples from 195 individuals were analyzed with five effective ISSR primers. POPGENE 1.31 and GenAlEx6 software was used for statistical processing. Basic genetic parameters were proportion (P_{95}) of polymorphic loci, expected (H_e) heterozygosity, number of alleles per locus (Na), effective number of alleles per loci (Ne), and number of rare alleles (R). The Bayesian method of population structure analysis was performed using STRUCTURE 2.3.4 software. Genetic structure of a population was characterized by proportion of heterozygous genotypes (H_T) in the entire population, the expected proportion of heterozygous genotypes (H_S) in the subpopulation, and the proportion of interpopulation genetic diversity (G_{ST}). As a result, a total of 128 ISSR-PCR markers were identified. The number of amplified ISSR-PCR markers ranged from 7 to 23 depending on the ISSR primer. It was found that the portion of polymorphic loci in *A. ruthenus* populations was high and amounted to 0,938. Genetic diversity was the highest in the Vyatka sterlet population (P_{95} = 0.876; H_e = 0.232; Ne = 1.402; R = 10) and the lowest in the Ob sterlet population (P_{95} = 0.634; H_e = 0.100; Ne = 1.175; R = 3). A total of 23 rare ISSR-PCR markers were identified for all the samples studied, and 10 of these markers were characteristic of the Vyatka river sterlets. This indicates the possibility of successful identification of these sterlets by population-specific markers. Genetic structure analysis showed that the expected proportion of heterozygous genotypes (H_T) for the total sample was 0.283, whereas H_S index was much lower making 0.173, therefore, G_{ST} value was high and amounted to 0.386. The studied populations were highly differentiated. The interpopulation component accounted for 38.6 % of genetic diversity, while intrapopulation component was responsible for 61.4 %. In each of the studied populations, the rare ISSR-PCR markers have been determined that can be used for identification of studied populations of this species. Thus, the efficiency of ISSR analysis for the identification of sterlets at population level has been proved. It has been established that polylocus ISSR-PCR markers can be used both for characterizing gene pools and for molecular genetic identification of populations and breeds, including sterlet populations and replacement broodstocks. Recommendations for genetic conservation of the Kama and Ob sterlet populations have been developed. These data should be used to manage replacement broodstocks in sterlet artificial reproduction for further release of the fry in a population with an identical gene pool.

Keywords: genetic diversity, gene pool, genetic structure, ISSR-PCR markers, molecular-genetic identification, *Acipenser ruthenus* L., sterlets

Polymorphism analysis of molecular-genetic markers is a compulsory development stage of programs for preservation of the genetic resources of sturgeons. Gene pool analysis with the use of molecular-genetic markers testifies the

expressed specific properties of genetic structure and the need for further development of genetic-based methods for preservation of biodiversity of rare fish species [1].

Inter-microsatellite DNA polymorphism analysis [inter simple sequence repeats, ISSR] is a method to investigate genetic diversity of plant and animal populations. It has good repeatability and is successfully applied in the global [2, 3] and national practice [4]. ISSR-methods may be used for identification of cross-species and intra-species genetic variability, identification of kinds, populations, lines, and in several cases for individual genotyping [5]. ISSR markers are breed and species specific [6]. ISSR-method was used by Yu.A Stolpovskiy et al. [7-9], L.V. Nesteruk et al. [10], and P.P. Srivastava et al. [11] for genotyping animal populations and breeds, and for identification of silkworm populations [12]. In this regard, it is perspective to apply multi-locus markers in molecular genetic identification of starlet populations and flocks.

Each year, sturgeon breeding in Russia becomes more extensive as one of the most important agricultural industries. Artificial reproduction allows compensating damages caused to water resources and taking measures for reintroduction of sturgeons [13]. Starlet (*Acipenser ruthenus* L.) is one of the mostly known sturgeon representatives in Russia with long evolutionary history [14]. Certain populations of such species are included in the Red Book of the Russian Federation [15], Perm Territory [16] and Kirov Region [1] Commercial value of sturgeons promotes intensive catching of such fishes. Construction of hydrotechnical facilities and location of industrial zones near water objects had resulted in sharp decrease of the number of natural starlet populations [18]. For its recovery and for compensation of damage caused by economic humankind activity, species raised at fish breeding farms are released to the natural environment. Nowadays, there is no information on effectiveness of such measures since it lacks information on recovery of populations which is one of the most important components of nature protection reacclimatization measures. Accounting of returns to the populations of the species raised at fish breeding farms requires conduction of studies involving identification of young fish at farms, as well as species being members of the natural population in the studied water reservoirs [19]. Due to the need for protection of wild capture and endangered fishes, it is especially important to study populations undergoing antropogenic loads [20]. Inter-microsatellite DNA polymorphism analysis of starlet populations of the Kama River and Ob River basins has not been performed until now.

Use of Inter-microsatellite DNA polymorphism analysis enabled us for the first time to obtain information on starlet populations in Kama, Ob, and Vyatka rivers. We have identified ISSR makers and proposed two approaches to identification of starlet populations. i.e. by unique markers and combination of polymorphous markers.

Purpose of present paper is studying genetic diversity of the natural starlet populations for ISSR marker polymorphism.

Techniques. The natural starlet populations (*Acipenser ruthenus* L.) aged 3-4 years were studied. Samples were collected in 2015-2016 from 195 individuals caught in five places: Vi — Vyatka River near Vishkil Urban Area (middle course), Sh — Vyatka River near Shurma Urban Area (lower course, 236 km from Vi sample down the stream), Vp — Vyatka River in area of Vyatskie Polyani city of Kirov Region (lower course, 138 km from Sh sample down the stream), Km — Kama River lower the stanch of Votkinsk Hydroelectric Station, CHM — Ob River in confluence region of Irtysh and Ob rivers.

Flipper fragments were collected in vivo. Afterwards, fishes were returned to basin. Flippers were fixed in 96 % alcohol. Total DNA was extracted from

100 mg samples by S.O. Rogers method [21]. DNA quality and concentration were controlled spectrophotometrically (NanoDrop 2000, (Thermo Fisher Scientific, USA) and diluted to 10 ng/μl.

Inter-microsatellite DNA polymorphism analysis protocol was as described [5]. A 25 μl PCR (polymerase chain reaction) mixture contained 2 units of Tag DNA-polymerase, 2.5 μl standard 10× PCR buffer, 25 pM of primer, 2.5 μM Mg^{2+}, 0.25 μM dNTPs, 5 μl of total DNA. DNA probes of fishes were analyzed with five earlier selected starlet-effective ISSR primers [22]. PCR (an amplifier GeneAmp Biosystem, Applied Biosystems, USA) was carried out as per usual ISSR protocol: 2 minute preliminary denaturation at 94 °C; 20 second denaturation at 94 °C, 10 second primer annealing at 6 °C, 10 second elongation at 72 °C (5 cycles); 5 second denaturation at 94 °C, 5 second primer annealing at 56 °C, 5 second elongation at 72 °C (30 cycles); 2 minute final elongation at of 72 °C. Annealing temperature depended on primer GC content and varied from 56 to 64 °C. In lieu of DNA, 5 μl de-ionized water was added to reaction mixture as negative control (K^-) to check purity of the reagents. To prove repeatability of the results, PCR analysis was repeated trice. Amplification products were separated by electrophoresis in 1.7 % agar gel with 1× TBE buffer. DNA fragment lengths were determined with molecular weight marker (100 bp + 1.5 + 3 Kb DNA Ladder, SibEnzyme-M LLC, Moscow) using Quantity One software in Gel-Doc XR system (Bio-Rad, USA).

Portion of polymorphic loci (P_{95}), expected heterozygosity (H_e), mean allele number (Na), effective allele number (Ne) per locus, and number of rare alleles (R) were calculated with the use of POPGENE 1.31 software [23] and special macros GenAlEx6 [24] for Microsoft Excel.

The following parameters were used to describe genetic structure of population [25, 26]: expected level of heterozigous genotypes across the entire population (H_T) as a measure of total genetic diversity; expected level of heterozigous genotypes in the subpopulation (H_S) as a measure of interpopulation diversity; proportion of interpopulation genetic diversity in total diversity, or population subdivision indicator (G_{ST}). Bayesian method of analysis of the population structure was carried out with the use of STRUCTURE 2.3.4 software. Reliability assessment of possible cluster groups and their visualization was carried out by STRUCTURE HARVESTER software [27]. Population structure was estimated by allocation of the studied species in the most probable number of clusters according to the algorithm of G. Evanno et al. [28]. Cluster number probability was determined within the range from 1 to 10.

Results. Total 128 ISSR-PCR markers of which 120 were polymorphic (P_{95} = 0.938) were revealed in five samples of the natural starlet populations. Number of amplified ISSR-PCR markers varied from 7 (CR-212 primer) to 23 (X9 primer) depending on the primer. The highest level of polymorphic loci was in sample Vp (P_{95} = 0.876), the least value was in CHM (P_{95} = 0.634). The expected heterozygosity (H_e) for the total sample was low (0.173) (Table 1). The expected heterozygosity was the highest in Vp population (H_e = 0.232), and the least in CHM (H_e = 0.100). We assume that high expected heterozygosity in Vp is due to systematic releases of young fish from the neighboring fish breeding farms where individuals from the Vyatka river natural populations are the basis for breeders' stock. Presence of unique ISSR-PCR markers (R) in only one of the studied populations is important to successfully affiliate starlet with certain population or to control geographic origin. We found 23 unique ISSR-PCR markers in the total sample of natural populations, provided that 10 of them were in Vp sample. This testifies on possibility of successful identification of

starlet belonging to such population.

1. Genetic diversity of the natural starlet (*Acipenser ruthenus* L.) populations (2015-2016)

Populations	P_{95}	H_e	Na	Ne	R
Vp	0.876	0.232 (0.018)	1.625 (0.486)	1.402 (0.381)	10
Km	0.768	0.162 (0.018)	1.476 (0.501)	1.282 (0.374)	5
CHM	0.634	0.100 (0.016)	1.258 (0.439)	1.175 (0.328)	3
Vi	0.835	0.198 (0.017)	1.625 (0.486)	1.325 (0.344)	5
Sh	0.805	0.174 (0.017)	1.523 (0.501)	1.295 (0.364)	0
Total	0.938	0.173 (0.110)	2.000 (0.000)	1.468 (0.340)	23

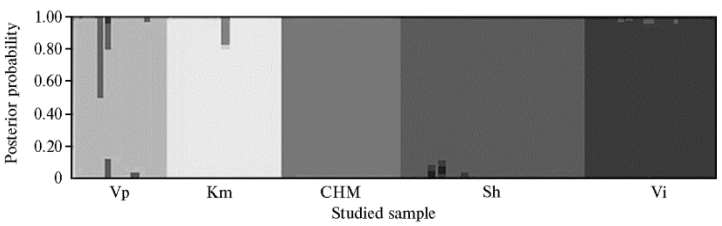
N o t e. P_{95} — portion of polymorphic loci, H_e — expected heterozygsity, Na — number of alleles per locus, Ne — effective number of alleles per locus, R — number of unique ISSR-PCR markers. Values are statistically significant at $P \leq 0.05$; standard deviations (SD) are in parenthesis. See description of populations in the section "Tehniques".

2. Genetic structure and differentiation of the studied starlet (*Acipenser ruthenus* L.) populations o Kama, Ob, and Vyatka rivers (2015-2016)

ISSR-primer	Nucleotide sequence (5′→3′)	H_T	H_S	G_{ST}
CR-212	$(CT)_8TG$	0.302 (0.028)	0.225 (0.017)	0.252
CR-215	$(CA)_6GT$	0.314 (0.033)	0.159 (0.085)	0.492
ISSR-9	$(ACG)_7G$	0.220 (0.022)	0.159 (0.012)	0.277
X9	$(ACC)_6G$	0.277 (0.022)	0.155 (0.008)	0.442
X11	$(AGC)_6G$	0.312 (0.022)	0.182 (0.009)	0.417
Per total sample		0.283 (0.026)	0.173 (0.011)	0.386

N o t e . H_T — expected level of heterozygous genotypes in the total sample, H_S — expected level of heterozygous genotypes in a separate population, G_{ST} — interpopulation genetic diversity in total diversity (population subdivision indicator). Values are statistically significant at $P \leq 0.05$; standard deviations (SD) are in parenthesis.

Analysis of the genetic structure and differentiation of the studied starlet populations (Table 2) revealed the expected heterozygosity H_T of 0.283 for the total sample. The expected level of heterozygous genotypes in certain population (H_S) was 0.173. Accordingly, in average 17 % of heterozygotes were in each population. G_{ST} value for population subdivision is 0.386, i.e. interpopulation genetic diversity component accounted for 38.6 % of diversity, intrapopulation diversity accounted for 61.4 %. That is, the studied starlet samples are highly differentiated.



Allocation of starlet (*Acipenser ruthenus* L.) genotypes from Kama, Ob, and Vyatka rivers (at K = 5) (2015-2016). STRUCTURE software. See description of populations in section "Methodology".

We have identified five genetically segregated groups and determined probability of affiliation of each of the 195 studied individuals with any of the proposed clusters (see Table).

The identified ISSR-PCR markers are stable and reproducible. Obtained data result in two approaches to identification of starlet population, i.e. by unique markers and by combination of polymorphic markers. Illustration of the first way that we suggest is the Vi sample with unique ISSR-PCR 780 bps marker $Ac_{un}780_{X9}$ which is amplified with primer $(ACC)_6G$ (denoted as X9) and present in Vi with frequency of 0.660. Simultaneous presence of two polymorphic markers, the $Ac_p640_{CR\text{-}212}$ (640 bps, frequency 0.706) amplified with primer $(CT)_8TG$, and Ac_p520_{X9} (520 bps, frequency 0.735) amplified with $(ACC)_6G$, implies the affiliation with Km population.

Earlier], ISSR-PCR markers were used to control genetic structure of agricultural mammal species and to identify breed-specific properties of their

gene pools [29]. Simple sequence repeats (SSR) loci were used to identify specific affiliation of sturgeons and to identify fish species of hybrid origin [30]. In this paper we have shown effectiveness of inter-microsatellite DNA polymorphism analysis (ISSR-PCR markers) in studying genetic structure and molecular identification of starlet populations. This is necessary to form replacing breeding stocks in which artificial starlet reproduction occurs with further release of young fish to population with identical gene pool. *A. ruthenus* population from Vyatka river (Vp) with high genetic diversity is recommended for such stocks. Molecular genetic formulae, bar codes, and genetic passports based on molecular markers, including rare ISSR-PCR markers, will provide control of affiliation of individuals to certain population and/or their geographic origin.

Therefore, we have found 128 ISSR-PCR markers in the studied populations of *Acipenser ruthenus* of Kama River and Ob River basins. Polymorphic loci revealed with the use of these markers in the total sample are very frequent (0.938). The *A. ruthenus* sample from Vyatka river (Vyatskie city) shows the highest genetic diversity indicators (P_{95} = 0.876; H_e = 0.232; Ne = 1.402; R = 10) compared to the sample from the same river close to headwaters. Sample from Ob river shows low genetic diversity (P_{95} = 0.634; H_e = 0.100; Ne = 1.175; R = 3). The expected heterozygosity (H_T) in total sample is 0.283, whereas in a separate population this indicator (H_S) is significantly lower (0.173). Accordingly, interpopulation genetic diversity component in starlet populations accounts for 38.6 %, and intrapopulation component accounts for 61.4 %. Studied populations are strongly diversified. Unique molecular markers are identified in each sample of *A. ruthenus*. Combinations of polymorphic markers can be used in molecular genetic identification of the starlet populations.

REFERENCES

1. Kharchenko P.N., Glazko V.I. *DNK-tekhnologii v razvitii agrobiologii* [DNA technology in the development of agrobiology]. Moscow, 2006 (in Russ.).
2. Chen Y., Peng Z., Wu C., Ma Z., Ding G., Cao G., Ruan S., Lin S. Genetic diversity and variation of Chinese fir from Fujian province and Taiwan, China, based on ISSR markers. *PLoS ONE*, 2017, 12(4): e0175571 (doi: 10.1371/journal.pone.0175571).
3. Wazid H., Surendra Nath B. Genetic characterization of microsporidians infection Indian non-mulberry silkworms (*Antheraea assamensis* and *Samia cynthia ricini*) by using PCR based ISSR and RAPD marker assay. *Int. J. Indust. Entomol.*, 2015, 30(1): 6-16.
4. Mel'nikova M.N., Senchukova S.D., Pavlov S.D. *Doklady akademii nauk*, 2010, 435(1): 138-141 (in Russ.).
5. Zietkiewicz E., Rafalski A., Labuda D. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*, 1994, 20: 176-183.
6. Glazko V.I., Feofilov A.V., Bardukov N.V., Glazko T.T. *Izvestiya Timiryazevskoi sel'skokhozyaistvennoi akademii*, 2012, 1: 18-125 (in Russ.).
7. Stolpovskii Yu.A. *Vavilovskii zhurnal genetiki i selektsii*, 2013, 17(4/2): 900-915 (in Russ.).
8. Stolpovskii Yu.A., Lazebnyi O.E., Stolpovskii K.Yu., Sulimova G.E. *Genetika*, 2010, 46(6): 1-9 (in Russ.).
9. Stolpovskii Yu.A., Kol N.V., Evsyukov A.N., Nesteruk L.V., Dorzha Ch.M., Tsendsuren TS., Sulimova G.E. *Genetika*, 2014, 50(10): 1163-1176 (doi: 10.7868/S0016675814100142) (in Russ.).
10. Nesteruk L.V., Makarova N.N., Evsyukov A.N., Svishcheva G.R., Lhasaranov B.B., Stolpovsky Yu.A. Comparative estimate of the sheep breed gene pools using ISSR-analysis. *Russian Journal of Genetics,* 2016, 52(3): 304-313 (doi: 10.1134/S102279541603011X).
11. Srivastava P.P., Kar P.K., Awasthi A.K., Raje Urs S. Identification and association of ISSR markers for thermal stress in polyvoltine silkworm *Bombyx mori. Russian Journal of Genetics*, 2007, 43(8): 858-864 (doi: 10.1134/S1022795407080042).
12. Bhuvaneswari G., Surendra Nath B. Molecular characterization and phylogenetic relationships of seven microsporidian isolates from different Lepidopteran pests cross infecting silkworm *Bombyx mori* based on intergenic spacer sequence analysis. *Journal of Entomology and Zoology Studies*, 2015, 3(2): 324-330.

13. Bazelyuk N.N., Kozlova N.V., Mukhamedova R.M. *Estestvennye nauki*, 2013, 2: 82-86 (in Russ.).
14. Kovalchuk O.M., Hilton E.J. Neogene and Pleistocene sturgeon (*Acipenseriformes*, *Acipenseridae*) remains from southeastern Europe. *J. Vertebr. Paleontol.*, 2017, 37(5): e1362644 (doi: 10.1080/02724634.2017.1362644).
15. *Krasnaya kniga Rossiiskoi Federatsii. Zhivotnye.* [The Red Book of the Russian Federation. Animals]. Moscow, 2001 (in Russ.).
16. *Krasnaya kniga Permskogo kraya* /Pod redaktsiei A.I. Shepelya [The Red Book of the Perm Krai. A.I. Shepel (ed.)]. Perm', 2008 (in Russ.).
17. *Krasnaya kniga Kirovskoi oblasti. Zhivotnye, rasteniya, griby* [The Red Book of Kirov Province. Anumals, plants, fungi]. Ekaterinburg, 2001 (in Russ.).
18. Timoshkina N.N., Vodolazhskii D.I., Usatov A.V. *Ekologicheskaya genetika*, 2010, 8: 12-24 (in Russ.).
19. Fopp-Bayat D., Kuzniar P., Kolman R., Liszewski T., Kucinski M. Genetic analysis of six sterlet (*Acipenser ruthenus*) populations — recommendations for the plan of restitution in the Dniester River. *Iran. J. Fish. Sci.*, 2015, 14(3): 634-645.
20. Adrianov A.V. *Biologiya morya*, 2004, 30(1): 3-19 (in Russ.).
21. Rogers S.O., Bendich A.J. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.*, 1985, 5: 69-76.
22. Komarova L.V., Kostitsyna N.V., Boronnikova S.V. *Materialy Mezhdunarodnoi konferentsii «Tendentsii innovatsionnykh protsessov v nauke»* [Proc. Int. Conf. «Trends in innovative processes in science. Part 1]. Moscow, 2015, chast' 1: 6-8 (in Russ.).
23. Yeh F.C., Mao J., Young R.C. *POPGENE, the Microsoft Windows-based user-friendly software for population genetic analysis of co-dominant and dominant markers and quantitative traits.* Alta, Department of Renewable Resources, University of Alberta, Edmonton, 1999.
24. Peakall R., Smouse P.E. GenAlEx6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes*, 2006, 6: 288-295.
25. Nei M. *Molecular population genetics and evolution.* Amsterdam, 1975.
26. Nei M., Li W.-H. Mathematical model for studying genetic variation in terms restriction endonucleases. *PNAS USA*, 1979, 76: 5269-5273 (doi: 10.1073/pnas.76.10.5269).
27. Earl D.A., Vonholdt B.M. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.*, 2012, 4(2): 359-361 (doi: 10.1007/s12686-011-9548-7).
28. Evanno G., Regnaut S., Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.*, 2005, 14(8): 2611-2620 (doi: 10.1111/j.1365-294X.2005.02553.x).
29. Glazko V.I., Gladyr' E.A., Feofilov A.V., Bardukov N.V., Glazko T.T. ISSR-PCR and mobile genetic elements in genomes of farm mammalian species. *Agricultural Biology*, 2013, 2: 71-75 (doi: 10.15389/agrobiology.2013.2.71eng).
30. Barmintseva A.E., Myuge N.S. *Genetika zhivotnykh*, 2013, 49: 1093-1105 (doi: 10.7868/S0016675813090038) (in Russ.).

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COMPARATIVE ANALYSIS OF RUMEN BACTERIAL COMMUNITY OF YOUNG AND ADULT *Rangifer tarandus* REINDEERS FROM ARCTIC REGIONS OF RUSSIA IN THE SUMMER-AUTUMN PERIOD

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Abstract

Reindeer husbandry is a strategically important industry in the Arctic regions of Russian Federation due to providing the native population with food stuffs. Observing the characteristics of rumen microorganisms' composition is necessary to deepen the information on the reindeer physiology. In this paper, the results of molecular genetic analysis of the rumen bacterial community composition of young and adult specimen *Rangifer tarandus* individuals from Arctic regions of Russia are presented for the first time. Samples of ruminal contents were collected from 3 animals of each age group in 2017 summer-autumn period in the Yamal-Nenets Autonomous District and the Murmansk Province. The bacterial community composition of the reindeer rumen was analyzed in the laboratory of the «BIOTROF+» company by T-RFLP method (terminal restriction fragment length polymorphism). According to the biodiversity indicators, the Yamal-Nenets Autonomous District reindeer ruminal microorganisms' diversity was significantly higher ($P < 0.05$) than that in the reindeers of Murmansk region. Young reindeers from the Yamalo-Nenets Autonomous District showed lower biodiversity indicators ($P < 0.05$) comparing to adults, whereas in the Murmansk region this was not observed. According to the taxonomic affiliation, it has been established that up to 83.50±5.07 % of the phylotypes belong to four bacterial phylums, the *Firmicutes*, *Bacteroidetes, Actinobacteria* and *Proteobacteria*, while *Tenericutes*, *Fusobacteria*, *Acidobacteria*, *Cyanobacteria* were less frequent. Ruminal microbiome of *Rangifer tarandus* reindeers showed much higher proportion of unidentified bacteria, as well as the *Eubacteriaceae* and *Clostridiaceae* bacteria, as compared to the most studied members of the *Bovidae* family. Note, that several *Eubacteriaceae* and *Clostridiaceae* members are capable of detoxification of usnic acid and other secondary metabolites produced by lichens. During the reindeer ontogenesis, noticeable changes in the ratio of phylotypes and taxonomic groups in rumen microbiota were found. The greatest age changes were noticed in the phylum *Firmicutes* composition. In adult reindeer rumen, the total counts of cellulosolytic bacteria of the *Clostridia* class, especially of the families *Eubacteriaceae*, *Clostridiaceae* and *Lachnospiraceae* potentially capable of hydrolysis of plant carbohydrates with the formation of volatile fatty acids (VFA), were significantly higher than in young group ($P < 0.05$). The inverse pattern was characteristic of bacteria with similar properties from the phylum *Bacteroidetes*, including the genera *Bacteroides*, *Prevotella*. Identification of a significant number of opportunistic and pathogenic microorganisms in the *Rangifer tarandus*

rumen bacterial community, with the dominance of the phylum *Fusobacteria*, families *Campylobacteriaceae* and *Enterobacteriaceae*, is also of interest. Up to date, this issue has been poorly observed. Direct regularity in changing ruminal pathogen profiles in reindeers of different age or from different habitat was not revealed. Perhaps the detected differences in the level of pathogenic and opportunistic microorganisms could be associated with other factors, e.g. specific pasture ration in different regions or the epizootic situation in the herd. Additional research will clarify the issues in question. In general, the obtained results can be used as a basis to develop recommendations for improving the efficiency of animals breeding.

Keywords: rumen microorganisms, molecular-genetic methods, reindeer, *Rangifer tarandus,* Arctic regions

Reindeer (*Rangifer tarandus*) is unique species specifically adapted to life in the context of North and, as such, deer farming serves an important animal breeding industry in arctic regions of Russia supplying food to the population. Reindeer diet has significant seasonal differences. In summer-autumn period, it is based on nearly 300 plant species, including grains, sedge, sallow and dwarf birch leaves. Lichen account for up to 15 %. During winter-spring period it is increase up to 70 %, while the remaining 30 % are represented by residues of green plants, moss, and various admixtures [1, 2].

Digestion of vegetable feed in reindeers occurs just like in other ruminants due to enzymes produced by rumen symbionts. It is known that reindeer rumen is inhabited by symbiont microorganisms: bacteria, fungi, archeas, and protozoans [3-5]. Its microbial community may reflect both regional properties of feeding pastoral diet, as well as total physiological state of animals. By today's estimates, diversity of microorganisms in rumen of ruminant animals reaches several thousand species, of which less than 100 were studied in detail. Majority of them are strictly anaerobic non-cultivated species [6-9] and, at that, the most informative methods of analysis of the microbial community in rumen are molecular-genetic aimed at studying of its structure in general, NGS-sequencing (next-generation sequencing) and T-RFLP-analysis (terminal restriction fragment length polymorphism). They allow detecting and determining the content of low-presented microorganisms in rumen community that is demonstrated in tests on cattle stock [10, 11], sheep [12, 13], deer [14], and goats [15, 16].

Nowadays, rumen microbiocenosis in reindeer is less studied among ruminants, although this is of significant concern due to the assessment of adaption-physiologic and anatomic adaption of a body to unfavorable ecosystem conditions and feeding conditions of such animal species. Only few papers on molecular-genetic analysis of rumen microbiocenosis in reindeers, who live in the territory of Northway [14, 17], as well as rumen microbial flora in other representatives of *Cervidae* family — axis deer [18, 19], were published.

In present paper, we have for the first time carried out molecular-genetic studies of rumen microbiome in reindeer living at the territory of two areas of Arctic Russia — Murmansk Region and Yamal-Nenets Autonomous District. Significant differences in content of bacterial community of rumen were established depending in the region and age of animals. The greatest age-specific changes were found in content of *Firmicutes* gens. No direct regularity characterizing age-specific changes in rumen content of pathogenic microorganisms were found.

Purpose of present study is comparative assessment of taxonomic content of bacterial community in rumen of young and mature *Rangifer tarandus* species from different ecosystems in summer-autumn period.

Technuques. Young (aged 1-2 years) and mature (aged 3-6 years) Nenets reindeer individuals were studied. Samples of rumen content were collected in summer-autumn in 2017 from 3 animals of each age-specific group in Yamal-Nenets Autonomous District (AD) (Harp Township, forest-tundra natural-climate area) and Murmansk Region (Loparskaya Station, tundra natural-climate area).

Content of bacterial community in rumen was analyzed bys T-RFLP method [20]. Total DNA was extracted from the samples by Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) subject to producer recommendations. PCR was carried out at a DNA amplifier Verity (Life Technologies, Inc., USA) with the use of eubacterium primers 63F (3′-CAGGCCTAACACATGCAAGTC-5′) with fluorophore WellRed D4 at 5′-end (Beckman Coulter, USA) and 1492R (3′-TACGGHTACCTTGTTACGACTT-5′). Amplification of 16S rRNA gene fragments was performed in the following mode: 3 minutes at 95 °C (1 cycle); 30 seconds at 95 °C, 40 seconds at 55 °C, 60 seconds at 72 °C (35 cycles); 5 minutes at 72 °C. Final concentration of total DNA in the solution was measured using a fluorimeter Qubit (Invitrogen, Inc, USA) with Qubit dsDNA BR Assay Kit (Invitrogen, Inc., USA) subject to producer recommendations.

Fluorescent labeled amplicones of 16S rRNA gene were purified by standard methodology [21]. Restriction of 30-50 ng DNA by HaeIII, HhaI and MspI was done according to producer recommendations (Fermentas, Inc., Lithuania) during 2 hours at 37 °C. Restriction products were harvested using ethanol. Afterwards, 0.2 μl of molecular weight marker Size Standart-600 (Beckman Coulter, USA) and 10 μl formamide Sample Loading Solution (Beckman Coulter, USA) were added to analyze sample patterns using CEQ 8000 (Beckman Coulter, USA), the device error was no more than 5 %. Peak sizes and area were calculated (Fragment Analysis software, Beckman Coulter, USA), prototypes (phylotypes) were identified with the acceptable study-based error of 1 nucleotide with calculation of their relative portion in microbial community. Bacteria affiliation with certain taxonomic group was determined with the use of databases (http://mica.ibest.uidaho.edu/trflp.php).

Results were processed by dispersion analysis (Microsoft Excel 2010 software). Mean (*M*) values and standard errors of the mean (±SEM) are presented in tables below. Reliability of differences between the mean values was assessed by Student's *t*-test. Shannon's and Simpson's biodiversity coefficients were estimated using software http://folk.uio.no/ohammer/past/.

Results. Data on averaged content of summer-based pastoral diet in reindeer is presented in table 1 below.

1. Composition (%) of summer pasture diet of reindeer (*Rangifer tarandus*) in two areas of Arctic Russia

Diet component	I	II
Lichen *Cladonia*	10	5
Lichen *Nephroma*	–	5
Salix borealis	20	5
Salix polaris	–	15
Blueberry *Vaccinium uliginosum*	–	10
Dwarf birch *Betula nana*	20	25
Ordinary birch *Betula pendula*	20	5
Mixture of longstanding grasses	30	30

Note. 1 — Loparskaya Station, Murmansk Region (forest-tundra), 2 — Harp, Yamal-Nenets Autonomous District (tundra). Dashes mean lack of component in the diet.

The highest death rates in reindeer are during the first years of life that is probably caused by nutritional deficiency in the habitats [22, 23].

Used primers allow us to amplify 16S rRNA gene nucleotide positions 63 to 1492 (numeration for *Escherichia coli* 16S rRNA gene).

By T-RFLP, we revealed in reindeer rumen a significant number of bacterial phylotypes, from 106.0±4.70 to 163.0±7.20 depending on the animal age and habitat (Table 2). This indicator varies during ontogenesis. Maximum phylotypes are in young individuals from Murmansk Region ($P < 0.05$). Animals from Yamal-Nenets Autonomous District show inverse pattern ($P < 0.05$).

Shannon's and Simpson's diversity indices for reindeer from Yamal-Nenets Autonomous District were higher ($P < 0.05$), i.e. these animals are more heterogeneous on rumen bacterial community compared to those of Murmansk Region. Younger deer from Yamal-Nenets Autonomous District had lower diversity ($P < 0.05$) compared to mature individuals. This testifies on less entropy and higher homogeneity of the rumen bacterial community. Diversity indices in *R. tarandus* from Murmansk Region during ontogenesis did not significantly vary (see Table 2).

2. Diversity indicators of rumen bacterial community in young (aged 1-2 years) and mature (aged 3-6 years) reindeer (*Rangifer tarandus*) from Murmansk Region (I) **and Yamal-Nenets Autonomous District** (II) (*M*±SEM, 2017)

Indicator	I		II	
	young	mature	young	mature
Shannon's index	2.89±0.32	2.61±0.23	5.40±0.18*	7.12±0.25*
Simpson's index	0.74±0.03	0.74±0.02	0.88±0.03*	0.90±0.04*
Phylotype number, units	150.0±5.40	106.0±4.70	109.5±4.15	163.0±7.20

N o t e. See description of groups in section "Methodology".
* Regional differences are statistically significant at $P < 0.05$.

3. Bacterial taxa (%) **found in the rumen of young (aged 1-2 years) and mature (aged 3-6 years) reindeer (*Rangifer tarandus*) from Murmansk** Region (I) **and Yamal-Nenets Autonomous District** (II) (*M*±SEM, 2017)

Taxon	I		II	
	young	mature	young	mature
Phylum *Bacteroidetes*	8.20±0.38	3,89±0,13*	18,32±0,84	13,45±0,64*
Phylum *Firmicutes*	17.00±0.75	46,59±2,08*	35,93±1,63	48,65±1,96*
class *Clostridia*	9.08±0.40	32,52±1,65*	15,12±0,65	26,86±1,21*
family *Thermoanaerobacteraceae*	2.42±0.11	1,11±0,04*	0,24±0,01	0,12±0,01
family *Lachnospiraceae*	0.52±0.03	6,83±0,31*	2,30±0,10	2,72±0,35
family *Eubacteriaceae*	1.30±0.05	16,90±0,74*	9,47±0,34	15,34±0,48*
family *Ruminococcaceae*	1.16±0.04	0,80±0,03*	0,19±0,01	–
family *Clostridiaceae*	3.68±0.17	5,16±0,19*	2,61±0,20	8,36±0,38*
genus *Peptococcus*	–	1,72±0,07	0,31±0,02	0,32±0,01
genus *Lactobacillus*	4.16±0.19	4,71±0,27	2,66±0,12	1,12±0,06*
genus *Bacillus*	1.56±0.06	5,97±0,24*	4,37±0,25	5,03±0,22
genus *Staphylococcus*	0.14±0.01	0,86±0,04*	0,10±0,01	0,31±0,02*
class *Negativicutes*	2.06±0.08	2,53±0,14	13,68±0,54	15,33±0,63
Phylum *Actinobacteria*	15.65±0.78	4,47±0,17*	12,20±0,52	7,91±0,30*
genus *Bifidobacterium*	0.25±0.02	0,15±0,01*	1,09±0,06	0,21±0,02*
other	15.40±0.65	4,32±0,16*	11,11±0,36	7,70±0,21*
Phylum *Proteobacteria*	7.63±0.29	13,11±0,63*	4,34±0,21	13,49±0,34*
family *Enterobacteriaceae*	0.64±0.03	7,59±0,12*	1,83±0,09	1,00±0,04*
family *Campylobacteriaceae*	6.08±0.28	3,04±0,10*	1,30±0,05	9,69±0,35*
family *Pseudomonadaceae*	0.91±0.03	1,94±0,43*	0,32±0,05	0,48±0,02
family *Burkholderiaceae*	–	0,29±0,01	0,89±0,04	2,32±0,08*
family *Succinivibrionaceae*	–	0,25±0,01	–	–
Phylum *Tenericutes* (genus *Mycoplasma*)	0.82±0.02	1,48±0,04*	–	–
Phylum *Fusobacteria*	1.05±0.04	0,97±0,06	0,18±0,01	1,65±0,05*
Phylum *Cyanobacteria*	–	–	0,70±0,03	0,75±0,02
Phylum *Acidobacteria*	–	–	0	0,33±0,01
Non-classified sequences	49.65±3.35	29,49±1,32*	28,33±1,12	13,77±0,95*

N o t e. See description of groups in section "Methodology". Dashes mean that values are below those validly determined by T-RFLP method.
* Differences between mature and young species within one region are statistically significant at $P < 0.05$.

Majority of identified phylotypes are of four bacterial phyla, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*, which in total, depending on the age and habitat, makes 48.48±4.19 to 83.50±5.07 % of the reindeer rumen bacteria community (see Table 3). Bacteria of *Tenericutes*, *Fusobacteria*, *Acidobacteria*, and *Cyanobacteria* phyla are less abundant. Significant part of phylotypes, from 13.77±0.95 to 49.65±3.35 %, could not be identified and refer to any taxon. This necessitates additional studies of their functional role.

Our research results are in line with contemporary understanding of ru-

men microbiota in ruminants [8, 20, 24], and particularly in reindeer [14, 17]. It was earlier reported on large quantity of unidentified taxa in rumen of reindeer *Rangifer tarandus* from Northway as compared to cattle stock and Thompson gazelles [5].

In the samples we collected the percentage of *Eubacteriaceae* and *Clostridiaceae* members of *Clostridia* having cellulose and sacharolythic properties was also significantly higher than that reported for more studied ruminants of *Bovidae*, in particular for cattle [8, 20, 24]. According to researchers, these anaerobic microorganisms, in particular *Eubacterium rangiferina*, in reindeer ensure detoxication of usnic acid and other secondary metabolites produced by lichen of *Cladonia*, *Usnea*, *Lecanora*, *Ramalina*, *Evernia*, *Parmelia*, *Alectoria* genera [25-27]. It is believed that due to specific properties of microbial community of reindeer rumen, consumption of significant quantity of lichen during winter (up to 70 % in total diet) does not have toxic effect on reindeer (as apart from elk or sheep). Note, it was reported on massive death of over 300 elks due to lichen consumption at lack of the alternative feed [25].

Microorganisms of phylum *Cyanobacteria* are present in reindeer rumen [17] that is entirely logic since cyanobacteria refers to lichen symbionts. Lichen cyanobionts are mostly the members of *Nostoc* genus, and to a lesser extent represent *Calothrix*, *Scytonema*, and *Fischerella* genera [28]. In out experiment, the number of cyanobacteria was minor among the individuals from Yamal-Nenets Autonomous District and did not reach the limits of valid determination by T-RFLP method in animals from Murmansk Region that is probably due to regional specific of summer pasture diet of reindeer.

We have identified a number of similar trends in age-specific changes of microbiome structure in reindeer from various Arctic regions. Thus, total percentage of unidentified bacterial phylotypes in mature animals is significantly lower ($P < 0.05$) than in young animals. The greatest age-specific changes among the identified taxa are noted for phylum *Firmicutes*. Total content of microorganisms of *Clostridia* class in rumen of mature species, especially members of *Eubacteriaceae*, *Clostridiaceae*, and *Lachnospiraceae* families potentially able to hydrolyze vegetable carbohydrates to volatile fatty acids, is higher compared to young animals ($P < 0.05$). Bacteria with similar properties from *Bacteroidetes* phylum (including *Bacteroides*, *Prevotella*) which ferment starch, cellulose, several other carbohydrates, proteins and deaminate amino acids shows opposite pattern.

Interestingly, *Ruminococcaceae* family cellulolytic bacteria found in cattle rumen in significant quantity [8, 20, 24] are fully absent in reindeer in our study.

Count of *Negativicutes* bacteria able to utilize acids (including acetic, propione, butyric, lactic, and etc.) after fermentation of mono-, oligo- and polysaccharides had some trend towards growth in mature individuals compared to young animals. Significant abundance of selenomonades in rumen, including *Selenomonas ruminantium*, which differ by appearance and biodiversity from those found in cattle is described by B.V. Tarakanov [7]. It was reported that acid-utilizing bacteria of *Megasphaera*, *Selenomonas*, *Dialister* genera are physiologically significant groups for cattle since they disallow formation of lactate in the rumen. This prevents a drop of pH followed by lactate acidosis [7, 20, 29]. Here, we want to draw attention to reliably low percent found for acid-forming *Lactobacillus* ($P < 0.05$) and high count of acid-utilizing members of class *Negativicutes* ($P < 0.05$) in reindeer from Yamal-Nenets Autonomous District compared to those from Murmansk Region. Identified differences are probably due to regional summer pasture diets in Murmansk Region (tundra) and Yamal-Nenets Autonomous District (forest-tundra area).

Rumen community is widely represented by conventionally pathogenic

microorganisms, majority of which is traditionally related to gastroenteritis. These are bacteria of *Enterobacteriaceae* and *Pseudomonadaceae* families. The percentage of actinomycetes of phylum *Actinobacteria* (*Coriobacteriaceae*, *Corynebacterium*) including causative agents of actinomycosis which affect different organs and tissues [20] was high. Among pathogenic bacteria, we identified agents of campylobacteriosis (*Campylobacteraceae* family), pasteurellosis (*Pasteurellaceae* family), mycoplasmosis (*Tenericutes* gens), necrobacteriosis (*Fusobacteria* genus), and purulo-necrotic infections (*Staphylococcus* genus). Counts of these pathogens in the studied samples were minor, except for enterobacteria, actinobacteria, campylobacteria, and fusobacteria. Importantly, in Northern reindeer only necrobacteriosis causing massive death of young animals is quite fully studied. In cattle, *Fusobacterium necrophorum*, the causative agent of necrobacteriosis, may penetrate into blood and then cause hepatic abscess, injury of hooves, skin, and mucosa [22, 23, 29].

In our study we did not found direct regularity characterizing age-specific changes in rumen composition of pathogenic microorganisms, including *Fusobacteria* gens, *Campylobacteriaceae*, *Enterobacteriaceae* families. The identified differences in emergence of these pathogenic and conventionally pathogenic microorganisms are probably related to nutrition and epizootic situation in stock that requires clarification in additional studies.

Therefore, the results of T-RFLP analysis evidence in notable changes of bacterial community in reindeer rumen during ontogenesis and differences in community composition in animals living in Murmansk Region and Yamal-Nenets Autonomous District. In general, over 80 % of identified microorganisms refer to four bacterial genera, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*. *Tenericutes*, *Fusobacteria*, *Acidobacteria* and *Cyanobacteria* taxa are less freguent. Total diversity of microorganisms is higher in reindeer from Yamal-Nenets Autonomous District ($P < 0.05$) as compared to animals from Murmansk Region. There are similar trends in composition of bacteria which are potentially able to hydrolyze vegetable carbohydrates. No direct regularity characterizing age-specific changes in rumen composition of pathogenic microorganisms are found. Obtained results extend the available information on reindeer physiology in Arctic Russia conditions. These results may be helpful to improve reindeer herding and decrease animal death.

REFERENCES

1. Orpin C.G., Mathiesen S.D., Greenwood Y., Blix A.S. Seasonal changes in the ruminal microflora of the high-arctic Svalbard reindeer (*Rangifer tarandus platyrhynchus*). *Appl. Environ. Microb.*, 1985, 50(1): 144-151.
2. Sundset M.A., Edwards J.E., Cheng Y.F., Senosiain R.S., Fraile M.N., Northwood K.S., Præsteng K.E., Glad T., Mathiesen S.D., Wright A.D.G. Molecular diversity of the rumen microbiome of *Norwegian reindeer* on natural summer pasture. *Microb. Ecol.*, 2009, 57: 335-348 (doi: 10.1007/s00248-008-9414-7).
3. Mathiesen S.D., Mackie R.I., Aschfalk A., Ringø E., Sundset M.A. Microbial ecology of the gastrointestinal tract in reindeer — changes through season. In: *Microbial ecology of the growing animal; Biology of the growing animals. V. 3.* W. Holzapfel, P. Naughton (eds.). Elsevier Press, Oxford: 73-100.
4. Sundset M.A., Edwards J.E., Cheng Y.F., Senosiain R.S., Fraile M.N., Northwood K.S., Praesteng K.E., Glad T., Mathiesen S.D., Wright A.D. Rumen microbial diversity in Svalbard reindeer, with particular emphasis on methanogenic archaea. *FEMS Microbiol. Ecol.*, 2009, 70(3): 553-562 (doi: 10.1111/j.1574-6941.2009.00750.x).
5. Sundset M.A., Præsteng K.E., Cann I.K.O., Mathiesen S.D., Mackie R.I. Novel rumen bacterial diversity in two geographically separated sub-species of reindeer. *Microb. Ecol.*, 2007, 54: 424-438 (doi: 10.1007/s00248-007-9254-x).
6. Orpin C.G., Joblin K.N. The rumen anaerobic fungi. In: *The rumen microbial ecosystem*. P.N. Hobsen, C.S. Stewart (eds.). Blackie Academic & Professional, London, 1997: 140-195.

7. Tarakanov B.V. *Metody issledovaniya mikroflory pishchevaritel'nogo trakta sel'skokhozyaistvennykh zhivotnykh i ptitsy* [Methods for studying microflora of the digestive tract of agricultural animals and poultry]. Moscow, 2006 (in Russ.).
8. Church D.C. *Ruminant animal: Digestive physiology and nutrition*. Prentice Hall, New Jersey, 1993.
9. Hungate R.E. *The rumen and its microbes*. Academic Press, NY, 1966.
10. Jami E., Mizrahi I. Composition and similarity of bovine rumen microbiota across individual animals. *PLoS ONE*, 2012, 7(3): e33306 (doi: 10.1371/journal.pone.0033306).
11. Veneman J.B., Muetzel S., Hart K.J., Faulkner C.L., Moorby J.M., Perdok H.B. Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? *PLoS ONE*, 2015, 10(10): e0140282 (doi: 10.1371/journal.pone.0140282).
12. Snelling T.J., Genç B., McKain N., Watson M., Waters S.M., Creevey C.J., Wallace R.J. Diversity and community composition of methanogenic archaea in the rumen of Scottish upland sheep assessed by different methods. *PLoS ONE*, 2014, 9(9): e106491 (doi: 10.1371/journal.pone.0106491).
13. de la Fuente G., Belanche A., Girwood S.E., Pinloche E., Wilkinson T., Newbold C.J. Pros and cons of Ion-Torrent next generation sequencing versus Terminal Restriction Fragment Length Polymorphism T-RFLP for studying the rumen bacterial community. *PLoS ONE*, 2014, 9(7): e101435 (doi: 10.1371/journal.pone.0101435).
14. Salgado-Flores A., Hagen L.H., Ishaq S.L., Zamanzadeh M., Wright A.-D.G., Pope P.B. Rumen and cecum microbiomes in reindeer (*Rangifer tarandus tarandus*) are changed in response to a lichen diet and may affect enteric methane emissions. *PLoS ONE*, 2016, 11(5): e0155213. (doi: 10.1371/journal.pone.0155213).
15. Han X., Yang Y., Yan H., Wang X., Qu L., Chen Y. Rumen bacterial diversity of 80 to 110-day-old goats using 16S rRNA sequencing. *PLoS ONE*, 2015, 10(2): e0117811 (doi: 10.1371/journal.pone.0117811).
16. Wang L., Xu Q., Kong F., Yang Y., Wu D., Mishra S., Li Y. Exploring the goat rumen microbiome from seven days to two years. *PLoS ONE*, 2016, 11(5): e0154354 (doi: 10.1371/journal.pone.0154354).
17. Zielińska S., Kidawa D., Stempniewicz L., Łoś M., Łoś J.M. New insights into the microbiota of the Svalbard Reindeer *Rangifer tarandus platyrhynchus*. *Front. Microbiol.*, 2016, 7: 170 (doi: 10.3389/fmicb.2016.00170).
18. Gruninger R.J., Sensen C.W., McAllister T.A., Forster R.J. Diversity of rumen bacteria in Canadian cervids. *PLoS ONE*, 2014, 9(2): e89682 (doi: 10.1371/journal.pone.0089682).
19. Zhi-Peng L., Na J., Han-Lu L., Xue-Zhe C., Yi J., Fu-He Y., Guang-Yu L. Analysis of bacterial diversity in rumen of sika deer (*Cervus nippon*) fed different forages using DGGE and T-RFLP. *China Agriculture Science*, 2014, 47(4): 759-768 (doi: 10.3864/j.issn.0578-1752.2014.04.016).
20. Laptev G.YU., Novikova N.I., Il'ina L.A., Iyldyrym E.A., Nagornova K.V., Dumova V.A., Soldatova V.V., Bol'shakov V.N., Gorfunkel' E.P., Dubrovina E.G., Sokolova O.N., Nikonov I.N., Lebedev A.A. *Normy soderzhaniya mikroflory v rubtse krupnogo rogatogo skota* Norms of microflora in rumen of cattle]. St. Petersburg, 2016 (in Russ.).
21. Maniatis T., Fritsch E.F., Sambrook J. *Molecular cloning: A laboratory manual*. Cold Spring Harbor, NY, 1982.
22. Samandas A.M., Laishev K.A., Samoilov S.G. *Sibirskii vestnik sel'skokhozyaistvennoi nauki*, 2011, 5-6: 92-96 (in Russ.).
23. Laishev K.A., Samandas A.M., Mityukov A.S., Prokudin A.V., Silkina E.V. *Izvestiya Sankt-Peterburgskogo gosudarstvennogo agrarnogo universiteta*, 2011, 24: 118-121 (in Russ.).
24. Nelson K.E., Zinder S.H., Hance I., Burr P., Odongo D., Wasawo D., Odenyo A., Bishop R. Phylogenetic analysis of the microbial populations in the wild herbivore gastrointestinal tract: insights into an unexplored niche. *Environ. Microbiol.*, 2003, 5: 1212-1220 (doi: 10.1046/j.1462-2920.2003.00526.x).
25. Roach J.A.G., Musser S.M., Morehouse K., Woo J.Y.J. Determination of usnic acid in lichen toxic to elk by liquid chromatography with ultraviolet and tandem mass spectrometry determination. *J. Agr. Food Chem.*, 2006, 54: 2484-2490 (doi: 10.1021/jf052767m).
26. Luzina O.A., Salakhutdinov N.F. *Bioorganicheskaya khimiya*, 2016, 3(42), 2016: 276 (doi: 10.7868/S0132342316030106) (in Russ.).
27. Sundset M.A., Kohn A., Mathiesen S.D., Præsteng K.E. *Eubacterium rangiferina*, a novel usnic acid-resistant bacterium from the reindeer rumen. 2008. *Naturwissenschaften*, 95: 741-749 (doi: 10.1007/s00114-008-0381-0).
28. Pankratov T.A., Kachalkin A.V., Korchikov E.S., Dobrovol'skaya T.G. *Mikrobiologiya*, 2017, 3(86): 265-283 (doi: 10.7868/S0026365617030156) (in Russ.).
29. Nocek J.E. Bovine acidosis: implications on laminitis. *J. Dairy Sci.*, 1997, 80: 1005-1028 (doi: 10.3168/jds.S0022-0302(97)76026-0).

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DIOXIN AND HEAVY METALS CONTAMINATION OF REINDEER OFFAL FROM RUSSIAN FAR NORTH REGIONS

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Abstract

Reindeer herding is vitally important agricultural sector in Russian Far North regions. Meat, liver, kidneys and other offal are highly consumed by indigenous people and go for export, therefore problem of reindeer products safety related to chemical contamination is of both scientific and practical interest. Here, we report levels of dioxins, dl-PCBs, cadmium and mercury in reindeer meat, liver and kidneys determined in 704 individual samples of meat, liver and kidneys of reindeers (*Rangifer tarandus tarandus* and *Rangifer tarandus sibiricus*) from 8 main reindeer-herding regions of Russia within the broad geographical range from western to eastern border of the country, including Kola Peninsula, Nenets Autonomny Orkrug and Yamalo-Nenets Autonomny Orkrugs, Taymir Peninsula, Kamchatka and Chukotka. Stable organic pollutants, including dioxins (polychlorinated dibenzo-para-dioxins and polychlorinated dibenzofurans) and dioxin-like polychlorinated biphenyls, were determined by high-resolution chromatography-mass spectrometry and expressed as WHO toxic equivalents (WHO-TEQ). Toxic elements were determined by mass spectrometry with inductively coupled plasma. Dioxin pollution has shown clear geographical distribution. It reaches the highest level at the Kola Peninsula and decreases to the East. Heavy metal pollution did not show any geographical trends. In particular, dioxins concentration in reindeer liver varied from 42.2±10.6 pg WHO-TEQ/g of fat in Kola Peninsula to 1.2±0.6 WHO-TEQ/g of fat in Chukotka. We have also investigated cadmium and mercury levels in different feed samples (lichen, mushrooms, compound feed and forage grain). Literature analysis showed a significant decrease of dioxin levels in reindeer liver over the last 15 years, while cadmium and mercury content in reindeer liver and kidneys has increased dramatically. Causes of clear geographical distribution of dioxin pollution and significant rise of heavy metal contamination need further research. It was shown that cadmium poses the highest health risk. Consumption of reindeer offal in six out of 8 investigated regions may lead to cadmium intake exceeding the tolerable intake level more than threefold. In the meantime, consumption of reindeer meat poses no health risk related to any of the investigated contaminants. Taking into account growing interest to reindeer liver processing for food production, it is usable to take into account our data on possible risk of longtime consumption of reindeer offal.

Keywords: *Rangifer tarandus* L., reindeer, dioxins, polychlorinated biphenyls, cadmium, mercury, persistent pollutants, Far North

Deer herding is an important agricultural industry of the Russian Federation. Total reindeer stock at the national territory reaches nearly 1.5 million of animals [1]. Deer meat is one of the main food products in core nations at the north of Russia and, thus, analysis of deer meat and sub-product contamination by dioxins and toxic elements is important [2-4]. It is believed that Northern ecosystems are apt to accumulation of sustainable contaminants since they have all necessary characteristics, including climate and food chain specificities. Contaminants reach the highest values in organisms being at the top of food

chain. Accumulation of xenobiotics depends on genus, feeding preferences, and metabolic activity of animals. As to reindeer, seasonal accumulation and mobilization of fat during winter plays an important role. Lipophilic compounds accumulated in fat tissue, in particular dioxins and dioxin-like polychlorinated biphenyls (dPCB), penetrate into other organs and tissues during fat mobilization [5-7]. Elective increase of dioxin accumulation occurs in liver of reindeer and sheep as compared to other productive animals (cows, pigs, poultry). Dioxins and dPCB are mainly penetrated into body in alimentary way (with feed and soils [8, 9].

Increased dioxin and dPCB accumulation in reindeer and sheep liver are caused by specific biochemical properties (in particular, lower activity of detoxification enzymes, including cytochrome CYP1A); frequent change of pastures which increases probability of local point contaminated by dioxins; eating soil particles at pasturing [8]. Young reindeer show active penetration of dioxin and dPCB with mother's milk [10]. Increased dioxin accumulation is specific only for reindeer liver; their content in other organs and tissues, including muscles, kidneys, fat, blood, brain, and spleen is insignificant [11, 12].

During the entire year, vascular plants and lichen serve the main food for deer, where reindeer moss is the feeding base. Lichen makes over 50 % feed during winter. Accumulation of heavy metals in lichen at atmosphere contamination and active consumption of lichen by deer during winter are deemed to be the key factors defining the presence of heavy metals in organism [13]. Contamination of far North occurs mainly due to cross-border atmosphere and hydrosphere transfer from other regions. For instance, only 13 % of mercuric contamination sources of Murmansk Region are located at the territory of the Kola Peninsula, 22 % — in other regions of Russia, and the other regions are Europe, China, America, Central Asia, etc. At the same time, industrial enterprises located at the territory of Far North, mainly mining ones (Severonikel of Kola Peninsula and Norilskiy Nikel of Krasnoyarsk Territory), also contribute to contamination by dioxin and toxic elements [2]). Polychlorinated biphenyls were actively produced in the past for different needs, for instance, for oil, paints, and lacquers [14]. During studies of Russian Far North within the International Program of Monitoring and Assessment of Arctic State it was concluded on presence of active unidentified dPCB sources at the territory of Russian Arctic, which were probably storage places of non-disposed PCB stocks [2].

Long-term effect of increased dioxin and dPCB concentrations negatively affects immune, nervous, and endocrine systems, sexual function and fetal development, and causes oncologic diseases [15]. Cadmium is first of all toxic for kidneys, may cause serious disorders in their function up to renal insufficiency. Accumulation of metal results in bone demineralization. Its effect is also due to growth of occurrence frequency of lung, endometrial, bladder, and breast cancer [16]. Mercury has toxic effect on developing nervous system, negatively affects the immune, blood, and sexual systems, liver and kidneys [17].

In present study we for the first time have identified content of toxic contaminants in reindeer meat and sub-products with reference to geographic regions and have assessed the risk at consumption of deer kidneys, liver, and meat. The highest dioxin contamination of reindeer liver was noted at Kola Peninsula, in furtherance its degree was decreasing when moving from West to East. We have not found similar dependence for cadmium and mercury.

Our purpose was assessment of the composition of dioxin, dioxin-like polychlorinated biphenyls, cadmium, and mercury in sub-products and muscular tissue of reindeers (*Rangifer tarandus tarandus* L. and *Rangifer tarandus sibiricus* Murray) from eight regions of Far North of Russia, studying of time-based con-

tamination dynamics by comparison with data from 15-year old literature sources, and estimation of xenobiotic penetration dosage to organism of the representatives of core northern nations.

Techniques. Reindeer tissue probes (704 individual samples of muscular tissue, liver, and kidneys) and feed (reindeer moss, mushrooms, combined feed, grain) were collected by inspectors of the Russian Service for Veterinary and Phytosanitary Surveillance within the scope of the state veterinary monitoring of food product and feed safety in years 2014-2016 in eight main deer breeding regions of the Russian Federation: Murmansk Region, Nenets Autonomous District (AD), Komi Republic, Yamal-Nenets Autonomous District, Taymyr Peninsula (Krasnoyarsk Territory), Sakha Republic (Yakutia), Kamchatka Territory and Chukotka Autonomous District. Tissues samples were frozen and delivered to the Russian State Center of Quality and Standardization of Medicinal Products for Animal Use and Feed (VGNKI, Moscow).

Dioxins (polychlorinated dibenzo-para-dioxins and poly-chlorinated dibenzofuranes) and dPCB were determined by approved methodology [18] with the use of a chromatography-mass-spectrometer Autospec Premier (Waters Corp., USA). Defrosted probes (50-100 g) were chopped and, after addition of internal standard solutions of isotope labeled dioxins and dPCB (Wellington Labs, Canada), wiped with sorbent Prep DE (Dionex, USA) until getting a homogenous mixtures. Afterwards, they were extracted under pressure by hexane-dichloride-methane solution 1:1 (vol.) (ASE 350, Dionex, USA). Extracts ere defatted (H_2SO_4/silica gel), purified on columns filed with 10 % activated carbon on celite, steamed and analyzed with the use of gas chromatograph with mass-spectrometric detection (GX-MK) in selected ion monitoring (SIM) mode at mass-spectrometric resolution no lower than 10 000 with capillary column VF-Xms (60 m×0.25 μm, Agilent, USA). Total concentrations of dioxin congeners and dPCB were expressed in toxic equivalent units of World Health Organization (WHO TEU) and recalculated for fat content in the original sample. Fat content in probes was determined gravimetrically (State Standard GOST 23042-86 “Meat and Meat Products. Methods of Fat Determination”).

Toxic elements in samples were assessed according to the approved methodology [19]. Content of toxic elements (cadmium and mercury) was determined by mass-spectrometry method with inductively coupled plasma (ISP-MS) (mass-spectrometer Varian 820 MS, Varian, Australia). For measurements, samples were dissolved in nitric acid by microwave deterioration.

Overall dosage (OD) of dioxins and dPCB entering the organism was calculated by formula: OD(d) = AC(d) × ACons(d), where AC(d) is average concentration of total dioxins and dPCB in reindeer liver, kidneys or meat, pg WHO TEU/g of the total weight; ACons(d) is average consumption of liver, kidneys or meat, g/pax. per week. Due to lack of information on consumption of deer liver by inhabitants of Far North of Russia, liver consumption value by Sami in Northway of 64 g/pax. per week [13] was used in calculation, accounting for similar diet in deer breeding nations. Annual volume of consumed deer meat was taken from data by reindeer-breeding Chukchi (Chukotka Autonomous District) and comprised 72 kg/pax. per year [20]. Cadmium and mercury overall entering dosage OD(t), μg/pax. per week, was calculated by formula: OD(t) = AC(t) × ACons(t), where AC(t) is average content of cadmium or mercury in deer liver, kidneys or meat, μg/kg; ACons(t) is average consumption of deer liver, kidneys or meat, g/pax. per week. Since there is no information on consumption of deer kidneys by core nations of Russian Far North, the above indicated value for liver was used. Also, according to data collected for Eskimos of Northern Canada, core deer-breeding nations consume more deer kidneys than deer liver [21]. Obtained

results were compared with values of acceptable transferable weekly dosages of dioxin and dPCB, cadmium and mercury sum. For sum of dioxin and dPCB and cadmium, we have used values of 14 pg/kg and 2.5 μg/kg of human body weigh per week as established by EU Scientific Committee on Food [15, 16]. For mercury, we used the WHO values of 4 μg/kg of human body weight per week [22]). WHO model average weight of 60 kg person was applied to recalculate values expressed per kilogram of body weight to the values per person [23].

Data on toxic element accumulation which were obtained for regions with sample number N > 10 were involved in statistical processing. Average concentrations in several samples were subjected to dispersion analysis (ANOVA) for identification of statistical differences between regions. Values were statistically significant at $P < 0.05$. Mean values (X) and standard errors of the mean (Δ) are shown in tables below.

Results. Content of dioxins and dPCB in muscular tissue of reindeer does not exceed permissible values (see Table 1).

1. Accumulation of dioxins and dioxin-like polychlorinated biphenyl (D and dPCB, pg WHO TEU/g of fat, $X \pm \Delta$) **in reindeer (*Rangifer tarandus tarandus* L. and *R. tarandus sibiricus* Murray) in regions of Russian Far North** (2014-2016)

Region/number of samples	D	D + dPCB	D:dPCB, %
	Liver		
Murmansk Region/34	42.20±10.55	145.60±23.99	29:71
Nenets AD/155	23.90±5.98	85.20±13.95	28:72
Republic of Komi/10	20.71±5.18	–	–
Yamal-Nenets AD/79	14.01±3.50	–	–
Taymyr peninsula (Krasnoyarsk Territory)/22	5.06±2.43	18.60±5.34	29:71
Republic of Sakha (Yakutia)/5	1.10±0.53	–	–
Kamchatka Territory/5	3.50±1.68	–	–
Chukotka AD/10	1.20±0.58	–	–
Finland (3)/–	42	84.1	50:50
	Muscular Tissue		
Murmansk Region /24	1.30±0.62	–	–
Nenets AD/5	0.67±0.32	–	–
Taymyr peninsula (Krasnoyarsk Territory)/35	< 0.5	–	–

Note. AD — autonomous district. WHO TEU — toxic equivalent unit of World Health Organization. Permissible dioxin content in liver is 6, in muscular tissue — 3 pg WHO TEU/g of fat [24]. Dashes mean lack of data.

At the same time, number of hepatic dioxins in reindeer from Murmansk Region, Nenets Autonomous District, Republic of Komi, and Yamal-Nenets Autonomous District exceeded the acceptable limits. The highest hepatic dioxins and dPCB (7 times above the acceptable level) were found in deer from Kola Peninsula. dPCB comprised nearly 70 % of the total dioxin and PCB toxicity expressed in WHO TEU for all three studied regions. Dioxin contamination of deer meat was decreased at moving from West to East. Average dioxin content values at the territory of Northern Finland established by Finnish scientists in years 2006-2011 [5] well correlate with data obtained by us for Kola Peninsula. The dPCB contribution to total toxicity of persistent organic pollutants (POPs) for liver of reindeer at Finnish territory was significantly lower (50 %) than at territory of Kola Peninsula and other regions of Russia.

Concentration of cadmium and mercury in muscular tissue in reindeer is within the acceptable level (Table 2). However, values significantly grew in liver and kidneys. At that, no expressed decrease in content of heavy metals in organs and tissues is found when moving from west to east similar to the trend observed for dioxins. Cadmium level in reindeer moss (feeding base of reindeer) and mushrooms does not significantly exceed that in feed for bovine animals and pigs. Mercury level in reindeer moss was higher than in feed grain and insignificantly exceeded that in combined feed. That is, the obtained results disallow explanation of deer sub product contamination by heavy metals. Possibly, as in case of dioxins [8], reindeer differences from other species of productive animals (pasturing at

locally polluted territory, consumption of soil particles) play the key role. High concentration of cadmium and mercury in other types of deer's feed is also possible. Accumulation of mercury in mushrooms, for instance, was significantly higher than in other types of feed products (see Table 2).

2. Content of cadmium and mercury in feed and organisms of reindeer (*Rangifer tarandus tarandus* L. and *Rangifer tarandus sibiricus* Murray) from different regions of Russian Far North (mg/kg, $X \pm \Delta$, years 2014-2016)

Specimen	Region	Number of specimen	Cadmium	Mercury
Liver	Murmansk Region	109	1.400±0.200	0.350±0.080
	Nenets Autonomous District	155	0.260±0.040	0.170±0.070
	Republic of Komi	10	0.620±0.090	0.260±0.060
	Yamal-Nenets Autonomous District	78	0.720±0.110	0.180±0.080
	Taymyr Peninsula (Krasnoyarsk Territory)	67	0.340±0.050	0.033±0.015
	Republic of Sakha (Yakutia)	5	0.960±0.140	0.200±0.050
	Kamchatka Territory	10	0.530±0.080	0.090±0.040
	Chukotkaka Autonomous District	8	0.830±0.120	0.090±0.040
Kidneys	Murmansk Region	67	4.400±0.700	0.600±0.140
	Nenets Autonomous District	48	1.500±0.200	0.700±0.170
	Republic of Komi	10	1.700±0.300	0.380±0.090
	Yamal-Nenets Autonomous District	49	4.300±0.600	0.580±0.140
	Taymyr Peninsula (Krasnoyarsk Territory)	10	0.810±0.120	0.060±0.026
	Republic of Sakha (Yakutia)	4	7.600±1.100	0.950±0.230
	Kamchatka Territory	10	2.500±0.400	0.410±0.100
	Chukotka Autonomous District	8	5.600±0.800	1.100±0.300
Muscular tissue	Murmansk Region	6	0.012±0.004	0.110±0.050
	Nenets Autonomous District	5	< 0.005	0.020±0.009
	Yamal-Nenets Autonomous District	10	< 0.005	< 0.01
	Taymyr Peninsula (Krasnoyarsk Territory)	35	< 0.005	< 0.01
Reindeer moss	Murmansk Region	20	0.029±0.009	0.031±0.014
Mushrooms (birch boletus)		3	0.042±0.013	0.084±0.037
Reindeer moss	Republic of Sakha (Yakutia)	6	0.071±0.021	0.032±0.014
Feed grain	Different regions	6	0.035±0.011	0.013±0.010
Combined feed for cattle and pigs		10	0.055±0.017	0.027±0.012

N o t e. AD — autonomous district. Permissible level (PL) of cadmium is 0.3 mg/kg for liver, 1.0 mg/kg for kidneys, and 0.05 mg/kg for muscular tissue; PL of mercury is 0.1 mg/kg for liver, 0.2 mg/kg for kidneys, and 0.03 mg/kg for muscular tissue [24].

The figure compares our results with data obtained in 2001 within the Program of Monitoring and Assessment of the Arctic State [2]. These data show is that dioxin content in organs and tissues of reindeer for the last 15 years had significantly decreased, whereas content of cadmium and mercury had significantly increased.

Calculation of the dosage of pollutants consumed by a person via reindeer meat, liver, and kidneys as compared to weekly permissible dosage are provided in tables 3, 4, and 5.

Consumption of reindeer liver resulted in entering of significantly higher dosages of dioxins and dPCB as compared to meat.

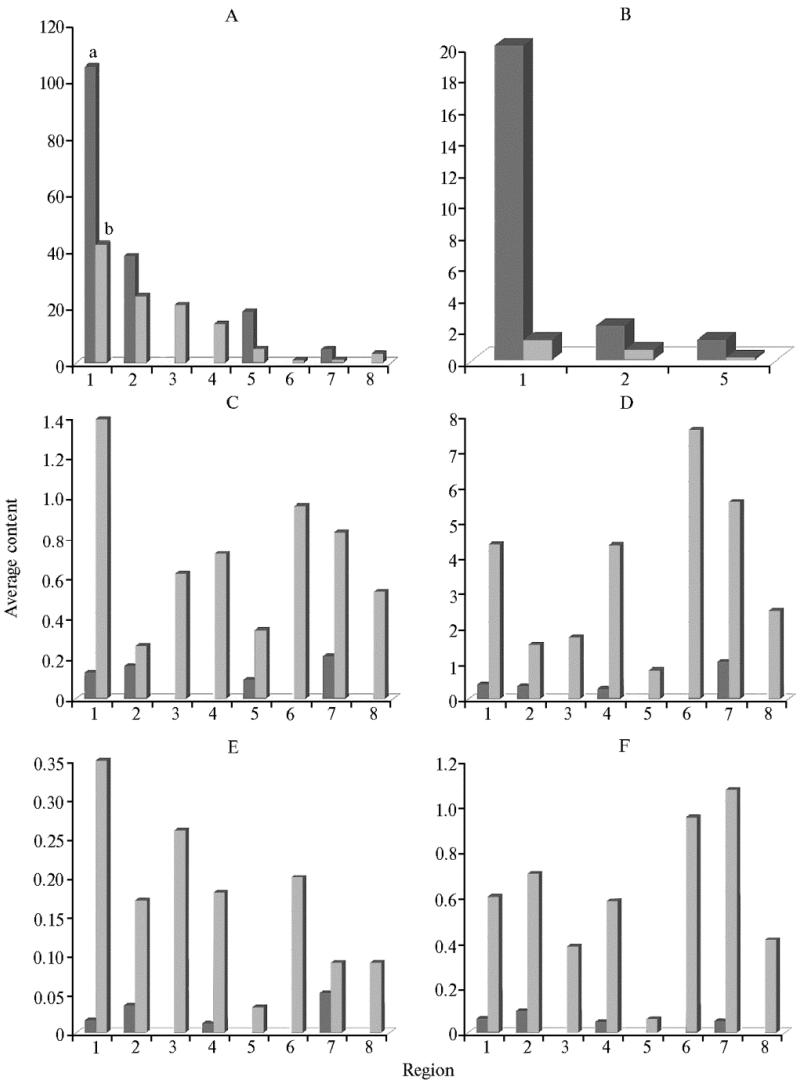
3. Dosages of cadmium and mercury entering the body at consumption of reindeer (*Rangifer tarandus tarandus* L. and *Rangifer tarandus sibiricus* Murray) sub products and meat as compared to weekly permissible levels

Region	Product, sub product	Dosage, mcg/(pax.·week)		% of PL	
		cadmium	mercury	cadmium	mercury
Murmansk Region, Nenets AD, Yamal-Nenets AD, Taymyr Peninsula	Meat	< 1	< 1	< 1	< 1
Murmansk Region	Liver	88.96	22.40	59	9
Nenets AD		16.64	10.88	11	5
Republic of Komi		39.68	16.64	26	7
Yamal-Nenets AD		46.08	11.52	31	5

Continued Table 3

Taymyr Peninsula (Krasnoyarsk Territory)		21.76	2.11	15	1
Republic of Sakha (Yakutia)		61.44	12.80	41	5
Kamchatka Territory		33.92	5.76	23	2
Chukotka AD		53.12	5.76	35	2
Murmansk Region	Kidneys	278.4	38.40	186	16
Nenets AD		97.28	44.80	65	19
Republic of Komi		110.72	24.32	74	10
Yamal-Nenets AD		277.12	37.12	185	15
Taymyr Peninsula (Krasnoyarsk Territory)		51.84	3.84	35	2
Republic of Sakha (Yakutia)		486.40	60.80	324	25
Kamchatka Territory		159.36	26.24	106	11
Chukotka AD		355.84	68.48	237	29

N o t e. AD — autonomous district. Permissible level (PL) of cadmium is 150 μg/(pax. · week), PL of mercury is 240 μg/(pax. · week).



Average dioxin content (pg WHO TEU/g fat; A, B)**, cadmium** (mg/kg; C, D) **and mercury** (mg/kg; E, F) **in liver** (A, C, E)**, muscular tissue** (B) **and kidneys** (D, F) **of reindeer (*Rangifer tarandus tarandus* L. and *Rangifer tarandus sibiricus* Murray) from different regions of Russian Far North:** 1 — Murmansk Region, 2 — Nenets Autonomous District (AD), 3 — Republic of Komi, 4 — Yamal-Nenets Autonomous District, 5 — Taymyr Peninsula (Krasnoyarsk Territory), 6 — Republic of Sakha (Yakutia), 7 — Chukotka AD, 8 — Kamchatka Territory; a — data obtained in 2001 (the Program of Monitoring and Assessment of Arctic State — AMAP 2001), b — results obtained in years 2014-2016.

In the most dioxin-polluted Murmansk region dioxin and dPCB dosage affecting people via reindeer meat and liver is over half of permissible dose. For mercury, the most load resultes from reindeer kidneys, and average does at consumption of liver and kidneys does not exceed one third of PL. Situation with cadmium is extremely unfavorable since at consumption of only kidneys PL is exceeded in majority of regions. Total load at consumption of reindeer liver and kidneys exceeds PL in six of eight studied regions, except for Taymyr and Nenets AD, and reaches the highest values in Republic of Sakha (Yakutia) (is more than 3.5 times higher than the PL). Dosages of cadmium and mercury entering human body with reindeer meat in all four studied regions are no more than 1 % of a daily permissible dose.

4. Total dosage of dioxin and dioxin-like polychlorinated biphenyl (D and dPCB, pg WHO TEU/g of total weigh) **entering human body at consumption of reindeer (*Rangifer tarandus tarandus* L. and *Rangifer tarandus sibiricus* Murray) meat and liver as compared to weekly permissible level** (PL)

Region	Product, sub product	Fat, %	D + dPCB	Load on body, pg/(pax. · week)	% of TD
Murmansk Region	Meat	~ 5 (18)	0.0650	98	12
Nenets AD			0.0335	50	6
Taymyr Peninsula (Krasnoyarsk Territory)			0.0085	13	2
Murmansk Region	Liver	4.5	6.55	419	50
Nenets AD		6.0	5.11	327	39
Taymyr Peninsula (Krasnoyarsk Territory)		5.0	0.93	60	7

N o t e . AD — autonomous district, WHO TEU —toxic equivalents of World Health Organization. TD — 840 pg WHO TEU/(pax. · week),

5. Total percentage of pollutants entering human body at consumption of reindeer (*Rangifer tarandus tarandus* L. and *Rangifer tarandus sibiricus* Murray) sub products and meat as compared to permissible level (PL)

Region	% of PL		
	D + dPCB (liver + meat)	cadmium (liver + kidneys)	mercury (liver + kidneys)
Murmansk Region	62	245	25
Nenets AD	45	76	24
Republic of Komi		100	17
Yamal-Nenets AD		216	20
Taymyr Peninsula (Krasnoyarsk Territory)	9	50	3
Republic of Sakha (Yakutia)		365	30
Kamchatka Territory		129	13
Chukotka AD		272	31

N o t e. AD — autonomous district, D and dPCB — dioxins and dioxin-like polychlorinated biphenyl. Dashes mean lack of data.

It should also be noted that indigenous peoples of Russian Far North is subjected to significant effect of dioxins, dPCB and toxin elements and from other sources, besides deer meat, including food products, in particular marine mammals and fish [26].

Recently, high nutritional value of reindeer meat and liver is shown [25]. Also, nutritional value of reindeer liver is significantly higher than that of beef [27]. In using reindeer liver for cooking, it is reasonable to account for its contamination by persistent organic pollutants and toxic elements.

Therefore, dioxin contamination of reindeer liver in Russian Far North has clear geographical distribution, the most contaminated region is Kola Peninsula, in furtherance dioxin content is decreased when moving from west to east. Contamination of reindeer sub products by cadmium and mercury does not display similar regularity. For the last 15 years, content of dioxins in reindeer liver had significantly decreased, with the increase of cadmium and mercury level in liver and kidneys. Consumption of reindeer meat from the studied regions does not bear significant risk for health. But regular and long-term eating of reindeer

liver and kidneys could by risky because of more than 3-fold exceeded permissible doses.

REFERENCES

1. *Ministerstvo sel'skogo khozyaistva Rossiiskoi Federatsii. Prikaz ot 14 yanvarya 2013 goda № 11. Ob utverzhdenii otraslevoi programmy «Razvitie severnogo olenevodstva v Rossiiskoi Federatsii na 2013-2015 gody» »* [Ministry of Agriculture of the Russian Federation. Order No. 11 of January 14, 2013. On approval of the sectoral program "Development of Reindeer Reindeer in the Russian Federation in 2013-2015"]. Available http://docs.cntd.ru/document/902393115. Accessed October 22, 2016 (in Russ.).
2. *Persistent toxic substances, food security and indigenous peoples of the Russian North. Final report. Arctic Monitoring and Assessment Programme (AMAP), Oslo, 2004.* Available https://www.amap.no/documents/doc/persistent-toxic-substances-food-security-and-indigenous-peoples-of-the-russian-north.-final-report/795. Accessed October 22, 2016.
3. Dudarev A.A., Alloyarov P.R., Chupakhin V.S., Dushkina E.V., Sladkova Y.N., Dorofeyev V.M., Kolesnikova T.A., Fridman K.B., Nilsson L.M., Evengerd B. Food and water security issues in Russia I: Food security in the general population of the Russian Arctic, Siberia and the Far East, 2000-2011. *Int. J. Circum. Heal.*, 2013, 72: 21848 (doi: 10.3402/ijch.v72i0.21848).
4. Nilsson L.M., Destonuni G., Berner J., Dudarev A., Mulvad G., Odland J.O., Rautio A., Tikhonov C., Evengerd B. A call for urgent monitoring of food and water security based on relevant indicators for the arctic. *Ambio*, 2013, 42: 816-822 (doi: 10.1007/s13280-013-0427-1).
5. Holma-Suutari A. *Harmful agents (PCDD/Fs, PCBs, and PBDEs) in Finnish reindeer (Rangifer tarandus tarandus) and moose (Alces Alces). Academic dissertation.* University of Oulu, Finland, 2014.
6. Laurent C., Marchand P., Feidt C., Le Bizec B., Rychen G. Tissue distribution and bioconcentration factors of PCDD/Fs in the liver and adipose tissue following chronic ingestion of contaminated milk in rats. *Chemosphere*, 2005, 60: 929-938 (doi: 10.1016/j.chemosphere.2005.01.041).
7. Ruokojärvi P., Suutari A., Hallikainen A., Laaksonen S., Nieminen M., Kiviranta H. Distribution of PCDD/Fs, PCBs and PBDEs in semidomesticated reindeer (*Rangifer tarandus tarandus* L.) meat and liver. *Organohalogen Compounds*, 2011, 73: 1321-1324.
8. Panel on Contaminants in the Food Chain. Scientific opinion on the risk to public health related to the presence of high levels of dioxins and dioxin-like PCBs in liver from sheep and deer. *The EFSA Journal*, 2011, 9(7): 2297 (doi: 10.2903/j.efsa.2011.2297).
9. Schröter-Kermani C., Rappolder M., Neugebauer F., Päpke O. PCDD, PCDF, and DLPCB in terrestrial ecosystem: Are there correlations of levels or patterns in soil and roe deer liver? *Organohalogen Compounds*, 2011, 73: 1325-1328.
10. Holma-Suutari A., Ruokojärvi P., Laaksonen S., Kiviranta H., Nieminen M., Viluksela M., Hallikainen A. Persistent organic pollutant levels in semi-domesticated reindeer (*Rangifer tarandus tarandus* L.), feed, lichen, blood, milk, placenta, foetus and calf. *Sci. Total Environ.*, 2014, 476-477(1): 125-135 (doi: 10.1016/j.scitotenv.2013.12.109).
11. Holma-Suutari A., Ruokojärvi P., Komarov A.A., Makarov D.A., Ovcharenko V.V., Panin A.N., Kiviranta H., Laaksonen S., Nieminen M., Viluksela M., Hallikainen A. Biomonitoring of selected persistent organic pollutants (PCDD/Fs, PCBs and PBDEs) in Finnish and Russian terrestrial and aquatic animal species. *Environmental Sciences Europe*, 2016, 28: 5 (doi: 10.1186/s12302-016-0071-z).
12. Hassan A.E.A. *Nutrients and toxic elements in semi-domesticated reindeer in Norway. Nutritional and food safety aspects. PhD dissertation.* University of Tromsø, 2012.
13. Hassan A.A., Rylander C., Brustad M., Sandanger T.M. Level of selected toxic elements in meat, liver, tallow and bone marrow of young semi-domesticated reindeer (*Rangifer tarandus tarandus* L.) from Northern Norway. *Int. J. Circumpol. Health*, 2012, 71: 1-7 (doi: 10.3402/ijch.v71i0.18187).
14. Komarov A.A. Dioxins and polychlorinated biphenyls in feeds (review). *Agrivultural Biology***,** 2003, 2: 20-37.
15. *European Commission, Scientific Committee on Food. Opinion on the risk assessment of dioxins and dioxin-like PCBs in food.* Update based on new scientific information, 30 May 2001. Available https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_catalogue_dioxins_out90_en.pdf. Accessed October 22, 2016.
16. EFSA Panel on Contaminants in the Food Chain (CONTAM). Cadmium in food — Scientific opinion of the Panel on Contaminants in the Food Chain. *The EFSA Journal*, 2009, 7(3): 980 (doi: 10.2903/j.efsa.2009.980).
17. EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. *The EFSA Journal*, 2012, 10(12): 2985 (doi: 10.2903/j.efsa.2012.2985).
18. *MU A-1/030. Metodicheskie rekomendatsii po arbitrazhnomu opredeleniyu stoikikh polikhlorirovannykh organicheskikh zagryaznitelei (dibenzodioksiny i dibenzofurany) s ispol'zovaniem khromato-mass-spektrometrii vysokogo razresheniya v pishchevoi produktsii* [MU A-1/030. Recommendations on arbitration estimation of persistent polychlorinated organic pollutants (dibenzodioxins and dibenzofurans)

in food stuffs using high-resolution chromatography-mass spectrometry]. Moscow, 2015 (in Russ.).
19. *MU A-1/006. Metodicheskie ukazaniya po opredeleniyu massovoi doli mysh'yaka, kadmiya, rtuti i svintsa v pishchevykh produktakh, kormakh i kormovykh dobavkakh metodom mass-spektrometrii s induktivno svyazannoi argonovoi plazmoi* [MU A-1/006. Guidelines for determining the mass fraction of arsenic, cadmium, mercury and lead in food, feed and feed additives by mass spectrometry with inductively coupled argon plasma]. Moscow, 2014 (in Russ.).
20. Dudarev A.A. Dietary exposure to persistent organic pollutants and metals among Inuit and Chukchi in Russian Arctic Chukotka. *Int. J. Circumpol. Health*, 2012, 71 (doi: 10.3402/ijch.v71i0.18592).
21. Schuster R.C., Gamberg M., Dickson C., Chan H.M. Assessing risk of mercury exposure and nutritional benefits of consumption of caribou (*Rangifer tarandus*) in the Vuntut Gwitchin First Nation community of Old Crow, Yukon, Canada. *Environ. Res.*, 2011, 111(6): 881-8877 (doi: 10.1016/j.envres.2011.05.025).
22. Evaluation of certain contaminants in food (Seventy-second report of the Joint FAO/WHO Expert Committee on Food Additives). *WHO Technical Report Series*, 2011, 959: 55.
23. *Joint FAO/WHO Expert Committee on Food Additives (JECFA). Procedures for recommending maximum residue limits residues of veterinary drugs in food*. Rome, 2000.
24. *Tekhnicheskii reglament Tamozhennogo soyuza o bezopasnosti pishchevoi produktsii. TR TS 021/2011* [Technical regulations of the Customs Union on food safety. TR TS 021/2011]. Available http://www.eurasiancommission.org/ru/act/texnreg/deptexreg/tr/Documents/TR%20TS%20PishevayaProd.pdf. Accessed October 22, 2016 (in Russ.).
25. Novak G.V., Bodrova L.F. *Izvestiya Orenburgskogo gosudarstvennogo agrarnogo universiteta*, 2014, 6: 120-122 (in Russ.).
26. *AMAP. AMAP Assessment 2002: Human health in the Arctic*. Arctic Monitoring and Assessment Programme (AMAP), Oslo, 2003. Available https://www.amap.no/documents/doc/amap-assessment-2002-human-health-in-the-arctic/95. Accessed October 22, 2016.
27. Turshuk E.G., Loboda E.A. *Tekhnika i tekhnologiya pishchevykh proizvodstv*, 2012, 1(24): 85-89 (in Russ.).

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MELANINE PROTEIN-ENERGY ADDITIVE FROM *Hermetia illucens* LARVAE IN NUTRITION OF CALVES

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Abstract

One of the natural sources of various biologically active substances, including melanin, are insects. In connection with the possibility of industrial breeding of some insect species for feed purposes and obtaining various products of processing their biomass, an assessment of the biological effectiveness of the substances obtained is necessary. The purpose of the present studies was to study the effectiveness of the use of the melanin protein-energy additive (MPEA) from the larva of the *Hermetia illucens* fly, in feeding black-and-white breed calves to enhance their safety and growth. Researches were carried out on 30 calves of black-and-white breed on the basis of experimental farm «Klenovo-Chegodaevo», Moscow. According to the principle of animal analogues, 3 groups of calves were formed, 10 animals in each. In the first period of the experiment (1-2 months old calves), the animals of the group 2 were fed individually with 5.0 ml of MPEA (6 mg melanin per head daily), and the animals of the group 3 were fed 7.5 ml of MPEA (9 mg melanin per head daily). Starting from 3 month age, a daily dose of the additive was increased to 7.5 and 10 ml per head, respectively, in the experimental groups. The duration of the experiment was 89 days. The study of the chemical composition of MPEA showed the absence of crude fiber (chitin), with a high content of protein, fat, minerals. In the experimental additive the concentration of melanin was 1.2 mg melanin/ml. MPEA practically did not contain pathogenic microorganisms and was recognized as non-toxic. During the experiment, the calves receiving the MPEA an average daily gain and gross gain were 4.13-2.43 kg and 46.44-27.34 g (or 4.23 and 2.49 %) higher, res[ectively, than those in the control group, with 4.1 and 2.4 % decrease in feed consumption per 1 kg of live weight gain. In the calves of the experimental groups, there was a tendency for lowering total blood protein level by 3.45 and 2.71 g/l compared to the control due to reducing the fractions of albumins by 1.86 and 1.29 g/l, and globulins by 1.60 and 1.43 g/l, respectively. Also, ALT activity decreases by 4.3 IU/l ($p < 0.05$ for group 3) and 2.38 IU/l ($p > 0.05$ for group 2). The content of lysozyme, per cent of lysis, the blood BA level of the calves from the experimental groups turned out to be practically the same, 0.47-0.49 μg/l, 27.27-28.28 %, and 80.39-82.35 %, respectively. However, the FA index of the calves from the group 2 and group 3 was 5.94 and 6.95 % higher, respectively, compared to the control. In the calves of the group 2 and group 3, the number of lacto- and bifidobacteria in the colonic intestine increased, by 2.23×10^5 and 10.3×10^5 CFU/g for *Lactobacillus*, and by 0.33×10^8 and 1.07×10^8 CFU/ml for *Bifidobacteria* compared to the control. There was a decrease in the amount of lactose-positive coliforms by 1.196×10^5 and 1.11×10^5 CFU/g, respectively, compared to the control animals. Calculation of the feeding efficiency of the MPEA during the experiment showed a profitability of (+)381.65 and (+)180.90 rubles, or (+)4.29 and(+) 2.03 rubles per head daily, in the experimental calves when compared to the control animals. MPEA dosage was established experimentally, and, possibly, is not definitively precise, since this is the first study of MPEA biological effectiveness for calves. Further research is needed to identify the biological effect of the *Hermetia illucens* larvae on various species of farm animals.

Keywords: calves, larvae, *Hermetia illucens,* melanin, immunity, microflora

Respiratory and gastrointestinal diseases of young animals result in low

viability, reduced growth rates and, often, death. These factors still remain serious challenges in livestock husbandry. Pre-weaning and the next transit growing period are most important in life of young animals since the need for nutritious substances due to the intensive animal growth is high, and development of fermentative systems of gastrointestinal tract has not been terminated yet. In pre-weaning period mother's milk plays important role in antibacterial protection of calves. In transit period the risk of diseases is especially high since milk in the diet gradually decreases in amount, while own immune system is still developing [1]. In new-born calves aged 1-30 days the core place takes digestion malfunction manifesting in diarrhea and, consequently, in sharp dehydration, anophthalmia, toxemia, and immune deficit [2]. Massive gastroenteritis in calves are mainly caused by infection agents, including viruses, microbes, protozoan, and fungi, virulence of which is increased under unfavorable breeding and keeping conditions [3].

Antimicrobial medicines are widely used for treatment and prevention of bacterial infections in animals but often do not provide the desired outcome, in particular, because of adaptive variability of microorganisms and immune deficit of animals under the effect of medicinal products [4]. More attention is drawn to bioactive feed additives able to stimulate non-specific immunity [5, 6] for prevention and treatment of mixed gastrointestinal infections and digestion disorders caused by malfunction of gut microbiocenosis [7, 8]. Natural herbal medicines and extracts of animal organs and tissues are perspective [9, 10]. Melanin is of significant interest [11]. Melanin, a condensed phenol compound, is irregular high-molecular biopolymer which contributes to dark color of insect cover, human hair, and cell wall of mushrooms, plants, and microorganisms [12]. Various functional groups, highly stable paramagnet centers, and conjugated double bonds in the molecule ensures diverse use of melanin as photo-, radio-protector and antioxidant [13].

Melanin refers to the most powerful natural antioxidants. Presence of melanin in feeds may promote their longstanding storage [14]. During digestion, melanin is partially used by intestinal microflora and also serves as enterosorbent and peristalsis regulator affecting intestinal microflora composition. Melanin is an active antidote at severe intoxications and effective removers of toxins from intestinal tract before their penetration into blood [15]. Melanin is used for treatment and prevention of hepatic diseases, stress, and tumors. Melanin is a powerful natural adaptogen and also has an anti-aging effect [16].

Insects are natural sources of various bioactive substances, including melanin [17]. Given possible farming of insects for various derivatives, the assessment of biological effectiveness of these substances is of interest [18].

Black soldier fly-larva (*Hermetia illucens*) deserves the attention. This species is considered the most perspective for industrial breeding and use of larva biomass as animal feed [19, 20] and in aquaculture [21, 22]. These insects do not accumulate pesticides or mycotoxins [23, 24] and are rich in protein and fat [25]. Chemical composition of larva partially depends on composition of growth medium [26]. Their lipid profile partially simulates the lipid profile of the substrate, at that, micronutrients such as minerals and vitamins are easily accumulated [27]. N.S. Liland et al. [28] confirm plasticity of the nutritional composition of larva allowing accumulation of lipids, as well as various water-soluble compounds.

Melanin and its effect on animal productivity and meat quality are not studied enough. A.I. Bastrakov et al. [29] had shown high anti-infection properties of melanin-chitosan complex from dead flies and empty covers of pupa cas-

es. Additive from larva of *Hermetia illucens* fly potentially accelerates the growth and development of young farm animals, increases their survivability and stress resistance. Feeding this additive to young animals allows them additional important nutrients, since fly larva contain full-value proteins and balanced ratio of mineral substances [30].

In this paper, melanin protein-energy additive (MPEA) from fly (*Hermetia illucens*) larvae was for the first time used in feeding farm animals. This results in improved composition of intestinal microbiocenosis, reduction of the diarrhea cases in calves during pre-weaning and transit periods, better feed conversion, higher daily growth rates, and increased survivability of young animals.

Our purpose was to study the effectiveness of use of melanin protein-energy additive from the larva of *Hermetia illucens* fly, in feeding Black-and-White calves to enhance their survivability and growth.

Techniques. Black-and-White pre-weaning calves (n = 30) aged 1-4 months were involved in study (experimental farming unit Klenovo-Chegodaevo, Moscow, 2017). Three groups, each of 10 analogue animals, were formed. Animals of group I (control) were fed basic ration (combined feed, whole and regenerated milk, cereal grass hay, maize silage and mineral additives) according to scheme accepted in the unit. Dietary Melanin Protein Energy Additive (MPEA) was used in groups II and III. Till 3-month age animals of group II were individually fed orally with 0.5 ml of MPEA (6 mg of melanin per animal daily), and animals of group III received 7.5 ml of MPEA (9 mg of melanin per animal daily). Starting from 3-month age, the dosage was increased up to 7.5 and 10 ml of melanin per animal daily. Studies lasted 89 days. Feed consumption and costs per living weight gain was estimated daily. Calves were weighted individually at beginning and at the end of test (in morning hours before feeding), as well as monthly during tests for determination of gross and average daily growth.

Feeds were sampled for chemical analysis subject to the State Standard GOST P ISO 6497-2011. Metabolic energy was expressed per raw nutritive substances [31], rations were calculated using software KormOptimaExpert (Version 2016.15.1.1, Kormoresurs LLC, Russia).

MPEA was produced from the mixture of larva and prepupa of *Hermetia illucens* flies (1:1) which were raised on cracked maize grain. Squeezed biomass was dissolved in distilled water (1:3) following by 2 hour sterilization of 250 ml suspension at 100 °C in vials. MPEA feeding dose was calculated based on content of water-soluble melanin in the additive. Melanin concentration was determined in 100 ml suspension aliquot after sedimentation with HCl and drying the residue. Suspension was preliminary centrifuged 15 minutes at 1200 g for separation of non-melanin component (centrifuge OPN-8, JSC DASTAN MNC, Russia). The obtained melanin residue was separated by centrifuging in the same mode, then neutralized, dried, and weighed. Melanin concentration in the test suspension was 1.2 mg/ml.

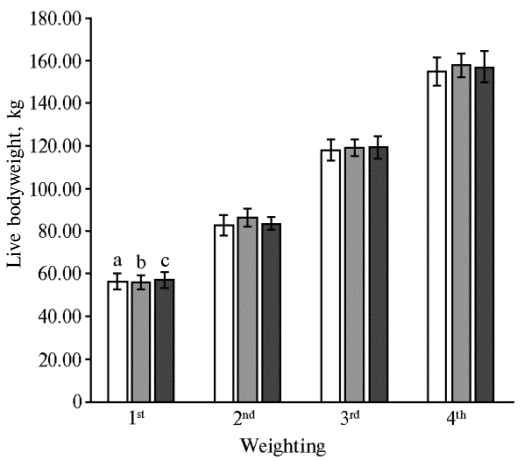
MPEA components were determined as follows: initial moisture content by GOST P 54951, air-dry substance by GOST 31640-2012, protein by GOST 32044.1-2012, fat by GOST 32905-2014, cellulose by GOST 31675-2012, nitrogen-free extractive substances by calculation, ash content by GOST 32933-2014, gross metabolic energy by calculation, calcium by GOST 32904-2014, and phosphorous by GOST P 51420-99). MPEA sample was also tested for *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Escherichia coli* (hemolytic), *Staphylococcus saprophyticus*, *Enterobacter* spp., *Citrobacter* spp., yeast-like fungi and mildew. Total toxic effect was assessed on 3-5-day culture of infusoria *Tetryhymena pyriformis*, toxicity degree was estimated as survivability

infusoria in the tested medium.

Excrements were individually collected from calves of each group (n = 3) at the end of the experiment. Microbiological profile was determined by serial dilution methods. Samples of gastrointestinal content were diluted 10-fold. Portions of 0.2 ml for spread-plating method and 1.0 ml for pour-plating method were used to isolate and identify microorganisms on selective and differential media with counting colony forming units (CFU/g or CFU/ml).

At isolation and determination of opportunistic pathogenic microorganisms, samples were incubated in liquid selective medium, and then plated on solid selective and diagnostic medium to confirm taxonomic affiliation of typical and atypical colonies. Identification criteria were morphology of colonies, microscopy, and biochemical properties on differential media (State Science Center of Applied Microbiology and Biotechnology, Russia) and with the use of test panels (BioMerieux, France). MRS and Bifidum media were used for identification of lactobacilli (lactobacteria and bifidobacteria), Endo-GRM agar for bacteria of *Escherichia* genus, meat peptone agar (MPA) with 5% sterile defibrinated sheep blood for hemolytic *Streptococcus* spp. and *E. coli* bacteria, and Sabouraud agar with 5 % potassium tellurite for yeast and yeast like fungi. Plates with 30-300 colonies were used for counting. Number of microorganisms (N) was calculated as $N = m \cdot V^{-1} \cdot d^{-1}$, where m stands for the average arithmetic number of colonies per two Petrie dishes, V stands for volume of inoculate in a dish (cm^3), and d stands for dilution coefficient.

At the end of test, blood was collected from animals of each group (n = 3). In samples of whole and stabilized blood, total protein, albumin, globulin, creatinine, urea, total bilirubin, total cholesterol, calcium, phosphorous, glucose, activity of alkaline phosphatase (AP), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), hemoglobin, erythrocytes, lymphocytes, hematocrit value were determined by approved methods. Blood bactericide activity (BA) was measured photonephelometrically, blood lysozyme level was assayed by V.I. Mutovin (1974), phagocytic activity (PA) — by endocytosis and digestive ability of blood cells.



Living weight of Black-and-White pre-weaning calves fed dietary bioactive additive from larvae of *Hermetia illucens* fly: a — I group (control, n = 10), b — II group (n = 10), c — III group (n = 10). See description of groups in section "Methodology" ($M \pm m$, experimental farming unit "Klenovo-Chegodaevo", Moscow, 2017).

Expected economic effect of the additive used in calf preweaning period was calculated from the data on feed consumption, costs, and growth gain.

Results were processed by dispersion analysis method (ANOVA) with the use of Statistica 10 software (StatSoft, Inc., USA, 2011; http://www.statsoft.com). Average arithmetic mean values (M), error of mean square ($\pm m$) and significance level (p) were calculated. Statistically, results were deemed highly reliable at $p < 0.001$ and significant at $p < 0.01$ and $p < 0.05$. Trend towards reliability was recognized at $p < 0.1$, but $p > 0.05$. The difference was invalid at $p > 0.1$.

Results. According to some authors' opinion [32], MPEA mani-

fests biological effect at 0.1 mg/kg of living weight. This was the base value to calculated feed additive dosage in our tests.

The obtained biomass of black solder fly larva contains 36 % protein and 45 % fat. After defatting, the outcome of squeezed mass with 70 % moisture was 775 g from 1 kg of raw biomass.

Comparison of MPEA and protein concentrate compositions (Table 1) had shown high content of raw chitin in air-dry substance (ADS) of protein concentrate (PC) and its complete lack in MPEA ADS. MPEA ADS is high in protein, fat, and mineral elements, with melanin concentration of 1.2 mg/ml. The additive is also free from pathogenic microorganisms and non-toxic.

Initial weight of all calves was practically equal (from 56.0 to 56.90 kg) (Table 2, Fig.). Dietary MPEA accelerates growth of the animals, and the final indicators in groups II and III were 2.8 and 2.0 kg higher (or +1.81 and +1.29 %), respectively, compared to the control group.

1. Chemical composition of melanin protein energy additive (MPEA) and protein concentrate produced from larva of *Hermetia illucens* fly

Indicator	Feed specimen			
	MPEA		protein concentrate	
	NS	ADS	NS	ADS
Initial moisture, %	71.06		1.89	
Air-dry substance, %	28.94	100.00	98.11	100.00
Protein, g/kg	94.46	326.40	533.62	543.90
Fat, g/kg	126.84	438.29	50.82	51.80
Raw chitin, g/kg	–	–	183.07	186.60
NFES, g/kg	17.68	61.09	92.62	94.40
Ash, g/kg	38.20	132.00	76.13	77.60
Gross energy, MJ/kg	7.36	25.43	19.99	20.38
Metabolic energy, MJ/kg	5.45	18.83	14.55	14.83
Calcium, g/kg	4.68	16.17	15.00	15.29
Phosphorus, g/kg	3.58	12.37	5.10	5.20

N o t e. NS — natural substance, ADS — air-dry substance; NFES — nitrogen-free extractive substances. Dashes mean that no chitin was detected.

2. Growth, survival and feed consumption by Black-and-White pre-weaning calves fed dietary Melanin Protein Energy Additive (MPEA) from *Hermetia illucens* fly larvae ($M \pm m$, experimental farming unit "Klenovo-Chegodaevo", Moscow, 2017)

Indicator	Group		
	I (control, $n = 10$)	II ($n = 10$)	III ($n = 10$)
Living weight, kg:	56.30±4.02	56.00±3.45	56.90±3.79
at beginning of test			
at the end of test	155.00±6.67	157.80±5.76	157.00±7.42
To control, %	100.00	101.81	101.29
Gross growth rate, kg	97.67±5.18	101.80±5.25	100.10±7.64
Average daily growth rate, g	1097.38±58.23	1143.82±58.98	1124.72±85.82
To control, %	100.00	104.23	102.49
Survival, %	90.00	100.00	100.00
Gross consumption of ME, MJ per animal daily	2830.20	2830.20	2830.20
Consumption of EFU per animal daily	3.18	3.18	3.18
Feed consumption, EFU per kg weight gain	2.90	2.78	2.83
To control, %	100.00	95.90	97.60

N o t e. ME — metabolic energy, EFU — energy feeding unit. See description of groups in section "Methodology".

Average daily growth rate in calves of groups II and III was 46.44 g and 27.34 g, or by 4.23 % and 2.49 %, higher than in control group. The animals of group II showed the highest growth rates. Feed consumption per 1 kg gain in groups II and III decreased by 4.1 % and 2.4 % as compared to control due to increase of average daily gain. Animals were willingly consuming MPEA without any rejections. Calves from trial groups fell ill less frequently, no diarrhea and lethal cases occurred, while in group I one calf died.

Although studied blood values do not significantly differ between groups

remaining within the physiological limits, there are some specific differences (Table 3). Total blood protein in groups II and III decreases by 3.45 and 2.71 g/l as compared to control because of a decrease in albumin level by 1.86 and 1.29 g/l and in globulin level by 1.6 and 1.43 g/l. Total protein level is not enough to fully assess diet quality. Blood ALAT and ASAT activity additionally indicates on whether the diet is high-grade and allows characterization of protein metabolism intensity and hepatic function. We have confirmed reliable decrease of ALAT activity (by 4.3 IU/l, $p < 0.05$)) in calves of group III as compared to control. In group II this indicator was 2.38 IU/l less than in control. ASAT activity shows the same trend and is 7.63 and 7.93 IU/l less in groups II and III compared to control. Decrease in activity of these enzymes indirectly indicates a stabilizing effect of the additive on free amino acids which are less subjected to catabolism and more effectively involved in protein biosynthesis.

In this experiment dietary MPEA does not reliably affect alkaline phosphatase and blood Ca and P concentrations in calves, at slight increase in Ca/P ratio in animals of group III. Blood lysozyme level, lysis percentage, and bactericidal activity were practically similar, i.e. 0.47 and 0.49 μg/l, 27.27 and 28.28 %, 80.39 and 82.35 %. PA was 5.94 and 6.95 % higher in calves of groups II and III, respectively, at $p < 0.05$ for group III, as compared to control.

3. Hematological indicators and resistance parameters in Black-and-White preweaning calves fed dietary Melanin Protein Energy Additive (MPEA) from *Hermetia illucens* fly larvae ($M \pm m$, experimental farming unit Klenovo-Chegodaevo, Moscow, 2017)

Indicator	Group		
	I (control, $n = 3$)	II ($n = 3$)	III ($n = 3$)
Total protein, g/l	78.56±2.17	75.11±1.74	75.85±1.05
Albumin, g/l	33.08±1,16	31.22±0.64	31.79±1.36
Globulin, g/l	45,49±3.33	43.89±1.12	44.06±1.43
Albumin/globulin coefficient	0.74±0.08	0.71±0.01	0.72±0.05
Cholesterol, μmol/l	3.37±0.03	3.60±0.38	3.12±0.36
Creatinine, μmol/l	68.58±6.99	70.39±3.27	62.76±4.40
Urea, μmol/l	5.40±0.41	5.10±0.50	6.49±0.39
Total bilirubin, μmol/l	8.10±3.06	8.46±0.66	7.17±0.84
ALAT, IU/l	15.86±1.03	13.48±1.54	11.56±0.71*
ASAT, IU/l	65.52±5.69	57.89±5.67	57.59±3.04
Triglicerides, μmol/l	0.94±0.04	0.95±0.03	0.86±0.11
Alkali phosphatase, IU/l	361.33±24.35	435.48±56.05	244.71±65.05
Glucose, μmol/l	5.17±0.14	5.15±0.21	4.66±0.85
Calcium, μmol/l	2.84±0.08	2.87±0.07	2.83±0.08
Phosphorous, μmol/l	2.93±0.03	3.12±0.11	2.72±0.33
Ca/P ratio	1.25±0.02	1.19±0.02	1.40±0.21
Iron, μmol/l	35.64±2.03	34.55±2.39	31.64±2.27
Leucocytes, $\times 10^9$/l	12.92±0.25	13.26±0.88	12.30±0.51
Erythrocytes, $\times 10^{12}$/l	10.74±0.32	10.83±0.31	11.17±0.43
Hemoglobin, g/l	105.37±5.39	102.40±3.77	108.77±2.28
Hematocrit, %	41.21±2.18	41.92±0.81	41.98±0.83
Lysis, %	28.28±1.01	27.27±1.75	27.27±1.75
Lysozyme:			
mkg/ml blood	0.49±0.02	0.47±0.03	0.47±0.03
s.u.a, un.a/mg of protein	1.59±0.10	1.60±0.13	1.59±0.12
BSBA, %	82.35±0.00	80.39±1.30	80.39±1.30
PA, %	48.20±1.45	54.14±1.84	55.15±1.83*
PI	3.39±0.18	2.98±0.09	3.28±0.14
PN	1.63±0.10	1.61±0.06	1.81±0.09

N o t e. A/G — albumin/globulin, ALAT — alaninaminotransferase, ASAT — aspartaaminotransferase, Ca/P — calcium/phosphorous, BSBA — blood serum bactericidal activity, PA — phagocytic activity, PI — phagocytic index, PN — phagocyte number; s.u.a, un.a/mg — specific units of activity. See description of groups in section "Methodology".

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

In large intestine of animals from groups II and III counts of lactobacteria increased by 2.23×10^5 and 10.3×10^5 CFU/g, of bifidobacterium — by 0.33×10^8

and 1.07×10^8 CFU/ml, respectively, as compared to control (Table 4). Abundance of lactose-positive *E. coli* forms decreases by 1.196×10^5 and 1.11×10^5 CFU/g ($p < 0.05$ for group III). No lactose-negative *E. coli* forms were found in animals of trial groups, whereas these forms were present in one calf of the control group (see Table 3).

4. Qualitative and quantitative composition of microbial flora of large intestine in Black-and-White pre-weaning calves fed dietary Melanin Protein Energy Additive (MPEA) from *Hermetia illucens* fly larvae ($M\pm m$, experimental farming unit Klenovo-Chegodaevo, Moscow, 2017)

Microorganisms	Group		
	I (control, $n = 3$)	II ($n = 3$)	III ($n = 3$)
Lactobacillus, $\times10^5$ CFU/g	3.10±1.35	5.33±0.99	13.4±3.68*
Bifidobacillus, $\times10^8$ CFU /ml	1.33±1.04	1.66±0.84	2.40±1.30
Hemolytic microorganisms, CFU g:			
streptococci, $\times10^3$	2.20±0.08	2.30±0.53	2.35±0.69
Escherichia coli	Not found	Not found	Not found
E.coli, CFU/g:			
lactose-positive, $\times10^5$	1.20±1.20	0.004±0.001	0.09±0.09
lactose-negative	Found in 1 specimen	Not found	Not found
Fungi of *Candida* genus, CFU/g	Not found	Not found	Not found

N o t e. CFU — coli form unit. See description of groups in section "Methodology".
* Differences from control were statistically significant at $p < 0.05$.

MPEA amounts per animal in groups II and III during the experiment were 520.0 ml and 742.5 ml. Cost of 1 l of the additive is 100.00 rubles, costs per animal in tests are 52.00 and 74.25 rubles. Revenue from conventional sale is (+)1355.35 rubles for control group I, (+)1737.00 rubles for group II, and (+)1536.25 rubles for group III. In the trial groups the indicator increases because of living weigh gain due to dietary MPEA. Additional revenue per each calf of groups II and III during tests is also in line with these findings. During the entire period total revenue, as compared to the control, is (+)381.65 and (+)180.90 rubles, or (+)4.29 and (+)2.03 rubles per animal dayly,.

Other authors report on anti-stress effect of melanin (0.1 mg/kg) derived from yeast *Nadsoniella nigra* X-1 [33], with no diseases and deaths of post-weaned pigs. Melanin in stressed animals promotes normal proteinase inhibition and prevents cytolytic syndrome of pancreatic gland [34, 35].

Thus, in this experiment differences in growth rates of calves fed dietary Melanin Protein Energy Additive (MPEA) from larva of *Hermetia illucens* fly were not statistically significant that allows for an increase of daily MPEA dosage. Interestingly, MPEA micro dose influences positively non-specific immunity and intestinal microbiocenosis. Biological effect of MPEA correlates with higher growth, animal survival rates, and better fed conversion. The MPEA doses established herein empirically are not ultimately recommended since biological effectiveness of the additive is studied for the first time. Further studies will clarify biological effect of both *Hermetia illucens* fly larva and larva-derived extracts of physiologically active substances on health and productivity of farm animals, poultry, and fish.

REFERENCES

1. Hulbert L.E., Moisa S.J. Stress, immunity, and the management of calves. *J. Dairy Sci.*, 2016, 99(4): 3199-3216 (doi: 10.3168/jds.2015-10198).
2. Lisitsyn V.V. *Veterinariya sel'skokhozyaistvennykh zhivotnykh*, 2013, 3: 6-12 (in Russ.).
3. Mishchenko V.A., Pavlov D.K., Dumova V.V., Nikeshina T.B., Getmanskii O.I., Kononov A.V., Lisitsyn V.V. *Veterinarnaya patologiya*, 2005, 3: 34-38 (in Russ.).
4. Krasochko P.A. *Biotekhnologicheskie osnovy konstruirovaniya i ispol'zovaniya immunobiologicheskikh preparatov dlya molodnyaka krupnogo rogatogo skota. Avtoreferat doktorskoi dissertatsii* [Biotechnolog-

ical aspects of design and use of immunobiologicals for young cattle. DSci. Thesis]. Shchelkovo, 2009 (in Russ.).
5. Tarakanov B.V. *Zootekhniya*, 1993, 8: 16-18 (in Russ.).
6. Abbas K.A. The synergistic effects of probiotic microorganisms on the microbial production of butyrate in vitro. *McNair Scholars Research Journal*, 2010, 2(1): 8.
7. Chu G.M., Lee S.J., Jeong H.S., Lee S.S. Efficacy of probiotics from anaerobic microflora with prebiotics on growth performance and noxious gas emission in growing pigs. *Anim. Sci. J.*, 2011, 82(2): 282-290 (doi: 10.1111/j.1740-0929.2010.00828.x).
8. Tairova A.R., Kuznetsov A.I. *Veterinarnyi vrach*, 2001, 4: 49-51 (in Russ.).
9. Teterev I.I. *Propolis v zhivotnovodstve i veterinarii* [Propolis in livestock and veterinary medicine]. Kirov, 1998: 54-58 (in Russ.).
10. Ushakova N.A., Naumova E.I., Pavlov D.S., Chernukha B.A. *Sposob polucheniya biologicheski aktivnoi kormovoi dobavki iz rastitel'nogo syr'ya. Patent 2202224 (RF) MPK7 A 23 K 1/165. Institut problem ekologii i evolyutsii im. A.N. Severtsova RAN (RF). 2001104906/13 Zayavl. 22.02.2001. Opubl. 20.04.2003. Byul. № 12* [Technology of manufacturing a plant derived biologically active feed additive. Patent 2202224 (RF) МПК7 A 23 K 1/165. Appl. 22.02.2001. Publ. 20.04.2003. Bul. № 12] (in Russ.).
11. Ostrovskii M.A., Dontsov A.E. *Fiziologiya cheloveka*, 1985, 11(4): 670-678 (in Russ.).
12. Baraboi V.A. *Uspekhi sovremennoi biologii*, 2000, 117: 86-92 (in Russ.).
13. Dontsov A.E. *Zashchitnoe deistvie melaninov pri okislitel'nom stresse* [Protective effect of melanins in oxidative stress]. Lambert Academic Publishing, Saarbrűcken, 2014.
14. Wang J.-M., Ao A.-H., Qiao C.-S., Zhong Y., Zhang Y.-Y. The research progress of melanin. *Adv. Mat. Res.*, 2011, 204-210: 2057-2060 (doi: 10.4028/www.scientific.net/AMR.204-210.2057).
15. Meredith P., Sarna T. The physical and chemical properties of eumelanin. *Pigm. Cell Res.*, 2006, 19: 572-594 (doi: 10.1111/j.1600-0749.2006.00345.x).
16. Lia H.-J., Lic J.-X., Zhao Z. Characterization of melanin extracted from apricot (*Armeniaca sibirica*) and its effect on hydrazine-induced rat hepatic injury. *ScienceAsia*, 2016, 42: 382-391 (doi: 10.2306/scienceasia1513-1874.2016.42.382).
17. Zueva O.Yu. *Razrabotka biotekhnologicheskikh protsessov polucheniya biologicheski aktivnykh soedinenii iz medonosnykh pchel i issledovanie ikh svoistv. Avtoreferat kandidatskoi dissertatsii* [A biotechnology for production of honeybee-derived biologically active compounds and their properties. PhD Thesis]. Shchelkovo, 2004 (in Russ.).
18. Bußler S., Rumpold B.A., Jander E., Rawel H.M., Schlüter O.K. Recovery and techno-functionality of flours and proteins from two edible insect species: Meal worm (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*) larvae. *Heliyon*, 2016, 2(12): e00218 (doi: 10.1016/j.heliyon.2016.e00218).
19. Makkar H.P.S., Tran G., Heuzé V., Ankers P. State-of-the-art on use of insects as animal feed. *Anim. Feed Sci. Tech.*, 2014, 197: 1-33 (doi: 10.1016/j.anifeedsci.2014.07.008).
20. Sánchez-Muros M.-J., Barroso F.G., Manzano-Agugliaro F. Insect meal as renewable source of food for animal feeding: a review. *J. Clean. Prod.*, 2014, 65: 16-27 (doi: 10.1016/j.jclepro.2013.11.068).
21. Barroso F.G., de Haro C., Sánchez-Muros M.-J., Venegas E., Martínez-Sánchez A., Pérez-Bacyn C. The potential of various insect species for use as food for fish. *Aquaculture*, 2014, 422-423: 193-201 (doi: 10.1016/j.aquaculture.2013.12.024).
22. Bondari K., Sheppard D.C. Soldier fly, *Hermetia illucens* L., larvae as feed for channel catfish, *Ictalurus punctatus* (*Rafinesque*), and blue tilapia, *Oreochromis aureus* (*Steindachner*). *Aquaculture and Fisheries Management*, 1987, 18(3): 209-220 (doi: 10.1111/j.1365-2109.1987.tb00141.x).
23. Bosch G., Fels-Klerx H.J.V., Rijk T.C., Oonincx D.G.A.B. Aflatoxin B1 tolerance and accumulation in black soldier fly larvae (*Hermetia illucens*) and yellow mealworms (*Tenebrio molitor*). *Toxins*, 2017, 9(6): 185 (doi: 10.3390/toxins9060185).
24. Wang Y.S., Shelomi M. Review of black soldier fly (*Hermetia illucens*) as animal feed and human food. *Foods*, 2017, 6(10): 91 (doi: 10.3390/foods6100091).
25. Nowak V., Persijn D., Rittenschober D., Charrondiere U.R. Review of food composition data for edible insects. *Food Chem.*, 2016, 193: 39-46 (doi: 10.1016/j.foodchem.2014.10.114).
26. Čičková H., Newton G.L., Lacy R.C., Kozánek M. The use of fly larvae for organic waste treatment. *Waste Manage.*, 2015, 35: 68-80 (doi: 10.1016/j.wasman.2014.09.026).
27. Van Huis A. Potential of insects as food and feed in assuring food security. *Annu. Rev. Entomol.*, 2013, 58: 563-583 (doi: 10.1146/annurev-ento-120811-153704).
28. Liland N.S., Biancarosa I., Araujo P., Biemans D., Bruckner C.G., Waagbш R., Torstensen B.E., Lock E.-J. Modulation of nutrient composition of black soldier fly (*Hermetia illucens*) larvae by feeding seaweed-enriched media. *PLoS ONE*, 2017, 12(8): e0183188 (doi: 10.1371/journal.pone.0183188).
29. Bastrakov A.I., Dontsov A.E., Ushakova N.A. *Izvestiya Ufimskogo nauchnogo tsentra RAN*, 2016, 4: 77-79 (in Russ.).
30. van Huis A., van Itterbeeck J., Klunder H., Mertens E., Halloran A., Muir G., Vantomme P. *FAO Forestry Paper 171. Edible insects: future prospects for food and feed security.* FAO, Rome, Italy, 2013.
31. Kirilov M.P., Makhaev E.A., Pervov N.G., Puzanova V.V., Anikin A.S. *Metodika rascheta obmennoi energii v kormakh na osnove soderzhaniya syrykh pitatel'nykh veshchestv (dlya krupnogo*

rogatogo skota, ovets, svinei) [Calculation of exchange energy in feeds for cattle, sheep, pigs based on the content of raw nutrients]. Dubrovitsy, 2008: 32 (in Russ.).
32. *Svinovodstvo dlya vsekh* [Pig breeding for everybody]. Available http://svinovodstvo.blogspot.ru/2014/06/dobavki-dlja-porosjat.html#more. Accessed April 1, 2018 (in Russ.).
33. Chyizhanska N., Beregova T. Effect of melanin isolated from Antarctic yeasts on preservation of pig livestock after ablactation. *UAZH*, 2009, 8: 382-385.
34. Sloboguanyk N.M., Slobodyanyk V.M., Neporada K.S., Beregova T.V. *Visnyk problem biologii i medytsyny*, 2017, 4(1/139): 253-256.
35. Slobodyanyk N., Beregova T., Neporada K. Pancreatic enzymes activity under the conditions of acute stress and melanin administration depending on the stress resistance. *J. Pharm. Pharmacol.*, 2015, 3: 232-236 (doi: 10.17265/2328-2150/2015.05.004).

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MIXTURES OF BIOLOGICALLY ACTIVE SUBSTANCES OF OAK BARK EXTRACTS CHANGE IMMUNOLOGICAL AND PRODUCTIVE INDICATORS OF BROILERS

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Abstract

To date, numerous studies are focused on searching for alternatives to antibiotics with similar antimicrobial and growth-stimulating effects that do not cause bacterial resistance and potential side effects for animals. Promising phytogenic compounds have been also recognized as potential alternatives to antibiotics in feeds. One of the problems of phytogenic compound use is the unstable chemical composition of plant extracts, depending on the conditions of growth, distribution area and other factors, so the question arises of extracting some substances with the known properties or designing their compositions. In this paper, it has been shown for the first time that a dietary composition of biologically active substances of *Quercus* cortex helps to maintain productivity and improves the immunomodulating state of Smena 8 poultry cross broilers. In our experiment, a composition of substances (CS) extracted from *Quercus* cortex and chemically synthesized («Acros Organics B.V.B.A.», Belgium) was used, including 2-n-propylresorcinol (98 %, AVH27024); 4-hydroxy-3-methoxybenzaldehyde (99 %, AC14082-1000); 7-hydroxycoumarin (99 %, AC12111-0250); 3,4,5-trimethoxyphenol (98.5 %, AC18914-0050); scopoletin (95 %, AC30290-0010); coniferyl alcohol (98 %, AL22373-5) with a confirmed anti-QS effect. A total of 120 broiler chickens aged 7 days were divided into 4 groups (n = 30) by analogue method. Control group was fed with the basic diet (BD). BD + CS 1 (1 ml/kg of live weight), BD + CS 2 (2 ml/kg of lw), and BD + CS 3 (3 ml/kg lw) were used for group 1, group 2, and group 3, respectively. In the experimental groups, as compared to the control, the number of blood leukocytes increased by 19.2-28.5 % ($P \le 0.05$), blood lymphocytes were higher by 24.4, 36.2 % ($P \le 0.05$) and 44.0 % ($P \le 0.05$), blood monocytes were higher by 23.5, 23.5 and 29.4 % ($P \le 0.05$), and blood granulocyte counts were higher by 12.3 % ($P \le 0.05$), 5.7 and 9.5 %. The blood ALT activity in the group 2 and group 3 exceeded the control value by 13.2 % ($P \le 0.05$). The level of GGT tended to decrease in the group 2 and group 3, along with a significant decrease in LDH by 17.6-22.5 % ($P \le 0.05$). The intake of the CS as a feed additive was accompanied by an increase in blood SOD levels in the test groups, the highest concentration being observed in the group 1 (95.3 %). The catalase indices had similar patterns. Dietary composition of biologically active substances promoted a 16.4 % increase ($P \le 0.05$) in blood β-lysine levels in the group 1. Within 4 weeks, the dietary CSs led to an increase in the live weight of the poultry of the group 2 and groups 3 by 12.6-15.0 % ($P \le 0.05$) when compared to the group 1. In the group 1, the birds grew more rapidly with a 100 % survival rate of the herd, in contrast to the remaining groups with the survival index of 71 to 85 %. These contributed to an insignificant increase in the bird live weight at the end of the experiment (day 42) by 1.9 % ($P \ge 0.05$) compared to the control group. Thus, feeding broiler chickens with the composition of bioactive substances in the initial concentration helps to maintain productivity and improve the immunomodulating state of body.

Keywords: biologically active substances, oak bark, broiler chickens, biochemical and mor-

phological parameters of blood, blood enzymes, growth rate

Use of antibiotics (AB) as growth stimulants in animal feed is limited by growth of AB resistance of bacterial pathogens that poses a threat for the national health [1, 2]. To date, numerous studies are focused on searching for alternatives to antimicrobial substances with preventative and growth stimulating effects that do not cause bacterial resistance and potential side effects in animals.

Phytogenic compounds [1, 3-5] are widely recognized as potential antibiotic alternative in feeds. These are plant-based biologically active compounds with positive effect on animal growth and health, which are often used in form of essential oils and plant extracts [6]. Results of studies aimed at understanding of the phytogenic compound action mechanisms, possible side effects and economic effectiveness, are not convincing yet. There is information confirming the ability of phytogenic compounds, including extracts from alfalfa seeds [7], grasses [8], and essential oils [9, 10] to affect quorum sensing (QS) of bacteria. All of them are produced in studies in vitro and relate to food products. QS inhibition for prevention and combat with bacterial infections in farm animals remains the less studied problem, except for the aquaculture [11].

One of obstacles for use of phytogenic compounds is instable chemical composition of plant extracts depending on growing conditions, ecosystem, and other factors. Earlier, several compounds from oak bark extract (*Quercus* cortex) having both antibacterial and anti-QS activity [12, 13] was found. These substances were used in tests on agricultural poultry.

In present study we for the first time had shown that inclusion of the composition of biologically active compounds from *Quercus* cortex extract into the ration of broiler chickens cross Smena 8 promotes maintenance of productivity and strengthening of immune-modulating body state.

Purpose of our research is to study effect of various dosages of biologically active compounds from *Quercus* cortex extract on productive indicators and immunity in broiler chickens, and to assess perspectives of use of such combinations and possible mechanism of their action.

Techniques. Chemical substances were identified by gas chromatography with a mass-selective detector GQCMS 2010 Plus (Shimadzu, Japan) in tube HP-5MS. GCMS Solutions, GCMS PostRun Analysis software was used at interpretation of research results, set of mass spectra libraries CAS, NIST08, Mainlib, Wiley9 and DD2012 Lib was used for identification of compounds. Each identified component was quantified as the percentage of its peak area from the total area of the extract peaks.

In tests we used mixture of substances earlier extracted from the oak bark [12, 13] and artificially synthesized (Acros Organics B.V.B.A., Belgium). These are 2-n-propylresorcinol (98 %), 4-hydroxi-3-metoxibenzaldehyde (99 %), 7-hydroxi coumarine (99 %), 3,4,5-trimetoxiphenol (98.5 %), scopoletin (95 %), and coniferyl alcohol (98 %). Anti-QS-effect of this composition (SC) was tested on *Chromobacterium violaceum* strain CV026 by agar diffusion method (qualitatively) and by serial dilution method in liquid medium (quantitatively).

Broiler chickens aged 7 days (cross Smena 8, $n = 120$) divided by analogue method into 4 groups, $n = 30$ each, were selected for tests in vivarium conditions. During the tests, all poultry was in similar feeding and keeping conditions. Rations were made accounting for recommendations of the Russian Research and Technological Institute of Poultry (VNITIP) [14]. Control group ate the main diet (MD); group I (trial) was fed MD + substance composition (SC) 1 (1 ml/kg of live weight); group II (trial) was fed MD + SC 2 (2 ml/kg of live weight); group III (trial) — MD + SC 3 (3 ml/kg of live mass). Chemical substances in the composition for the group I were 2-n-propylresorcinol (1.5

mg/ml), 4-hydroxi-3-metoxibenzaldehyde (0.5 mg/ml), 7-hydroxi coumarine (0.5 mg/ml), 3,4,5-trimetoxi-phenol (2 mg/ml), scopoletin (0.3 mg/ml), coniferyl alcohol (4.5 mg/ml); for the group II — 3; 1; 1; 4; 0.6; 9 mg/ml, respectively, for the group III — 4.5; 1.5; 1.5; 6; 0.9; 13.5 mg/ml. Poultry was fed 2 times daily, feed consumption was accounted daily, SC solution was provided individually. Poultry was provided unlimitedly with water. Growth and development of chickens (inspection and individual weightings were carried out daily, in the same time in morning hours) was assessed. Decapitation was performed under nembutal ester on day 42. Housing and procedures during tests were in line with instructions and recommendations of the Russian regulation (Decree of the Ministry of Health of USSR№ 755 dated 12.08.1977) and The Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996). All endeavors were taken to minimize the agony in animals and to reduce the number of used samples.

Blood samples for hematologic studies were collected into vacuum vials with anticoagulant (EDTA-K3), for biochemical studies — in vacuum vials with coagulating activator (thrombin). Hematological indicators (number and types of leucocytes) were estimated (an automated analyzer URIT-2900 Vet Plus, URIT Medical Electronic Group Co., Ltd", China).

Data was statistically processed in software IBM SPSS Statistics Version 20 (https://www-01.ibm.com). Mean (*M*) and standard errors of the mean (±SEM) were calculated. Differences are statistically significant at $p < 0.05$.

Results. Analysis of morphological and biochemical blood indicators allows identification of changes in trial groups (Table 1). Number of leucocytes was 19.2-28.5 % higher ($P \leq 0.05$), counts of lymphocytes in groups I, II and III increased by 24.4; 36.2 ($P \leq 0.05$) and 44.0 % ($P \leq 0.05$), respectively, of monocytes — by 23.5; 23.5 and 29.4 % ($P \leq 0.05$), and of granulocytes — by 12.3 ($P \leq 0.05$); 5.7 and 9.5 %.

1. Count ($\times 10^9$/л) **of white cells in blood of cross Smena 8 broiler chickens fed bioactive composition (extract from oak bark + artificially synthesized substances)** (*M*±SEM, vivarium conditions)

Indicator	Group			
	control (n = 15)	I (n = 15)	II (n = 15)	III (n = 15)
Leucocytes	24.9±1.21	29.7±1.33*	30.5±1.08*	32.0±1.51*
Lymphocytes	12.7±0.91	15.8±1.18	17.3±1.58*	18.3±1.11*
Monocytes	1.7±0.77	2.1±1.81	2.1±0.54	2.2±0.53*
Granulocytes	10.5±0.91	11.8±0.21*	11.1±0.94	11.5±1.34

N o t e. See description of groups in section "Methodology".
* Differences with control are statistically significant at $P \leq 0.05$.

Hematologic parameters serve convenient indicator for assessment of physiological state and health in animals and may be useful to control effects of feed additives [15]. Our data correlates with earlier research results [16, 17]. Other tests on animals in vitro also showed that plant-based bioactive compounds increase immune activity by increase of phagocytosis [18]. This can also explain growth of blood indicators in trial groups.

Activity of blood alanine aminotransferase (ALAT) in groups II and III trial was 13.2 % higher ($P \leq 0.05$) compared to control. Activity of aspartate aminotransferase (ASAT) was the least in group I ($P \geq 0.05$) (Table 2).

In earlier paper [19] dietary polyphenols did not significantly affect ASAT and ALAT, however, the researchers did not performed exact chemical identification of the bioactive components. In the composition used in our experiment, the coniferyl alcohol which possesses cytotoxicity [20] is high in level and can affect hepatic cells, as follows from ASAT and ALAT indicators in groups II and III. Content of γ-glutamyl transpeptidase (γ-GT) decreases in

groups II and III at a decrease of lactate dehydrogenase (LDG) by 17.6-22.5 % ($P \leq 0.05$) as compared to control. Obtained data indirectly testify positive effect of the substance composition in the initial concentration on protein and carbohydrate metabolism. Similar findings are reported by H.Y. Qiao et al. [20]. In their study dietary 4-hydrxi-3,5-dimetoxi-cinnamic acid, a polyphenolic compound, had no effect ($P > 0.05$) on blood activity of creatine kinase and lactate dehydrogenase in broilers. Besides, as is known [21], oak bark extract promotes better glucose tolerance and significantly decreases its absorption through the gastric epithelium.

2. Activity of blood enzymes and non-specific immunity indicators in cross Smena 8 broiler chickens fed bioactive composition (extract from oak bark + artificially synthesized substances) (M±SEM, vivarium conditions)

Indicator	Group			
	control (n = 15)	I (n = 15)	II (n = 15)	III (n = 15)
ALAT, IU/l	3.8±0.76	4.0±0.36	4.3±0.43*	4.3±0.37*
ASAT, IU/l	228.8±21.84	219.6±12.53	229.9±13.23	231.8±15.51
γ-GT, IU/l	16.5±1.04	18.3±2.85	15.0±1.08	16.0±2.00
LDG, IU/l	14.2±3.68	14.0±5.57	11.7±2.99*	11.0±2.97*
SOD, %	218.6±54.02	427.1±52.52*	391.1±38.80*	378.1±51.33*
Catalase, μmol $H_2O_2 \cdot l^{-1} \cdot min^{-1}$	989.4±46.30	1438.0±57.73*	1701.6±53.33*	1468.5±52.91*
BSLA, %	47.1±0.39	45.3±0.91	44.9±1.2	45.1±0.55
β-Lysine, %	72.9±0.40	89.3±0.80*	69.6±0.52	84.5±0.51

N o t e. ALAT — alanine aminotransferase, ASAT — aspartate aminotransferase, γ-GT — γ-glutamyl transpeptidase, LDG — lactate dehydrogenase, SOD — superoxide dismutase, BSLA — blood serum lysozyme activity. See description of groups in section "Methodology".

* Differences with control are statistically significant at $P \leq 0.05$.

SC intake with feed leads to increased antioxidant activity, in particular, due to higher blood content of superoxide dismutase in chickens of trial groups, with the highest concentration in group I (95.3 %). Catalase content in poultry in trial groups was higher than in control by 45.3-71.9 % ($P \leq 0.05$). Antioxidant properties of oak bark extracts [22, 23] are also shown in our tests. Increased total antioxidant activity and SOD level in blood of broilers [24, 25] were due to feed supplementation with gallic acid, an oak bark component. Increased blood catalase concentration also occurred in broiler chickens after use of grape seed powder as a source of polyphenolic substances in diet [19].

SC in the diet promotes 16.4 % higher blood β-lysine content in group I as compared to control ($P \leq 0.05$). Weight gain in poultry of group I was also higher than in groups II and III, i.e. by 15.0 % ($P \leq 0.05$) in 1 week, by 14.3 % in 2 weeks and by 12.6-13.9 % in weeks 3 and 4. In group I, more rapid growth combined with 100 % survival while in other groups this indicator was 71-85 % (see Table 3).

3. Weigh (g) **dynamics in cross Smena 8 broiler chickens fed bioactive composition (extract from oak bark + artificially synthesized substances)** (M±SEM, vivarium conditions)

Group	Week 1	Week 2	Week 3	Week 4
Control	299.5±15.4	652.5±28.9	1164.0±21.5	1787.0±22.2
I	317.2±12.5	618.8±23.5	1134.4±17.2	1660.0±23.5
II	275.6±20.1	541.0±23.2*	1007.0±22.7	1571.5±26.8
III	291.6±10.7	558.4±11.3	1015.2±12.4	1456.8±19.5*

N o t e. See description of groups in section "Methodology".

* Differences with control are statistically significant at $P \leq 0.05$.

Use of bioactive substances at initial concentration (group I) resulted in 1.9 % ($P \geq 0.05$) weight gain at the end of test (on day 42) as compared to control group. This is in line with data on growth stimulation by dietary gallic acid and grape seeds rich in polyphenols [19, 20]. The composition we used also contains phenolic compounds and, thus, these results are objectively comparable.

However, there are studies in which β-resorcylic acid as antimicrobial feeding additive has no effect on weight gain in broiler chickens [26]. In our study, weight of poultry in groups II and III decreased as concentration of bioactive substances increased, and, note, propyl resorcinol close in origin to resorcylic acid was present in the composition. As to growth stimulation effect, several mechanisms are possible, e.g. a decrease in total bacterial load, suppression of pathogenic microorganisms, thinning mucosa layer, and direct immune modulation [27]. It is known that tannings may participate in modulation of the composition and activity of intestinal microflora and its interaction with the entering compounds. It is reported that gram-positive bacteria are more sensitive to plant-based extracts rich in tannin-like substances [28, 29]. It is also confirmed by properties of compounds earlier found in *Quercus* cortex extract [12, 13].

Therefore, dietary additive composition of oak bark extract + artificially synthesized substances at a dose of 1 ml/kg live weight favorably modulates immune state and antioxidant activity in vivo in broiler chickens that is followed by increased blood levels of β-lysine, superoxide dismutase and catalase, higher survival (up to 100 %) and improved productivity (up to 15 %).

REFERENCES

1. Randrianarivelo R., Danthu P., Benoit C., Ruez P., Raherimandimby M., Starter S. Novel alternative to antibiotics in shrimp hatchery: effects of the essential oil of *Cinnamosma fragrans* on survival and bacterial concentration of *Penaeus monodon* larvae. *J. Appl. Microbiol.*, 2010, 109: 642-650 (doi: 10.1111/j.1365-2672.2010.04694.x).
2. Allen H.K., Levine U.Y., Looft T., Bandrick M., Casey T.A. Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends Microbiol.*, 2013, 21: 114-119. (doi: 10.1016/j.tim.2012.11.001).
3. Windisch W., Schedle K., Plitzer C., Kroismayr A. Use of phytogenic products as feed additives for swine and poultry. *J. Anim. Sci.,* 2008, 86: e140-e148 (doi: 10.2527/jas.2007-0459).
4. Yang W.Z., Benchaar C., Ametaj B.N., Chaves A.V., He M.L., McAllister T.A. Effect of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. *J. Dairy Sci.*, 2007, 90: 5671-5678 (doi: 10.3168/jds.2007-0369).
5. Gong J., Yin F., Hou Y., Yin Y. Review: Chinese herbs as alternatives to antibiotics in feed for swine and poultry production: potential and challenges in application. *Can. J. Anim. Sci.*, 2014, 94: 223-241 (doi: 10.4141/cjas2013-144).
6. Puvača N., Stanačev V., Glamočić D., Lević J., Perić L., Stanaćev V., Milić D. Beneficial effects of phytoadditives in broiler nutrition. *World Poultry Sci. J.*, 2013, 69: 27-34 (doi: 10.1017/S0043933913000032).
7. Vikram A., Jayaprakasha G.K., Jesudhasan P.R., Pillai S.D., Patil B.S. Suppression of bacterial cell-cell signalling, biofilm formation and type III secretion system by citrus flavonoids. *J. Appl. Microbiol.*, 2010, 109: 515-527 (doi: 10.1111/j.1365-2672.2010.04677.x).
8. Truchado P., Gimenez-Bastida J.A., Larrosa M., Castro-Ibanez I., Espin J.C., Tomas-Barberan F.A., Garcia-Conesa M.T., Allende A. Inhibition of quorum sensing (QS) in *Yersinia enterocolitica* by an orange extract rich in glycosylated flavanones. *J. Agric. Food Chem.*, 2012, 60(36)*:* 8885-8894 (doi: 10.1021/jf301365a).
9. Choo J.H., Rukayadi Y., Hwang J.K. Inhibition of bacterial quorum sensing by vanilla extract. *Lett. Appl. Microbiol.*, 2006, 42: 637-641 (doi: 10.1111/j.1472-765X.2006.01928.x).
10. Zhou L., Zheng H., Tang Y., Yu W., Gong Q. Eugenol inhibits quorum sensing at sub-inhibitory concentrations. *Biotechnol. Lett.*, 2013, 35: 631-637 (doi: 10.1007/s10529-012-1126-x).
11. Defoirdt T., Boon N., Bossier P., Verstraete W. Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. *Aquaculture*, 2004, 240: 69-88 (doi: 10.1016/j.aquaculture.2004.06.031).
12. Deryabin D.G., Tolmacheva A.A. Antibacterial and anti-quorum sensing molecular composition derived from *Quercus* cortex (Oak bark) extract. *Molecules,* 2015, 20(9): 17093-17108 (doi: 10.3390/molecules200917093).
13. Tolmacheva A.A., Rogozhin E.A., Deryabin D.G. Antibacterial and quorum sensing regulatory activities of some traditional Eastern-European medicinal plants. *Acta Pharmaceutica*, 2014, 64(2): 173-186 (doi: 10.2478/acph-2014-0019).
14. Fisinin V.I., Egorov I.A., Lenkova T.N., Okolelova T.M., Ignatova G.V., Shevyakov A.N., Panin I.G., Grechishnikov V.V., Vetrov P.A., Afanas'ev V.A., Ponomarenko Yu.A. *Metodicheskie uka-*

zaniya po optimizatsii retseptov kombikormov dlya sel'skokhozyaistvennoi ptitsy [Guidelines for the optimization of animal feed recipes for poultry]. Moscow, 2009 (in Russ.).
15. Togun V.A., Oseni B.S.A. Effect of low level inclusion of biscuit dust in broiler finisher diet on pre-pubertal growth and some haematological parameters of unsexed broilers. *Res. Comm. Anim. Sci.*, 2005, 1: 10-14.
16. Khalaji S., Zaghari M., Hatami K., Hedari-Dastjerdi S., Lotfi L., Nazarian H. Black cumin seeds, *Artemisia leaves* (*Artemisia sieberi*), and *Camellia* L. plant extract as phytogenic products in broiler diets and their effects on performance, blood constituents, immunity, and cecal microbial population. *Poultry Sci.*, 2011, 90(11): 2500-2510 (doi: 10.3382/ps.2011-01393).
17. Abou-Elkhair R., Ahmed H.A., Selim S. Effects of black pepper (*Piper nigrum*), Turmeric Powder (*Curcuma longa*) and Coriander Seeds (*Coriandrum sativum*) and their combinations as feed additives on growth performance, carcass traits, some blood parameters and humoral immune response of broiler chickens. *Asian Austral. J. Anim.*, 2014, 27(6): 847-854 (doi: 10.5713/ajas.2013.13644).
18. Geetha R.V., Lakshmi T., Roy A. A review on nature's immune boosters. *Intl. J. Pharm. Sci. Rev. Res.*, 2012, 13: 43-52.
19. Abu Hafsa S.H., Ibrahim S.A. Effect of dietary polyphenol-rich grape seed on growth performance, antioxidant capacity and ileal microflora in broiler chicks. *J. Anim. Physiol. Anim. Nutr.*, 2017, 102(1): 268-275 (doi: 10.1111/jpn.12688).
20. Qiao H.Y., Dahiya J.P., Classen H.L. Nutritional and physiological effects of dietary sinapic acid (4-hydroxy-3,5-dimethoxy-cinnamic acid) in broiler chickens and its metabolism in the digestive tract. *Poultry Sci.*, 2008, 87(4): 719-726 (doi: 10.3382/ps.2007-00357).
21. Rtibi K., Hammami I., Selmi S., Grami D., Sebai H., Amri M., Marzouki L. Phytochemical properties and pharmacological effects of *Quercus ilex* L. aqueous extract on gastrointestinal physiological parameters in vitro and in vivo. *Biomed. Pharmacother.*, 2017, 94: 787-793 (doi: 10.1016/j.biopha.2017.08.008).
22. Popović B.M., Štajner D., Ždero R., Orlović S., Galić Z. Antioxidant characterization of oak extracts combining spectrophotometric assays and chemometrics. *Sci. World J.,* 2013: 134656 (doi: 10.1155/2013/134656).
23. Youn S.H., Kwon J.H., Yin J., Tam L.T., Ahn H.S., Myung S.C., Lee M.W. Anti-inflammatory and anti-urolithiasis effects of polyphenolic compounds from *Quercus gilva* Blume. *Molecules*, 2017, 22(7): 1121 (doi: 10.3390/molecules22071121).
24. Samuel K.G., Wang J., Yue H.Y., Wu S.G., Zhang H.J., Duan Z.Y., Qi G.H. Effects of dietary gallic acid supplementation on performance, antioxidant status, and jejunum intestinal morphology in broiler chicks. *Poultry Sci.*, 2017, 96(8): 2768-2775 (doi: 10.3382/ps/pex091).
25. Shirzadegan K., Falahpour P. The physicochemical properties and antioxidative potential of raw thigh meat from broilers fed a dietary medicinal herb extract mixture. *Open Vet. J.*, 2014, 4(2): 69-77.
26. Wagle B.R., Upadhyay A., Arsi K., Shrestha S., Venkitanarayanan K., Donoghue A.M., Donoghue D.J. Application of β-resorcylic acid as potential antimicrobial feed additive to reduce *campylobacter* colonization in broiler chickens. *Front. Microbiol.,* 2017, 8: 599 (doi: 10.3389/fmicb.2017.00599).
27. Engels C., Schieber A., Gänzle M.G. Inhibitory spectra and modes of antimicrobial action of gallotannins from mango kernels (*Mangifera indica* L.). *Appl. Environ. Microb.*, 2011, 77(7): 2215-2223 (doi: 10.1128/AEM.02521-10).
28. Karimov I., Duskaev G., Inchagova K., Kartabaeva M. Inhibition of bacterial Quorum sensing by the ruminal fluid of cattle. *International Journal of GEOMATE*, 2017, 13(40): 88-92 (doi: 10.21660/2017.40.65948).
29. Nohynek L.J., Alakomi H.-L., Kähkönen M.P., Heinonen M., Helander I.M., Oksman-Caldentey K.M., Puupponen-Pimiä R.H. Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. *Nutr. Cancer*, 2006, 54(1): 18-32 (doi: 10.1207/s15327914nc5401_4).

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COMPARATIVE TESTS OF VARIOUS SOURCES OF MICROELEMENTS IN FEEDING CHICKEN-BROILERS

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Abstract

Animals of modern breeds and crosses need more dietary minerals to realize more of their genetic potential but that leads to an increase in the ecological load. So the development of new sources of essential chemical elements with relatively less toxicity and higher bioavailability of the components are of relevance. Ultra-dispersed particles (UDP) are among prospective preparations. This is the first report on a comparative study of the effects of dietary Cu and Zn additives as UDP of the alloy, asparaginates and sulfates on performance and productivity of Smena 7 broiler chicks. The study showed greater availability, a more pronounced positive effect of Cu/Zn-UDP and the various impact of the forms studied on mineral metabolism. Dietary Cu/Zn-UDP accelerated bird growth by 3.9 % ($P \le 0.05$) compared to Cu and Zn mineral salts and by 4.7 % ($P \le 0.01$) compared to Cu and Zn asparaginates. Administration of Cu/Zn-UDP led to an increase in blood NO metabolites by 9.8 % ($P \le 0.05$), 21.0 % ($P \le 0.01$), 13.0 % ($P \le 0.05$), and 11.0 % ($P \le 0.05$) compared to the control on days 7, 14, 21 and 28, respectively. By the end of the study, blood erythrocytes and hemoglobin was 6.27 % higher ($P \le 0.05$) and 19.40 % higher ($P \le 0.001$) compared to the control and also 5.21 % higher and 12.60 % higher when compared to Cu and Zn asparaginates used. Replacement of copper mineral salt with dietary Cu/Zn-UDP and Cu asparaginate was accompanied by an increase in this element pool in the body of 42-day old broiler chickens by 51.6 % ($P \le 0.01$) and 13.2 %, respectively. By the end of the study, the zinc pool, on the contrary, decreased by 22.9 % compared to the control when Zn asparaginate was fed but exceeded the control by 12.5 % ($P \le 0.05$) when using Cu/Zn-UDP. Copper and zinc preparations used in various ways influenced on the exchange of a number of chemical elements in the body. Feeding with Cu/Zn-UDP and Cu and Zn asparaginates resulted in lower pools of Ni, Al, Sn and a significant increase in iodine and cobalt pools compared to control. A distinctive feature of Cu/Zn-UDP action from that of the asparaginates was an increase in Pb and Cd pools which could result from a change of the load on transport systems in the intestine when using Cu/Zn-UDP.

Keywords: ultra-dispersed particles of Cu and Zn alloy, Cu and Zn asparaginates, broiler chicks, productivity, chemical element composition, biochemical and morphological blood parameters

Some estimates suggest that development of nanotechnologies by 2020 will result in establishment of industrial and agricultural productions with turnover from $3.0 tln [1] to $3.4 tln [2]. Yet today actual production of nanomaterials exceeds 100 ths. t per annum [3]. Along with wider use in medicine and biology [4-6], nanomaterials become prevalent in agriculture [7, 8], food and pro-

cessing industry [9, 10]. Only in USA, annual growth rate in such sector comprises 25 % ($1.08 bln.) [11]. Use of nanomaterials in agriculture as microelement medications is characterized by their less toxicity (12, 13) and higher biological availability [14, 15]. The later, in particular in the context of PCR, allows decreasing pollution of environment at production and use of feeds [16].

Opportunities for use of nanosized microelement forms were demonstrated in medicine. In particular, it allowed creating medicines for treatment of anemia. Thus, Ferumoxytol (Feraheme®, AMAG Pharmaceuticals, Inc., USA) containing superparamagnetic iron oxide nanoparticles (SPION) was approved by US Food and Drug Administration (FDA) for iron replacement therapy primarily in patients with chronic renal disease [17] and got widespread use at MRT tests [18].

Today, literature sources suggest using various nanostructural microelement sources in animal breeding industry, including selenium [19], iron [20], chrome [21] zinc [22], copper (23), etc. Usually, these are medicines containing one microelement in form of nanoparticles. However, with development of the concept of synthesis and use of such substances prospects for microelement complexes, including antagonists, become evident [24].

Science had acquired a great deal of knowledge on the nature and mechanisms of antagonist relations between chemical elements and other elements [25-27], phytate [28], amino acids and their salts [29], polyphenols and peptides [30], etc. in human and animal body, especially at absorption stage in gastrointestinal tract. Necessity for studying of such relations is determined by the need for tackling the challenges of prenosological diagnostics and treatment of elementosis, correction of diets [31], and estimation of actual nutritional value of diets [32]. Antagonism may reduce availability of some elements which requires increasing their input norms and may negatively affect the environment. Earlier, separate feeding of antagonist substances was proposed to exclude antagonist relations at soaking (Patent of Invention RUS 2195269 14.02.2001).

In this paper, we had for the first time shown that productive and biological action of various forms of two essential microelements — zinc and copper (alloy in form of ultrafine particles, asparaginates and sulphates) and their effect on mineral metabolism in broiler chickens cross Smena 7 is different; herewith, ultrafine particles were more available in general and had more expressed positive effect.

Purpose of this paper was to study the effectiveness of ultrafine particles of copper and zinc alloy as a mineral additive in feeding of broiler chickens as compared to mineral salts and organic forms of such elements.

Techniques. Copper and zinc asparaginates (V-Min+ LLC, Sergiev Possad, Russia), mineral salts $ZnSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$ (Lenreactiv, Saint Petersburg, Russia) and powder from ultrafine particles of Cu-Zn alloy (UFP Cu-Zn) made by Peredovie Poroshkovie Tehnologii LLC (Tomsk, Russia) were used as microelement sources. Cu-Zn UFPs were produced by method of electric explosion of conductor in argon atmosphere. Material attestation of UFPs involved scanning and transmission microscopy at JSM 7401F and JEM-2000FX (JEOL, Japan). X-ray phase analysis was carried out at a diffractometer DRON-7 (Scientific Industrial Enterprise Burevestnik, Russia). Based on attestation results, size of particles (d) 65±15 μm with ratio Cu^o 60±3.5 %, Zn^o 40±2.9 %; Z-potential 12±0.4 mV, specific surface 5±1.6 m^2/g. At production of lyosols, water suspensions of Cu-Zn UFPs were treated by ultrasound at dispergator UZDN-2T (Scientific Industrial Enterprise Academpribor, Russia) at 35 KHz, 300/450 W, 10 mcA during 30 minutes. Lyosol was introduced into the combined feed by gradual mixture method.

Studies were carried out on broiler chicken (cross Smena 7) in vivarium conditions of the Institute of Bioelementology of Orenburg State University. Poultry keeping and procedures followed during tests were in line with instructions and recommendations of the Russian Regulations (Decree of the Ministry of Health of USSR Nr. 755 dated 12.08.1977) and The Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, 1996). All endeavors were taken to minimize the agony in animals and to reduce the number of used specimen. 90 chickens aged 1 day were selected for tests. Chickens assigned with individual numbers (leg plastic labels), were weighted and in furtherance were kept in equal conditions. Three groups aged 2 weeks were formed based on individual daily weighting data and estimated feed costs by paranalogues: one control and two trial (n = 24 each). Chickens were fed full-value combined feeds formed accounting for the recommendations [33] according to age periods. Composition of the main diet (MD) (g/kg) during the period from 7 to 28 days: wheat grain — 475, barley grain — 30, maize — 80, soybean meal — 250, sunflower meal— 70, sunflower oil — 50, premix (made according to the effective recommendations) — 20, cooking salt — 3.4, moncalcium phosphate — 13, limestone meal — 5, DL-methionine 98.5 % — 1,6, lysine monochlohydrate 98 % — 1, cooking soda — 1; aged 28-42 days: wheat grain — 435, maize — 226, soybean meal — 150, sunflower meal — 100, sunflower oil — 50, premix — 20, cooking salt — 3, moncalcium phosphate 10.5, limestone meal — 1, DL- methionine 98.5 % — 1.2, lysine monochlohydrate 98 % — 2.3, baking soda — 1. During the tests chickens were on the Main Diet, in which copper and zinc were introduced in form of $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ as part of premix including all standardized microelements. Copper and zinc sulphates in the premix for chickens from trial groups during the period from 14 to 42 days were replaced by Cu-Zn UFPs in dosage of 2.84 mg/kg of feed (group I) or by Cu and Zn asparaginates in the same dosage (group II). Chickens from all groups were supplied with distilled water.

Poultry growth was daily controlled by individual weighting in morning hours before feeding.

Blood was collected from axillary vein on empty stomach in morning hours before killing of chickens aged 21, 28, 35 and 42 days (for morphologic studies — in vacuum vials with anticoagulant EDTA, for biochemical — in vacuum vials with thrombin as coagulating stimulant). Blood serum was analyzed within no later than 3 hours following sampling.

Morphologic indicators were determined by automated hematologic analyzer URIT-2900 Vet Plus (URIT Medial Electronic Co., Ltd, PRC). Biochemical analysis of blood serum was conducted with the use of an automated analyzer CS-T240 (DIRUI Industrial Co., Ltd, PRC) and commercial veterinary kits (DiaVetTest, DIAKON-DS CJSC, Russia; Randox Laboratories, Ltd, United Kingdom).

Metabolism of chemical elements was studied by comparative slaughter method. Mass was calculated, and tissue and organ specimen were collected upon slaughter for assessment of the elementary composition frozen and stored at temperature of –18 °C. Specimens were analyzed for 25 chemical elements (Ca, Cu, Fe, Li, Mg, Mn, Ni, As, Cr, K, Na, P, Zn, I, V, Co, Se, Ti, Al, Be, Cd, Pb, Hg, Sn, and Sr). Total element pool in vivo upon slaughter was calculated as total content in organs and tissues, retention was determined as pool difference at the end and beginning of the experiment.

Elemental composition of organs and tissues was analyzed by nuclear-emission spectrometry methods with inductively bound plasma (Optima 2000 V, PerkinElmer, USA) and mass-spectrometry (Elan 9000, PerkinElmer,

USA). Ashing of biosubstrates was performed in microwave decomposition system Multiwave-3000 (Anton Paar, Austria).

Data was statistically processed by Statistica 10.0 software (StatSoft, Inc., USA) and MS Excel 2000 software package. Mean (M) and standard errors of the mean (±SEM) values were determined. Differences assessed by Student's t-test are deemed statistically significant at $P \leq 0.05$.

Results. Control species by 4.5 % left behind the species from II group by consumption of combined feeds during tests. At that, feed consumption to surplus of 1 kg live mass in the control comprised 1.73 kg, that is by 3.40 and 4.04 % more than in I and II groups. Feeding of broiler chickens by Cu-Zn UFPs was accompanied by more intensive growth (Fig. 1).

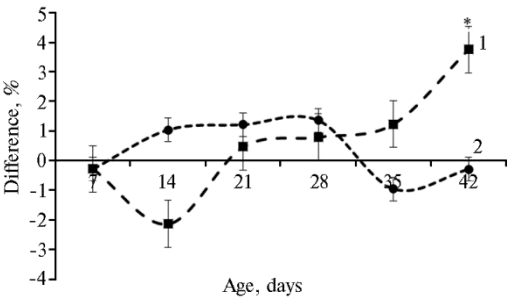

Fig. 1. Difference in live weight between cross Smena 7 broiler chickens in group I fed Cu-Zn alloy ultrafine particles (1) **and in group II fed Cu and Zn asparaginates** (2)**, as compared to control group where Cu and Zn in the ration were regulated by sulphates of the elements** (groups of n = 24 each, testing in vivarium conditions). Star means that deviations from control are statistically significant at $P \leq 0.05$.

During the main accounting period (14-42 days of life), live weight surplus of 2349.9 g was noted in group I that exceeded similar indicator in control and in group II by 3.9 ($P \leq 0.05$) and 4.7 % ($P \leq 0.01$), respectively. The results correlate to results of other studies describing growth stimulating effects of UFP metal medicines as compared to traditional microelement sources [34, 35]. It should be noted that valid intergroup difference in growth intensity in our test was noted at high growth rate in studied poultry (80.8-83.9 g/d). It is hard to explain by only greater biodiversity of microelements from UFP medicines since poultry, before commencement of the main accounting period, was kept on a balanced diet and accumulated pool of assessed microelements, which is quiet sufficient for further active growth, especially that their required quantity was supplied during the entire test.

1. Content of blood NO metabolites (μmol/l) **in cross Smena 7 broiler chickens depending on chemical formula of Cu and Zn used for microelement-based regulation of ration** (M±SEM, n = 6, testing in vivarium conditions)

Group	Age, days			
	21	28	35	42
I	28.9±0.27*	33.8±1.05**	33.5±1.27*	31.0±0.59*
II	24.7±0.70	28.6±0.77	30.5±0.49	32.4±1.96*
Control	26.4±0.20	27.9±0.44	29.6±0.78	27.8±2.36

N o t e. See description of groups in section "Methodology".
*, ** Differences from control are statistically significant at $P \leq .0.05$ and $P \leq 0.01$.

In our opinion, growth stimulating effect of UFPs was determined by specific nanoparticle action mechanism on animal organism [36], of which through strengthening of arginine metabolism and synthesis of nitrogen oxide. This hypothesis is supported by data (Table 1) demonstrating growth of NO metabolite concentrations in blood serum of chickens from group I during the entire test on days 7, 14, 21 and 28, respectively, by 9.8 ($P \leq 0.05$), 21.0 ($P \leq 0.01$), 13.0 ($P \leq 0.05$) and 11.0 % ($P \leq 0.05$) as compared to control.

Differences in action mechanisms of the studied medicines on poultry were also confirmed by assessment of hematologic parameters. Thus, use of UFP medicines and asparaginates promoted erythropoiesis. It was well demonstrated

during the first 3 weeks of additive use (Table 2).

2. Age-specific dynamics of several hematologic indicators in cross Smena 7 broiler chickens depending on chemical form of Cu and Zn used for microelement-based regulation of diet (M±SEM, n = 6, testing in vivarium conditions)

Group	Age, days			
	21	28	35	42
	Erythrocytes, $\times 10^{12}$/l			
I	2,76±0,095*	2,39±0,179*	2,27±0,083	2,71±0,139*
II	2,95±0,041**	2,40±0,182*	2,35±0,018	2,27±0,076*
Control	2,41±0,635	2,16±0,081	2,26±0,145	2,55±0,030
	Hemoglobin, g/l			
I	116,7±1,84	135,7±1,36**	134,3±2,67	146,7±1,57**
II	117,3±1,76	125,0±1,72	139,7±1,96*	130,3±5,93
Control	97,5±2,50	125,0±5,00	132,0±6,03	139,3±2,19
	Hematocrit, %			
I	21,83±0,067**	28,10±1,854*	26,40±0,529	27,53±1,780*
II	24,50±0,321*	28,40±1,629*	27,97±0,463*	26,60±1,790
Control	20,05±0,150	25,10±0,529	25,47±1,017	25,17±0,467
	Leucocytes, $\times 10^9$/l			
I	28,10±0,290	39,37±0,406	36,73±0,687	30,43±0,767
II	32,97±0,373	33,50±0,139	40,93±0,476	35,40±0,312
Control	28,45±0,850	34,30±0,921	36,33±0,998	35,73±0,307
	Lymphocytes, $\times 10^9$/l			
I	12,73±0,024	18,20±0,781*	15,73±0,210	13,13±0,820
II	11,20±0,195	12,97±0,730	18,20±0,318*	15,63±0,524
Control	11,00±0,600	10,87±0,822	15,87±0,513	15,77±0,724

Note. See description of groups in section "Methodology".
*, ** Differences from control are statistically significant at $P \leq .0.05$ and $P \leq 0.01$.

Commencement of changes in erythrocytes is reflected on hematocrit that within 7 days after commencement of tests was increased in groups I and II by 8.8 and 22.0 %, respectively, above the control group. In furtherance, the difference had changed within the range from 3.5 to 11.0 %. By the end of tests on chickens from group I, erythrocytes, hemoglobin, and hematocrit indicators were higher than in control and in group II by 6.27 ($P \leq 0.05$) and 19.4 % ($P \leq 0.001$), 5.21 and 12.6 %, 8.66 ($P \leq 0.05$) and 3.4 %. Similar effect of copper UFPs on hemoglobin and erythrocyte concentration is described earlier [37, 38].

Studying of effects from introduction into the chicken's ration of various microelements demonstrates their effect on blood morphology and leucogram in the context of stimulation of oxidation-reduction processes, which, in its turn, promoted more intensive metabolism [39-41]. In our research, quantity of white blood cells in broiler chickens from all groups was within the physiological limits. Indicators of chickens aged 28 days from group I moved towards the upper limit norm (within 2 weeks after commencement of the research), provided 14.7 % difference from the control. Upon introduction of asparaginate mixture, similar result (12.6 % difference with control) was noted only in chickens aged 35 days, i.e. 3 weeks following commencement of the research. Growth of leucocyte population in groups I and II had occurred mainly due to 67.0 % and 15.2 % difference in lymphocytes as compared to control). Effect of growth of the number of white blood cells under the effect of ultrafine medicine is not described here for the first time. Earlier, V.B. Borisevich and V.G. Kaplunenko [42] have identified moderate leucocytosis and strengthening of phagocytic activity in leucocyte cells of broilers due to dietary copper UFPs and mixture of nano-aquachelates of Ag, Cu, Zn, Mg, Co.

Introduction of various forms of copper and zinc sources had resulted in different changes in biochemical blood indicators of broiler chickens (Tables 3).

Total blood protein tends to increase as compared to control groups from day 28 to day 35 at feeding with UFPs. By the end of test (day 42) statistically significant difference from the control is 11.2 % ($P \leq 0.05$), which presupposes positive effect of the additive on protein metabolism. The same is con-

firmed by increase of urea indicators as compared to control during the entire research. No critical changes in creatinine appear that confirms the lack of nephrotoxic action. Blood concentration of glucose in chickens of group I exceeded control values with maximum differences of 39.1 % ($P \leq 0.05$, day 28) and 21.8 % ($P \leq 0.05$, day 42). Also, triglyceride concentration has a tendency towards growth (by 11.7-53.3 %, $P \leq 0.05$).

3. Age-specific dynamics of blood biochemical indicators in cross Smena 7 broiler chickens depending on chemical form of Cu and Zn used for microelement-based regulation of diet (M±SEM, $n = 6$, testing in vivarium conditions)

Group	Age, days			
	21	28	35	42
	Alanine aminotransferase, IU/l			
I	1.07±0.064*	2.83±0.129**	2.07±0.178	4.77±0.296**
II	1.87±0.069*	3.17±0.437*	2.80±0.289	2.37±0.110
Control	3.35±0.150	1.30±0.189	1.97±0.198	2.83±0.189
	Aspartate aminotransferase, IU/l			
I	281.0±11.40	232.7±9.60	261.1±13.10	353.5±9.40
II	231.0±9.60	244.6±18.10	221.9±12.70	272.0±8.70
Control	252.2±10.80	225.9±6.00	241.4±10.60	299.9±13.40
	Lactate dehydrogenase, IU/l			
I	3098.3±36.50*	2693.3±121.40	3104.3±25.00**	2008.0±24.10**
II	3316.3±200.40	2992.3±462.30	2852.0±111.50	2801.3±11.30*
Control	3851.0±54.00	2512.0±71.10	2444.3±15.80	3252.0±33.80
	γ-Glutamyle transferase, IU/l			
I	13.33±0.882**	19.67±1.764	18.67±0.333*	22.67±1.480
II	19.00±1.155*	17.00±0.142	22.33±1.202	21.67±0.333
Control	28.50±1.500	14.67±0.882	20.00±1.646	19.33±1.404
	Creatinine, μmol/l			
I	15.9±1.34	19.7±1.34	16.1±1.12	16.9±0.79
II	16.5±0.72	17.2±0.38	15.9±1.39	17.2±1.28
Control	24.9±1.65	16.3±1.39	17.8±0.67	16.3±1.83
	Glucose, mmol/l			
I	10.9±0.77*	10.3±0.37*	9.9±0.53	10.8±0.13**
II	9.3±0.64	7.9±0.60	8.9±0.01*	5.9±0.43
Control	6.1±0.74	7.4±0.85	9.8±0.82	8.9±0.65
	Total protein, g/l			
I	30.6±2.75	33.3±0.48*	31.4±1.33	33.1±1.49*
II	33.3±1.22	32.8±0.24*	32.6±0.37	28.2±2.09
Control	30.9±1.14	29.4±0.85	30.1±1.85	29.8±1.17
	Cholesterole, mmol/l			
I	4.5±0.08*	4.3±0.07	2.9±0.01**	2.1±0.11**
II	4.3±0.39	4.1±0.25	2.0±0.01	2.2±0.19*
Control	4.3±0.52	4.3±0.18	2.1±0.01	1.2±0.06
	Triglicerides, mmol/l			
I	0.51±0.03	0.16±0.029	0.23±0.036*	0.19±0.021
II	0.36±0.08	0.18±0.069	0.27±0.028*	0.10±0.024
Control	0.65±0.07	0.19±0.023	0.15±0.038	0.17±0.007
	Urea, mmol/l			
I	1.07±0.09	1.00±0.058	1.00±0.058	1.10±0.000*
II	1.37±0.07	1.07±0.033	0.93±0.088	0.97±0.033
Control	1.55±0.35	0.93±0.033	0.87±0.176	0.93±0.033

Note. See description of groups in section "Methodology".
*, ** Differences from control are statistically significant at $P \leq .0.05$ and $P \leq 0.01$.

Replacements of copper mineral salt with UFP and asparaginate were followed by 51.6 % ($P \leq 0.01$) and 13.2 % growth in Cu pool in chickens aged 42 days (Fig. 2). Zinc pool decreased by 22.9 % in group II, but exceeded control values by 12.5 % ($P \leq 0.05$) in group I. Possibly, for whatever reasons biological availability of copper from asparaginate (as compared to zinc) was higher. In such a case, Cu and Zn antagonism will result in reduced digestion of zinc. For the same reason, use of separate copper- and zinc-based diets is successful [43].

Elemental status analysis in the tested poultry indicates different effects of UFP and asparaginates on chemical metabolism. This clearly follows from comparison of pools of metals. Total concentrations of Zn, Pb, Cd, Ni, Al, and Sn decrease in chickens of group II (see Fig. 2). The same is true with Ni, Al,

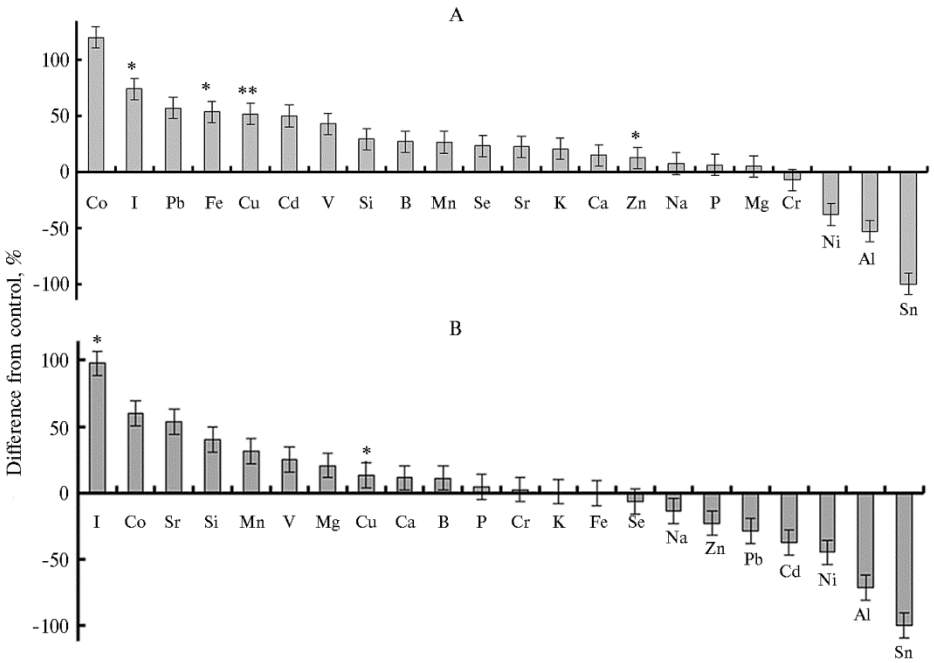

Fig. 2. Difference in pools of chemical element in cross Smena 7 broiler chickens aged 42 day from groups I (A) **and II** (B) **depending on formula of dietary Cu- and Zn-based additives.** See description of groups in section "Methodology". Star means that differences from control are statistically significant at $P \leq 0.05$.

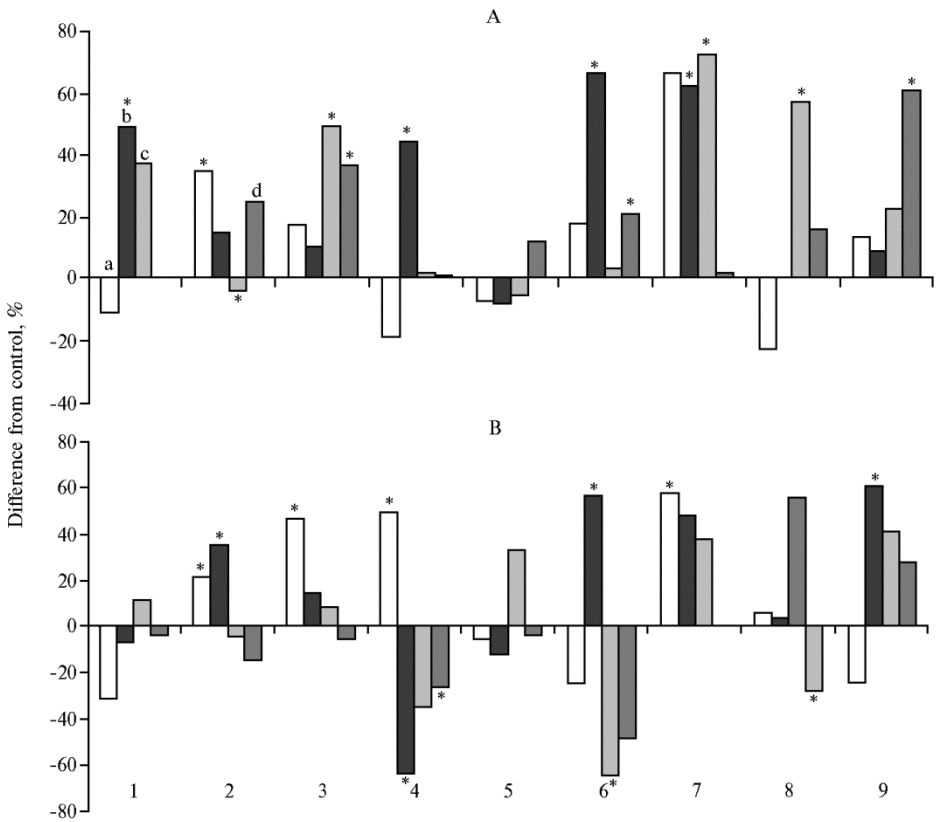

Fig. 3. Difference in accumulation of Cu as compared to control in cross Smena 7 broiler chickens from groups I (A) **and II** (B) **depending on formula of dietary Cu- and Zn-based additives:** 1 — hearth, 2 — brain, 3 — liver, 4 — spleen, 5 — kidneys, 6 — muscles, 7 — bursa of Fabricius, 8 —

thymus, 9 — feather; a, b, c, d — age of 21, 28, 35 and 42 days, respectively. See description of groups in section "Methodology". Star means that differences from control are statistically significant at $P \leq 0.05$.

and Sn in group I, while Pb and Cd levels rise. We explain this fact by competition for common transporters of copper, zinc, and other bivalent metals in intestines [44, 45]. Nanoparticles of metals, due to high penetration ability, may enter intestine cells not linking up with transportation proteins [46]. Then, transportation systems defining transfer of Zn and Cu could be used by other bivalent analogues (Pb, Cd) in group I but are more specific in group II.

Different mechanisms of entering and use of metals from UFPs and asparaginates is also supported by concentration dynamics of such elements in specific tissues and organs (Fig. 3).

Provision of Cu asparaginate during week 1 decreased Cu accumulation in feather by 24.5 % compared to control, however, afterwards, Cu excess over control reached 60.9 % after day 14, 41.4 % after day 21, and 28.1 % after day 28. In group I, this indicator during the entire research was higher than control values by 13.5, 8.8, 22.3 and 60.9 %. Feather is a marker biosubstrate to assess mineral status in poultry (Patent of the Russian Federation RU2478956C1). To our opinion, data we report in this paper show permanent change in availability of microelements in growing chickens. UFP-based diet results in more even entering copper to organism.

Therefore, comparison of Cu- and Zn-based dietary additives in the preparation forms of mineral salts, asparaginates, and ultrafine particles indicates that UFP-based forms increase biological availability of the microelements and has more expressed positive effect on productive properties of broiler chickens.

REFERENCES

1. Roco M.M. The long view of nanotechnology development: the national nanotechnology initiative at 10 years. In: *Nanotechnology research directions for societal needs in 2020. Science Policy Reports, V. 1.* Springer, Dordrecht, 2011: 1-28 (doi: 10.1007/978-94-007-1168-6_1).
2. Hooley G., Piercy N.F., Nicoulaud B. *Marketing strategy and competitive positioning.* London, 2012.
3. Makarov D.V. *Vestnik KRAUNTS. Fiziko-matematicheskie nauki*, 2014, 1(8): 97-102 (in Russ.).
4. Wang L., Hu C., Shao L. The antimicrobial activity of nanoparticles: present situation and prospects for the future. *Int. J. Nanomed.*, 2017, 12: 1227-1249 (doi: 10.2147/IJN.S121956).
5. Wahajuddin, Arora S. Superparamagnetic iron oxide nanoparticles: magnetic nanoplatforms as drug carriers. *Int. J. Nanomed.*, 2012, 7: 3445-3471 (doi: 10.2147/IJN.S30320).
6. Chatterjee D.K., Diagaradjane P., Krishnan S. Nanoparticle-mediated hyperthermia in cancer therapy. *Ther. Deliv.*, 2011, 2(8): 1001-1014.
7. Prasad R., Bhattacharyya A., Nguyen Q.D. Nanotechnology in sustainable agriculture: recent developments, challenges, and perspectives. *Front. Microbiol.*, 2017, 8: 1014 (doi: 10.3389/fmicb.2017.01014).
8. Mishra S., Keswani C., Abhilash P.C., Fraceto L.F. and Singh H.B. Integrated approach of agri-nanotechnology: challenges and future trends. *Front. Plant Sci.*, 2017, 8: 471 (doi: 10.3389/fpls.2017.00471).
9. Sekhon B.S. Nanotechnology in agri-food production: an overview. *Nanotechnology, Science and Applications*, 2014, 7: 31-53 (doi: 10.2147/NSA.S39406).
10. Bumbudsanpharoke N., Ko S. Nano-food packaging: an overview of market, migration research, and safety regulations. *J. Food Sci.*, 2015, 80: 910-923 (doi: 10.1111/1750-3841.12861).
11. Sabourin V., Ayande A. Commercial opportunities and market demand for nanotechnologies in agribusiness sector. *Journal of Technology Management & Innovation*, 2015, 10: 40-51 (doi: 10.4067/S0718-27242015000100004).
12. Zhang J., Spallholz J. Toxicity of selenium compounds and nano-selenium particles. In: *Handbook of systems toxicology.* D. Casciano, S.C. Sahu (eds.). John Wiley and Sons, West Sussex, UK, 2011: 787-801.
13. Zhang J. Biological properties of red elemental selenium at nano size (Nano-Se) in vitro and in vivo. In: *Nanotoxicity: from in vivo and in vitro model to health risks.* S.C. Sahu, D. Casciano (eds.). John Wiley and Sons, West Sussex, UK, 2009: 97-114.
14. Glushchenko N.N., Bogoslovskaya O.A., Baitukalov T.A., Ol'khovskaya I.P. *Mikroelementy v*

meditsine, 2008, 9(1-2): 52 (in Russ.).
15. Mishra B., Patel B.B., Tiwari S. Colloidal nanocarriers: a review on formulation technology, types and applications toward targeted drug delivery. *Nanomedicine*, 2010, 6: 9-24 (doi: 10.1016/j.nano.2009.04.008).
16. Tang H.Q., Xu M., Rong Q., Jin R.W., Liu Q.J., Li Y.L. The effect of ZnO nanoparticles on liver function in rats. *International Journal of Nanomedicine*, 2016, 31(11): 4275-4285 (doi: 10.2147/IJN.S109031).
17. Kowalczyk M., Banach M., Rysz J. Ferumoxytol: a new era of iron deficiency anemia treatment for patients with chronic kidney disease. *J. Nephrol.*, 2011, 24(6): 717-722 (doi: 10.5301/jn.5000025).
18. Weinstein J.S., Varallyay C.G., Dosa E., Gahramanov S., Hamilton B., Rooney W.D., Muldoon L.L., Neuwelt E.A. Superparamagnetic iron oxide nanoparticles: diagnostic magnetic resonance imaging and potential therapeutic applications in neurooncology and central nervous system inflammatory pathologies, a review. *J. Cereb. Blood Flow Metab.*, 2010, 30: 15-35 (doi: 10.1038/jcbfm.2009.192).
19. Zhou X., Wang Y. Influence of dietary nano elemental selenium on growth performance, tissue selenium distribution, meat quality, and glutathione peroxidase activity in Guangxi Yellow chicken. *Poultry Sci.*, 2011, 90(3): 680-686 (doi: 10.3382/ps.2010-00977).
20. Nikonov I.N., Laptev G.Y., Folmanis Y.G., Folmanis G.E., Kovalenko L.V., Egorov I.A., Fisinin V.I., Tananaev I.G. Iron nanoparticles as a food additive for poultry. *Dokl. Biol. Sci.*, 2011, 1: 328-331 (doi: 10.1134/S0012496611050188).
21. Zha L.Y., Zeng J.W., Chu X.W., Mao L.M., Luo H.J. Efficacy of trivalent chromium on growth performance, carcass characteristics and tissue chromium in heat-stressed broiler chicks. *J. Sci. Food Agric.*, 2009, 89: 1782-1786 (doi: 10.1002/jsfa.3656).
22. Yong Z., Lan L., Peng-Fei Z., Xin-Qi L., Wei-Dong Z., Zhao-Peng D., Shi-Wen W., Wei S., Ling-Jiang M., Zhi-Hui H. Regulation of egg quality and lipids metabolism by zinc oxide nanoparticles. *Poultry Sci.*, 2016, 95(4): 920-933 (doi: 10.3382/ps/pev436).
23. Ognik K., Stępniowska A., Cholewińska E., Kozłowski K. The effect of administration of copper nanoparticles to chickens in drinking water on estimated intestinal absorption of iron, zinc, and calcium. *Poultry Sci.*, 2016, 95(9): 2045-2051 (doi: 10.3382/ps/pew200).
24. Miroshnikova E., Arinzhanov A., Kilyakova Y., Sizova E., Miroshnikov S. Antagonist metal alloy nanoparticles of iron and cobalt: impact on trace element metabolism in carp and chicken. *HVM Bioflux*, 2015, 7(4): 253-259.
25. Goyer R.A. Toxic and essential metal interactions. *Annu. Rev. Nutr.*, 1997, 17: 37-50 (doi: 10.1146/annurev.nutr.17.1.37).
26. Kelleher S.L., Lönnerdal B. Zinc supplementation reduces iron absorption through age-dependent changes in small intestine iron transporter expression in suckling rat pups. *J. Nutr.*, 2006, 136(5): 1185-1191.
27. Hossain M.B., Kelleher S.L., Lönnerdal B. Maternal iron and zinc supplementation during pregnancy affects body weight and iron status in rat pups at weaning. *J. Nutr.*, 2011, 141(5): 798-804 (doi: 10.3945/jn.110.135681).
28. Oberleas D., Harland B.F. Treatment of zinc deficiency without zinc fortification. *Journal of Zhejiang University SCIENCE B*, 2008, 9(3): 192-126. (doi: 10.1631/jzus.B0710632).
29. Xin W., Xugang S., Xie C., Li J., Hu J., Yin Y.L., Deng Z.Y. The acute and chronic effects of monosodium L-glutamate on serum iron and total iron-binding capacity in the jugular artery and vein of pigs. *Biol. Trace Elem. Res.*, 2013, 153(1-3): 191-195 (doi: 10.1007/s12011-013-9668-x).
30. Hurrell R., Egli I. Iron bioavailability and dietary reference values. *Am. J. Clin. Nutr.*, 2010, 91(5): 1461S-1467S (doi: 10.3945/ajcn.2010.28674F).
31. Kudrin A.V., Skal'nyi A.V., Zhavoronkov A.A., Skal'naya M.G., Gromova O.A. *Immunofarmakologiya mikroelementov* [Immunopharmacology of microelements]. Moscow, 2000 (in Russ.).
32. Huang R.L., Yin Y.L., Wu G.Y., Zhang Y.G., Li T.J., Li L.L., Li M.X., Tang Z.R., Zhang J., Wang B., He J.H., Nie X.Z. Effect of dietary oligochitosan supplementation on ileal digestibility of nutrients and performance in broilers. *Poultry Sci.*, 2005, 84(9): 1383-1388.
33. Fisinin V.I., Egorov I.A., Lenkova T.N., Okolelova T.M., Ignatova G.V., Shevyakov A.N., Panin I.G., Grechishnikov V.V., Vetrov P.A., Afanas'ev V.A., Ponomarenko Yu.A. *Metodicheskie ukazaniya po optimizatsii retseptov kombikormov dlya sel'skokhozyaistvennoi ptitsy* [Guidelines for the optimization of animal feed recipes for poultry]. Moscow, 2009 (in Russ.).
34. Nikonov I.N., Folmanis Yu.G., Folmanis G.E., Kovalenko L.V., Laptev G.Yu., Egorov I.A., Fisinin V.I., Tananaev I.G. *Doklady Akademii nauk*, 2011, 440(4): 565-569 (in Russ.).
35. Il'ichev E., Nazarova A., Polishchuk S., Inozemtsev V. *Molochnoe i myasnoe skotovodstvo*, 2011, 5: 27-29 (in Russ.).
36. Yausheva E., Miroshnikov S., Sizova E., Miroshnikova E., Levahin V. Comparative assessment of effect of cooper nano and microparticles in chicken. *Oriental Journal of Chemistry*, 2015, 31(4): 2327-2336 (doi: 10.13005/ojc/310461).
37. Vishnyakov A.I., Ushakov A.S., Lebedev S.V. *Vestnik myasnogo skotovodstva*, 2011, 2(54): 96-

102 (in Russ.).
38. Ghahnavieh M.Z., Ajdary M., Naghsh N. Effects of intraperitoneal injection of gold nanoparticles in male mice. *Nanomed. J.*, 2014, 1(3): 121-127.
39. Shatskikh E.V. *Agrarnyi vestnik Urala*, 2008, 11(53): 83-84 (in Russ.).
40. Skorkina M.Yu., Fedorova M.Z., Sladkova E.A., Derkachev R.V., Zabinyakov N.A. *Yaroslavskii pedagogicheskii universitet*, 2010, 2: 101-106 (in Russ.).
41. Yausheva E.V., Miroshnikov S.A., Kvan O.V. *Vestnik Orlovskogo gosudarstvennogo universiteta*, 2013, 12(161): 203-207 (in Russ.).
42. Borisevich V.B., Kaplunenko V.G. *Nanomaterialy i nanotekhnologii v veterinarnoi praktike* [Nanomaterials and nanotechnologies in veterinary practice]. Kiev, 2012: 512 (in Russ.).
43. Hind T., Honnerdal B., Stenlund H., Gamayanti I., Ismail D., Seswandhana R., Persson L.A. A community based randomized controlled trial of iron and zinc supplementation in Indonesian infants: effects on growth and development. *Am. J. Clin. Nutr.*, 2004, 80: 729-736 (doi: 10.1093/ajcn/80.3.729).
44. Watts D.L. The nutritional relationships of Iron. *J. Orthomol. Med.,* 1988, 3(3): 110-116.
45. Ranganathan P.N., Lu Y., Jiang L., Kim C., Collins J.F. Serum ceruloplasmin protein expression and activity increases in iron-deficient rats and is further enhanced by higher dietary copper intake. *Blood*, 2011, 118(11): 3146-3153.
46. Bárány E., Bergdahl I.A., Bratteby L.-E., Lundh T., Samuelson G., Skerfving S., Oskarsson A. Iron status influences trace element levels in human blood and serum. *Environ. Res.*, 2005, 98(2): 215-223.

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PHENOTYPIC, BIOCHEMICAL AND MOLECULAR ANALYSIS OF *Bacillus anthracis* STRAINS ISOLATED DURING THE OUTBREAKS OF ANTHRAX IN THE RUSSIAN FEDERATION, 2014-2016

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Abstract

In 2014 to 2016, despite effective measures to prevent an introduction and transmission of Anthrax in the Russian Federation, there were seven outbreaks of Anthrax in Volgograd, Rostov, Belgorod, Saratov regions, the Republic of Tatarstan, and also six outbreaks in reindeer population in two districts of Yamal-Nenets Autonomous Okrug where 2657 reindeers died. In this article we present some results of comprehensive characterization of genetic, biological features and phylogenetic relationship of *Bacillus anthracis* strains isolated during the outbreaks in Volgograd region, Yamal-Nenets Autonomous Okrug and from the soils of burial in Chuvash Republic during last 3 years. Here, we differentiated 11 strains as followed from growth morphology, mobility, Gram stain procedure, capsule in vivo and in vitro formation, sporulation, proteolytic, hemolytic, lecithinase, phosphatase, glycolytic activity, protocatechuic acid production, Congo red sorption from the medium, phage sensitivity, toxicity in vitro, plasmid profile, sensitivity to antibiotics recommended for use in veterinary medicine, virulence for mice. MLVA-typing of the anthrax strains was performed for 20 VNTR loci. It was shown that the main phenotypic and diagnostic features of anthrax strains differed insignificantly and, in general, corresponded to those of a typical *B. anthracis* strain. The most significant phenotypic differences were found in asporogenous and avirulent strain *B. anthracis* № 6017 isolated in 2016 from a Lappish reindeer dog. The *B. anthracis* strains isolated during one outbreak were grouped into separate clusters, and within the cluster some strains had insignificant differences in 1-2 loci. The strains isolated from the soils of burials in the Republic of Chuvashia and from the Lappish reindeer dog during the Yamal outbreak formed separate clusters. *B. anthracis* strains showed high epizootic risk due to pathogenicity factors expressed in vitro. The tests identified the presence of capsula and toxins, high hemolytic and proteolytic activity, protocatechuic acid synthesis, and high virulence for laboratory mice (at 6-1000 spores). These results confirm the necessity of continuous monitoring and evaluation of epizootic caution of anthrax burials and case sites (frost fields), and specific preventive anti-anthrax measures.

Keywords: *Bacillus anthracis*, anthrax, strains, phenotypic properties, genotypic properties, virulence, MLVA

Single cases of anthrax in animals are annually registered in the Russian Federation, except for 2016, despite effective and quite wide measures to prevent this infection [1-4]. According to the information of analytic center of the Federal State Veterinary and Phytosanitary Surveillance (http://www.fsvps.ru/fsvps/iac/mes-

sages), seven anthrax foci were in 2014-2016 in Volgograd, Rostov, Belgorod, Saratov regions, Republic of Tatarstan, and six outbreaks were in reindeer populations at the territory of two regions of Yamal-Nenets Autonomous District (in the latter case, 2657 deer died).

Identification of new infectious strains during monitoring of animal and human diseases considering phenotype, biochemical and molecular-genetic characterization of isolates gives knowledge of spreading clones of the disease agent and their origin [5-8]. Biological properties characterize immunological identity of bacterial strains circulating in animals and vaccine strains, diagnostic features of bacterial pathogens, and the ways for emergence of resistance to medicines and biocides of various chemical classes among pathogens [9-12]. These are basic for epidemiological and epizootic passports reflecting the degree of epidemiological/epizootic danger of strains and must be used to improve effectiveness of specific and nonspecific prevention and eradication of animal and human infections [13, 14].

In present paper we have described properties and phylogenetic relations between the isolates of anthrax agents identified in the burial soil and in dead animals during disease outbreaks within Russia in 2014-2016, including reindeer epizooty which was the largest for the few past decades.

Purpose of this research is comprehensive characterization and certification of the anthrax strains isolated in the Russian Federation.

Techniques. Strains (11 *Bacillus anthracis* cultures) were isolated in 2014-2016 from bovine animals, Lappish reindeer dog, and reindeer, as well as from burial soils during the anthrax outbreaks in Volgograd Region and Yamal-Nenets Autonomous District, including burial soils in the Chuvash Republic. All strains are deposited in the State Collection of Microorganisms causing dangerous and extremely dangerous animal diseases, including zooanthroponoses and diseases not found in Russia (State Collection of Microorganisms, Federal Research Center of Virology and Microbiology (GKM-FICViM)).

Diagnostic traits were studied according to methodological guidelines MUK 4.2.2413-08 (Moscow, 2009). Phenotypes associated with pathogenicity (proteolytic, hemolytic, lecithinase, phosphatase, glycolytic activities, synthesis of protocatechuic acid, absorption of Congo Red dye from the medium) were identified according to recommendations [15].

Growth and differential media were medium for anthrax microbe isolation and culture (State Research Center of Applied Microbiology and Biotechnology, Russia); nutritious semi-liquid agar (BioCompas LLC, Russia); Hottinger agar based medium for determination of capsule and toxin formation on (RF Patent No 2204607); casein agar [16]; medium based on 10 % emulsion of chicken yolk in physiological solution; L-agar with 25 μg/ml Congo Red; two-layer blood agar (RF Patent No 2238316); Mueller Hinton Agar (HiMedia Laboratories Pvt. Ltd, India). Spores of anthrax cultures were produced on potato agar according to methodological guidelines MU 3.5.2435-09 (Moscow, 2009) and stored in 30 % glycerol.

Sensitivity of *B. anthracis* strains to antibacterial agents was assessed according to MUK 4.2.1890-04 (Moscow, 2004) using Mueller Hinton Agar and disks for veterinary laboratories (Scientific Research Center of Pharmacology, Saint Petersburg).

Capsule and toxin formation of *B. anthracis* strains in vitro was assessed by presence of mucous colonies and precipitation under 10 % CO_2 in air. For in vivo identification of capsule, 2 mice of 18-20 g in weight were intraperitoneally infected with 0.5 $cm^3$1-day broth culture of each strain. In case the mice did not

died within 10 days they were subjected to CO_2 euthanasia. To confirm capsule formation, imprint smears were made from mucous colonies and organs of dead mice. Preparations were fixed by alcohol:ester mixture (1:1) during 30 minutes and stained with methylene blue by Loeffler or Romanovsky-Giemsa according to guidelines for use of dyes. Preparations were examined under microscope at magnification of ×900. Rose color capsules around cells testified production of the capsular polypeptide. Test-system for Bacillus and allied species Microgen® Bacillus-ID (MID-66) (Microgen Bioproducts, United Kingdom) was used to identify biochemically the isolates.

LD_{100} and LD_{50} of the strains were assessed in mouse virulence tests using clinically healthy white outbreeds (18-20 g mice of both sexes). Animal feeding and keeping were subject to the accepted regulations. Animals were used according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986). For each isolate tested, 5 groups of 6 mice per each were subcutaneously injected with 0.5 cm^3 of sporous suspension (2×10^8, 4×10^7, 8×10^6, 1.6×10^6, and 3.2×10^5 spores/cm^3). Survival and death of mice were recorded during 10 days. LD_{50} values were calculated by Kerber formula modified by I.P. Ashmarin and A.A. Vorobyev [17]. Minimum number of spores causing 100 % death of mice was referred to as LD_{100}.

DNA-sorb-S.M variant 50 (Central Research Institute of Epidemiology, Moscow) was used for total (chromosome and plasmid) DNA extraction.

Test system AmpliSens® Bacillus anthracis-FRT (Central Research Institute of Epidemiology, Moscow) was used in PCR to identify genetic determinants of capsular polypeptide and toxin, the main pathogenies factors. Multilocus variable number tandem repeat analysis (MLVA) was carried out for 20 chromosomal and plasmid VNTR (variable number tandem repeat) loci with PCR primers [18] of a reagent kit for genetic typing anthrax strains by fragment analysis (OM-Anthrax-Genotype, Sintol LLC, Moscow). PCR and MLVA protocols were as per guidelines for test systems (an amplifier C1000 Touch Thermal Cycler with module for PCRq CFX96 Real-Time System, Bio-Rad, USA). At performance of MLVA [18], sequencing of each locus was done in an 8-capillary automated genetic analyzer NANOFOR 05 (Trial Plant of Science Instrument Engineering RAS, Chernogolovka).

UPGMA was used to plot MLVA data based dendrogram.

Results. Geographic origin of the studied isolates are given in Table 1.

1. Strains of *Bacillus anthracis* isolated in Russia 2014-2016

Strain	Inventory No.	Origin
		Volgograd Region
		(substracted in Volgograd Regional Veterinary Laboratory, 2014)
6246	370	Bovine spleen
3158/317-318	371	Soil
3184/410	372	Biomaterial (Dubtsovsky District)
		Chuvash Republic
		(substracted in Chuvash Republican Veterinary Laboratory, 2016)
5833	373	Burial soil
		Yamal-Nenets Autonomous District
		(substracted in All-Russia R&D Institute of Veterinary Virusology and Microbiology at Russian Academy of Agricultural Sciences, 2016)
5875	374	Reindeer ear
5885	375	Reindeer ear
5886	376	Reindeer ear
6017	377	Discharges from nose of Lappish reindeer dog
6019	378	Reindeer ear
6063	379	Reindeer ear
6064	380	Reindeer ear

2. Manifestation of phenotype, serological, and biological properties associated with pathogenicity in strains of anthrax agents *Bacillus anthracis* substracted at the Russian territory in 2014-2016

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
6246	T	R	–	+	+	+	+	+	+	–	+++	+	+++	α	+	±	+
3158/317-318	T	R	–	+	+	+	+	+	+	–	+++	+	+++	α	+	±	+
3184/410	T	R	–	+	+	+	+	+	+	–	+++	+	+++	α	+	±	+
5875	T	R	–	+	+	+	+	+	+	–	+++	–	+++	α	+	+	+
5885	T	R	–	+	+	+	–	+	+	–	+++	–	+++	α	+	+	+
5886	T	R	–	+	+	+	–	+	+	–	+++	–	+++	α	+	+	+
6019	T	R	–	+	+	+	+	+	+	–	+++	–	+++	α	+	+	+
6063	T	R	–	+	+	+	–	+	+	–	+++	–	+++	α	+	+	+
6064	T	R	–	+	+	+	–	+	+	–	+++	–	+++	α	+	+	+
6017	T	R	–	–	+	–	–	–	–	–	+++	+	+++	α	+	+	+
5833	T	R	–	+	+	+	+	+	+	–	+++	+	+++	α	+	+	+

N o t e. 1 — cell morphology, 2 — colony morphology, 3 — phosphatase activity, 4 — spore formation, 5 — presence of plasmid pXO1, 6 — presence of plasmid pxo2, 7 — toxin production in vitro, 8 — capsulation in vitro, 9 — capsulation in vivo, 10 — lecithinase activity, 11 — protease expression, 12 — synthesis of protocatechuic acid, 13 — hemolysine expression, 14 — hemolysis type, 15 — absorption of Congo Red, 16 — lysis by phage Fah-VNIIVViM, 17 — lysis by phage RD-ph-6. T — standard morphology, R — R type colonies (rough); «+/–» — manifestation of the trait is positive/negative, «±» — weak sensitivity to phage.

3. Biochemical activity in strains of anthrax agents *Bacillus anthracis* substracted at the Russian territory 2014-2016

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
6246	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
3158/317-318	–	+	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
3184/410	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
5875	–	–	–	-	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
5885	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
5886	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	+	+
6019	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
6063	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
6064	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
6017	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
5833	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+

N o t e. 1, 2, 3, 4, 5, 6 — fermentation of arabinose, mannitol, ramnose, saccharose, adonitol, methyl-D-glucoside; 7 — production of indole, 8 — utilization of citrate; 9, 10, 11, 12, 13, 14 — fermentation of cellobiose, mannose, salicine, tregalose, galactose, inulin; 15 — production of ONPG (ortho-nitrophenyl-β-D-galactopyranoside), 16 — Voges-Proskauer test, 17, 18, 19, 20, 21, 22 — fermentation of inositol, raffinose, sorbitol, xilose, methyl-D-mannoside, melicitose; 23 — arginine decomposition, 24 — reduction of nitrates to nitrites; «+/–» — manifestation of the trait is positive/negative.

At studying of the main identification traits of *B. anthracis* strains, it was established that working collection was represented by 10 standard virulent cultures and one strain atypical for capsule and spore formation. Comprehensive assessment of strain properties shown insignificant differences in phenotype (growth morphology in liquid and on solid nutritious mediums, motility, Gram staining, capsulation in vivo and in vitro, spore formation), biochemical activity (proteolytic, hemolytic, lecithinase, phosphatase, glycolytic activities, synthesis of protocatechuic acid, Congo Red absorption), antigen properties (Ascoli's thero precipitation test), sensitivity to anthrax bacteriophages Fah-VNIIVViM and RD-ph-6, toxin production in vitro, plasmid profile (PCR detection of fragments of genes for capsule and toxin formation), sensitivity to antibiotics recommended for veterinary use (Tables 2, 3). Except for strain No 6017 from Lappish reindeer dog, all cultures have highly expressed hemolysine and proteases, produce capsule polypeptide in vitro (mucous colonies of S, M or SM type on medium for capsulation and toxin production) and in vivo (capsulated rods in cytological preparations from organs of died mice), cause α-type hemolysis of erythrocytes, and absorb Congo Red. Distinctive property of *B. anthracis* isolates from Yamal-Nenets Autonomous District is lack or weak production of protocate-

chuic acid. Strains were visually divided into producing toxin in vitro (No 6246, 3158/317-318, 3184/410, 5833, 5875, 6019) and non-producing (No 5885, 5886, 6017, 6063, 6064). Studied cultures also differed in sensitivity to anthrax phages: all were lysed by phage RD-ph-6, however strains No 6246, 3158/317-318 and 3184/410 were poorly sensitive to phage Fah-VNIIVViM.

Strains were divided into four groups (Table 4) subject to classification (19) for LD_{50} in outbreed white mice which is deemed the most objective value.

4. Virulence of *Bacillus anthracis* strains isolated in Russia during 2014-2016 in outbreed white mice

Strain	Indicator, spores per animal		Virulence assessment
	LD_{100}	LD_{50}	
6246	5.4×10^2	8	Highly virulent
3158/317-318	6	Not titered	Highly virulent
3184/410	1.2×10^2	5	Highly virulent
5833	30	6	Highly virulent
5875	20	5	Highly virulent
5885	1.1×10^3	7	Highly virulent
5886	1.7×10^2	12	Virulent
6017	Not titered	Not titered	Avirulent
6019	4.7×10^2	19	Moderately virulent
6063	150	10	Virulent
6064	60	21	Moderately virulent

Six of the studied strains are highly virulent, two are virulent, and two are moderately virulent. *B. anthracis* isolated from Lappish reindeer dog (No 6017) do not cause death in outbreed white mice and is referred to as avirulent. LD_{50} for the studied strains was of 5 to 37 spores per animal, LD_{100} of 6 to 2000 spores per animal. Determination of LD_{50} in strain No 3158/317-318 was impossible since total value of LD_{100} was only 6 spores per animal.

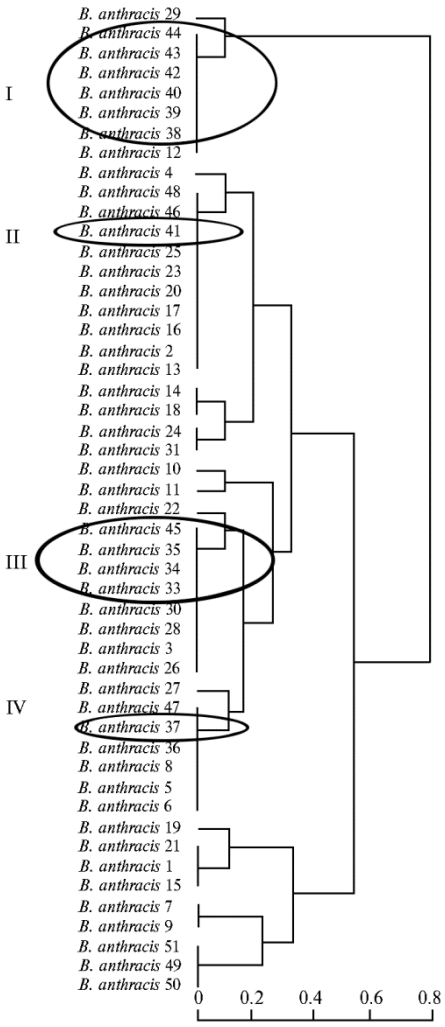
Nine of 11 studied strains are naturally resistant to polymyxin B, sensitive and highly sensitive to levomycetine, kanamycin, penicillin, tylosin, strepto-mycin, neomycin, tetracycline, ampicillin and enrofloxacin. Strain No 6246 is resistant to kanamycin and streptomycin (Table 5).

5. Antibiotic sensitivity of *Bacillus anthracis* strains isolated in Russia during 2014-2016

Strain	1	2	3	4	5	6	7	8	9	10
6246	S	S	MS	S	MS	MS	R	R	MS	R
3158/317-318	S	S	S	S	S	S	S	MS	S	R
3184/410	S	S	S	S	S	S	S	MS	S	R
5875	S	S	S	S	S	S	S	S	S	R
5885	S	S	S	S	S	MS	S	MS	S	R
5886	S	S	S	MS	MS	R	MS	MS	S	R
6019	S	S	R	S	MS	MS	MS	MS	S	R
6063	S	S	S	S	S	MS	S	MS	S	R
6064	S	MS	S	S	MS	MS	S	MS	S	R
6017	S	S	S	S	S	MS	S	S	S	R
5833	S	S	S	S	S	MS	S	S	S	R

Note. 1 — ampicillin, 2 — penicillin, 3 — neomycin, 4 — enrofloxacin, 5 — tylosin, 6 — levomycetine, 7 — streptomycin, 8 — kanamycin, 9 —tetracycline, 10 — polymixin B; R (resistance) — poorly sensitive; MS (medium sensitive) — medium sensitive; S (sensitive) — sensitive.

B. anthracis is one of the most genetically homogenous pathogens that challenges identification. Multilocus variable number tandem repeat analysis was used to tackle the problem. MVLA ensures PCR identification of bacterial DNA areas which are shortened or elongated during erroneously copying caused by slipping replication fork [20]. MLVA typing was performed for 20 VNTR loci at multiplex DNA amplification with estimation of lengths of fluorescently labeled products for each VNTR locus. Number of repeats in each locus was used to calculate the distances between strains (Fig.).



Dendrogram of phylogenetic relations of the studied *Bacillus anthracis* isolates (MLVA, UPGMA method)**:** cluster I — strains denoted as 38-40 are No 5875, 5885, 5886), 42-44 are No 6019, 6063, 6064); cluster II — strain denoted as 41 is No 6017); cluster III — strains denoted as 33-35 (are No 6264, 3158/317-318, 3184/410); cluster IV — strain denoted as 37 is No 5833 (State Collection of Microorganisms, Federal Research Center of Veterinary and Microbiology; see description of strains in Table 1).

MLVA data divide the strains into four clusters. Strains isolated from reindeer during outbreak of anthrax in Yamal-Nenets Autonomous District in 2016 form cluster I, one strain (No 6017, isolated from Lappish reindeer dog, Yamal-Nenets Autonomous District, 2016) is in cluster II, strains from Volgograd Region (2014) form cluster III, and cluster IV includes *B. anthracis* isolates from burial soils in Chuvash Republic (see Fig., Table 6).

This information confirms previously established facts that strains isolated from reindeer and humans in 2016 in Yamal-Nenets Autonomous District have one and the same MLVA genotype [21], and that different anthrax strains circulating in certain territories have geographically affiliated genotypes [22-25].

Significant differences are found in strains from clusters I and III by 1-2 loci inside cluster that may evidence on their subculture in sensitive animals.

6. MLVA-based genotyping of *Bacillus anthracis* strains isolated in Russia during 2014-2016 for 20 VNTR loci

Strain	1	2	3	4	5	6	7[a]	8	9	10	11	12[b]	13[b]	14	15	16[b]	17	18	19	20
Cluster III																				
6246	30	31	14	9	45	13	8[B]	20	13	57	78	4	20	5	20	14[B]	4	4	14	17
3158/317-318	30	31	14	9	45	13	10	20	13	57	78	4	20	5	20	12	4	4	14	17
3184/410	30	31	14	9	45	13	10	20	13	57	78	4	20	5	20	12	4	5	14	17
Cluster I																				
5875	27	27	15	10	45	14	9	16	14	53	17	3	21	3	23	9	3	5	14	15
5885	27	27	15	10	45	14	9	16	14	53	17	3	21	3	23	9	3	5	14	15
5886	27	27	15	10	45	14	9	16	14	53	17	3	21	3	23	9	3	5	14	15
6019	27	27	15	10	45	14	9	16	14	53	17	3	21	3	23	9	3	5	14	15
6063	27	27	15	10	45	14	9	16	14	53	17	3	21	3	23	9	3	5	14	15
6064	27	27	15	10	45	14	8[B]	16	14	53	17	3	21	3	23	9	3	5	14	15
Cluster II																				
6017	24	30	16	12	45	13	8	20	13	57	75	–	–	4	20	–	4	4	14	16
Cluster IV																				
5833	30	30	14	9	45	13	10	20	13	57	75	4	20	4	20	13	4	4	14	16

Note. VNTR — variable-number tandem-repeat; number of repeats in locus are indicated (from 1 to 45). Loci: 1 — Geb-Bams3, 2 — Geb-Bams13, 3 — Geb-Bams22, 4 — Geb-Bams23, 5 — Geb-Bams15, 6 — VNTR32, 7 — pXO1 aat, 8 — vrrC2, 9 — Geb-Bams1, 10 — vrrC1, 11 — Geb-Bams30, 12 — VNTR17, 13 — VNTR16, 14 — vrrA, 15 — vrrB1, 16 — CL33, 17 — VNTR23, 18 — VNTR35, 19 — vrrB2, 20 — CL12; [a] — locus located on plasmid pX01 *B. anthracis*, [b] — polymorphic loci located on plasmid pX02 *B. anthracis*; dashes mean that locus in not found.

Discovery in one anthrax outbreak (Yamal-Nenets Autonomous District, 2016) of the strains relating to two different clusters (I and II) allows assumption on presence of at least two sources of animal infection. However, this hypothesis requires further confirmation.

Thus, by main phenotypic properties and diagnostic features, anthrax strains isolated during 2014-2016 in three Russian Federation districts do not practically differ from the typical strain. Our findings indicate that these isolates belong to four MLVA20 genotypes having different geographic affiliation. The exception is *Bacillus anthracis* strain No 6017 (isolated from Lappish reindeer dog) on the position between strains from Volgograd Region and Chuvash Republic. The studied isolates possess high epizootic danger which is confirmed by the presence of pathogenic factors in vitro and high virulence in laboratory animals. This necessitates microbiological monitoring of anthrax burials and places of ani-mal death (together with improved preventive measures).

REFERENCES

1. *Ladnyi V.I., Yushchenko G.V.* Epidemiologiya i infektsionnye bolezni, *2009, 2: 36-40 (in Russ.).*
2. *Ryazanova A.G., Aksenova L.Yu., Buravtseva N.P., Golovinskaya T.M., Eremenko E.I., Tsygankova O.I., Varfolameeva N.G., Kulichenko A.N.* Problemy osobo opasnykh infektsii, *2016, 2: 24-27 (doi: 10.21055/0370-1069-2016-2-2427) (in Russ.).*
3. *Ryazanova A.G., Eremenko E.I., Aksenova L.Yu., Semenova O.V., Buravtseva N.P., Golovinskaya T.M., Kulichenko A.N.* Problemy osobo opasnykh infektsii, *2017, 1: 21-23 (doi: 10.21055/0370-1069-2017-1-21-23) (in Russ.).*
4. Dugarzhapova Z.F., Chesnokova M.V., Gol'dapel' E.G., Kosilko S.A., Balakhonov S.V. *Problemy osobo opasnykh infektsii*, 2017, 1: 59-64 (doi: 10.21055/0370-1069-2017-1-59-64) (in Russ.).
5. Lekota K.E., Hassim A., Mafofo J., Rees J., Muchadeyi F.C., Van Heerden H., Madoroba E. Polyphasic characterization of Bacillus species from anthrax outbreaks in animals from South Africa and Lesotho. *J. Infect. Dev. Ctries.*, 2016, 10(8): 814-823 (doi: 10.3855/jidc.7798).
6. Fouet A., Smith K.L., Keys C., Vaissaire J., Le Doujet C., Levy M., Mock M., Keim P. Diversity among French Bacillus anthracis isolates. *J. Clin. Microbiol.*, 2002, 40(12): 4732-4734 (doi: 10.1128/JCM.40.12.4732-4734.2002).
7. Kolton C.B., Podnecky N.L., Shadomy S.V., Gee J.E., Hoffmaster A.R. *Bacillus anthracis* gamma phage lysis among soil bacteria: an update on test specificity. *BMC Res. Notes*, 2017, 10(1): 598 (doi: 10.1186/s13104-017-2919-8).
8. Antonation K.S., Grützmacher K., Dupke S., Mabon P., Zimmermann F., Lankester F., Peller T., Feistner A., Todd A., Herbinger I., de Nys H.M., Muyembe-Tamfun J.-J., Karhemere S., Wittig R.M., Couacy-Hymann E., Grunow R., Calvignac-Spencer S., Corbett C.R., Klee S.R., Leendertz F.H. *Bacillus cereus* biovar *anthracis* causing anthrax in Sub-Saharan Africa — chromosomal monophyly and broad geographic distribution. *PLoS Neglected Tropical Diseases*, 2016, 10(9): e0004923 (doi: 10.1371/journal.pntd.0004923).
9. Saperkin N.V., Alebashina L.A., Kvashnina D.V. *Sovremennye problemy nauki i obrazovaniya*, 2016, 5. Available https://www.science-education.ru/ru/article/view?id=25429. Accessed December 24, 2017.
10. Tahoun A.B.M.B., Abou Elez R.M.M., Abdelfatah E.N., Elsohaby I., El-Gedawy A.A., Elmoslemany A.M. Listeria monocytogenes in raw milk, milking equipment and dairy workers: molecular characterization and antimicrobial resistance patterns. *J. Glob. Antimicrob. Resist.*, 2017, 10: 264-270 (doi: 10.1016/j.jgar.2017.07.008).
11. Morvan A., Moubareck C., Leclercq A., Hervé-Bazin M., Bremont S., Lecuit M., Courvalin P., Le Monnier A. Antimicrobial resistance of *Listeria monocytogenes* strains isolated from humans in France. *Antimicrob. Agents Chemother.*, 2010, 54(6): 2728-2731 (doi: 10.1128/AAC.01557-09).
12. Granier S.A., Moubarek C., Colaneri C., Roussel S., Courvalin P., Brisabois A. Antimicrobial susceptibility among *Listeria monocytogenes* isolates from non-human sources in France over a ten year period. *Lirvo de actas do congress ISOPOL XVII.* Porto, 2010: 63.
13. Somov G.P., Zaitseva E.A., Buzoleva L.S., Glazyrina T.A., Terekhova V.E. *Epidemiologicheskie i infektsionnye bolezni*, 2002, 1: 47-49 (in Russ.).
14. Egorova I.Yu., Sevskii T.A., Selyaninov YuO. Immunobiological properties of new unencaspsulated *Bacillus anthracis* 363/11 strain. *Appl. Biochem. Microbiol.*, 2016, 52(8): 733-738 (doi: 10.1134/S0003683816080044).
15. Smirnov A.M., Gulyukin M.I., Subbotin V.V., Kolbasov D.V., Donnik I.M., Skira V.N., Suvorov A.V., Babyshova L.V., Sribnyi N.I. V sbornike: *Novye metody issledovanii po problemam veterinarnoi meditsiny* [New research methods in veterinary medicine]. Moscow, 2008: 177-186 (in

Russ.).
16. Shevchenko O.V. *Proteoliticheskaya aktivnost' vozbuditelya sibirskoi yazvy. Kandidatskaya dissertatsiya* [Proteolytic activity of the anthrax causative agent. PhD Thesis]. Saratov, 1999 (in Russ.).
17. Ashmarin I.P., Vorob'ev A.A. *Statisticheskie metody v mikrobiologicheskikh issledovaniyakh* [Statistical methods in microbiological studies]. Leningrad, 1962 (in Russ.).
18. Lista F., Faggioni G., Valjevac S., Ciammaruconi A., Vaissaire J., Doujet C., Olivier G., De Santis R., Carattoli A., Ciervo A., Fasanella A., Orsini F., D'Amelio R., Pourcel C., Cassone A., Vergnaud G. Genotyping of *Bacillus anthracis* strains based on automated capillary 25-loci multiple locus variable-number tandem repeats analysis. *BMC Microbiol.*, 2006, 6: 33 (doi: 10.1186/1471-2180-6-33).
19. Shlyakhov E.N., Gruz E.V. V sbornike: *Dostizheniya i perspektivy bor'by s sibirskoi yazvoi v SSSR* [Achievements and prospects of combating anthrax in the USSR]. Moscow, 1978: 97-99 (in Russ.).
20. Keim P., Price L.B., Klevytska A.M., Smith K.L., Schupp J.M., Okinaka R., Jackson P.J., Hugh-Jones M.E. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. *J. Bacteriol.*, 2000, 182(10): 2928-2936 (doi: 10.1128/JB.182.10.2928-2936.2000).
21. Kulichenko A.N., Eremenko E.I., Ryazanova A.G., Aksenova L.Yu., Kovalev D.A., Pisarenko S.V., Varfolomeeva N.G., Zhirov A.M., Volynkina A.S., Buravtseva N.P., Golovinskaya T.M., Koteneva E.A., Tsygankova O.I., Dyatlov I.A., Timofeev V.S., Bogun A.G., Bakhteeva I.V., Kislichkina A.A., Mironova R.I., Titareva G.M., Skryabin Yu.P., Selyaninov Yu.O., Egorova I.Yu., Kolbasov D.V. *Problemy osobo opasnykh infektsii*, 2017, 1: 94-99 (doi: 10.21055/0370-1069-2017-1-94-99) (in Russ.).
22. Thierry S., Tourterel Ch., Le Flиche Ph., Derzelle S., Dekhil N., Mendy Ch., Colaneri C., Vergnaud G., Madani N. Genotyping of French *Bacillus anthracis* strains based on 31-loci multi locus VNTR analysis: epidemiology, marker evaluation, and update of the internet genotype database. *PLoS ONE*, 2014, 9(6): 95-131 (doi: 10.1371/journal.pone.0095131).
23. Fasanella A., Van Ert M., Altamura S.A., Garofolo G., Buonavoglia C., Leori G., Huynh L., Zanecki S., Keim P. Molecular diversity of *Bacillus anthracis* in Italy. *J. Clin. Microbiol.*, 2005, 43: 3398-340 (doi: 10.1128/JCM.43.7.3398-3401.2005).
24. Merabishvili M., Natidze M., Rigvava S., Brusetti L., Raddadi N., Borin S., Chanishvili N., Tediashvili M., Sharp R., Barbeschi M., Visca P., Daffonchio D. Diversity of *Bacillus anthracis* strains in Georgia and of vaccine strains from the former Soviet Union. *Appl. Environ. Microbiol.*, 2006, 72: 5631-5636 (doi: 10.1128/AEM.00440-06).
25. Antwerpen M., Ilin D., Georgieva E., Meyer H., Savov E., Frangoulidis D. MLVA and SNP analysis identified a unique genetic cluster in Bulgarian *Bacillus anthracis* strains. *Eur. J. Clin. Microbiol. Infect. Dis.*, 2011, 30(7): 923-930 (doi: 10.1007/s10096-011-1177-2).

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USE OF PROBIOTICS GIPROLAM AND SIMBITER-2 TO CORRECT THE VAGINA BIOCENOSIS IN DOWN-CALVING COWS

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Abstract

Postpartum endometritis prophylaxis in high producing dairy cows in the intensive animal husbandry is an urgent one because of great economic losses resulted from the productive function disorder, decreased performance and premature culling. Opportunistic pathogenic microflora and disbacteriosis may cause postpartum endometritis along with specific infections. Potentially dangerous pathogenic bacteria from cows with gynecological pathology contaminate the calves at birth, causing gastrointestinal diseases. Various pharmaceuticals and biologicals could be used to prevent postpartum endometritis, including those providing antioxidative effects, immunomodulatory activity, normalization of hormonal and metabolic status in cows' genitals and uterine involution. Antibiotics are in most common use, though their frequent application is accompanied by microecological disorders. Probiotics are considered as a perspective alternative to antibiotics for correcting genital microflora in calving cows. In the paper we report a study of the impact of probiotics Giprolam and Simbiter-2 on microflora of down-calving cows' birth canal in view to prevent postpartum endometritis and intestinal disorders in calves. Thirty-six red-and-white cows with milk yield of 5100-5400 kg for previous lactation were chosen. The cows of the group 1 (n = 12) and group 2 (n = 12) have received Giprolam and Simbiter-2, respectively, for 5-7 days prior to calving every 24 hours, 100 cm^3 intravaginally. The cows of the group 3 (n = 12) served as a control (no probiotics). Clinical observations were carried out in mother cows for 14 days after calving, and in the calves during colostrum period. Indigenous and opportunistic microflora was studied in the birth canal before and after calving, in colostrum and in large intestine in the calves. Bacteriologic examination of cervical mucus, reproductive tract discharge, colostrum, excrement, as well as cultural, morphological and biochemical study were performed traditionally. The efficacy of probiotic treatment has been stated. The experiments showed that Giprolam and Simbiter-2 in 71.4 % and 85.8 % cases, respectively, could effectively provide a physiological level of postpartum indigenous microflora, prevent colonization of the reproductive tract by opportunistic and pathogenic microflora and restrict postpartum endometritis. The mother cow treatment with Giprolam and Simbiter-2 could also prevent gastrointestinal diseases in 50.0 % and 41.7 % of the calves, respectively. The high potency of these probiotics is due to lactic acid bacteria capable of genital tract colonization, providing optimal indigenous microflora level and the resistance of the genital tract to harmful microflora.

Keywords: cows, microflora of maternal passages, microflora of colostrum, microflora of intestine in calves, probiotics, postpartum endometritis, prophylaxis

High incidence of acute postpartum endometritis in cows results in significant economic loss due to reproduction malfunction, milk production losses, and anticipated culling of animals [1-5]. Along with agents of infectious diseases [6-8], opportunistic pathogenic microbial flora and disbacteriosis manifested by persistent quantitative and qualitative changes in bacterial community of normal microbial flora plays an important role in the etiology and development of postpartum endometritis [9-11]. At gynecologic pathology in cows, the possible pathogenic bacteria may infect calves at birth causing gastrointestinal diseases [12].

Selenium medicines with antioxidant and immunomodulatory effect [13, 14], medicines from placenta normalizing postpartum hormonal-metabolic and involution processes in genitals [15, 16], preparations intensifying the uterine activity [17-19], and antimicrobial medicines [20-22] are recommended for use to prevent development of postpartum endometritis in cows. One of the main postpartum endometritis preventative measures is use of means eliminating inflammations [23, 24]. Use of antibacterial medicines during 3 days postpartum with preventative purpose does not have significant effect on vaginal microbiocenosis. At the same time, long-term treatment course decreases quantitative and qualitative characteristics of normoflora, increases pH of vaginal secret that prevents recovery of vaginal microbiocenosis postpartum and creates conditions for propagation of opportunistic pathogenic microbial flora [25, 26]. Number of lactobacillus and bifidobacterium is significantly decreased, and opportunistic pathogenic microbial flora increases in the genital tract postpartum causing postpartum purulent-septic diseases due to washing out of microorganisms from vagina by amniotic fluid and blood, traumatization of birth canal, and contamination of vagina by intestinal microflora [27, 28]. Recovery of protective microbial flora at disbiotic disorders without the use of biotherapeutic medicines is challenged as confirmed by disease recurrences [29-33].

Today, use of probiotics as the most essential competitors of pathogenic and opportunistic pathogenic microflora serves as an alternative to antimicrobial medicines [34-37]. However, their use in the first day postpartum does not always prevent occurrence and development of catarrhal endometritis that is due to disbiotic disorders in vaginal microbiotype before birth. In this regard, it is perspective to use probiotic medicines for correction of microbionenosis of birth canals in cows prior to calving.

In present study, we have for the first time shown the need for intravaginal use of probiotics to down-calving cows for prevention of postpartum endometritis that also promote optimization of intestinal microbiocenosis in newborn calves and their addiction to gastrointestinal diseases.

Purpose of this study is to learn corrective effect of Giprolam and Simbiter-2 probiotics on vaginal microbial flora in down-calving cows.

Techniques. Study was carried out in commercial dairy farm Vysokoye (EcoNivaAgro LLC, Liskinsky District, Voronezh Region) in 36 animals of red and pied breed with milk yield in the past lactation of 5100-5400 kg. During interlactation period cows were kept loose housing at deep litter. Groups were formed accounting for the expected calving term. Animals were moved to precalving section and, afterwards, to calving section during 10-15 days. Cows were divided into three groups. Within 5-7 days before calving, animals of trial group I (n = 12) were treated daily (with 24 hour interval) intravaginally by probiotic medicine Giprolam (Biotechagro LLC, Russia) by Janet's syringe and gynecologic pipette (100 cm^3, 5-7 injections). Multiprobiotic Simbiter-2 (Research Industrial Enterprise O.D. Prolisok, Ukraine) was injected intravaginally in cows of trial group II (n = 12) according to similar scheme [38]. Medicines were not provided to group III (control, n = 12).

Clinical surveillance was performed over cows within 14 days following calving and over calves in colostral period. State of birth canal microbiocenosis before and after calving, quantitative and qualitative bacterial composition of colostrum and large intestines in calves was assessed by quantity and frequency of occurrence of indigenous and opportunistic pathogenic microbial flora. Bacteriologic tests of cervical mucus, discharges from genital tracts, colostrum, excrements, studying of cultural and morphologic properties of identified microorganisms were carried out by commonly accepted methods [39].

Preventative effectiveness of probiotics was determined by formula:

$$E = 100 \times (B\text{-}A)/B,$$

where E stands for effectiveness, %; A and B are disease frequency amongst animals treated and not treated with medicine, % [40].

Obtained results are presented as mean values (M) and standard errors of the mean ($\pm m$).

Results. Giprolam medicine (registration № PVR 1-35.13/02987) is a suspension which contains viable strains of lactobacillus *Lactobacillus fermentum* 44/1 (Russian National Collection of Industrial Microorganisms B-2940) and *Lactococcus lactis* subsp. *lactis* 57_4 (Russian National Collection of Industrial Microorganisms B-3145), nor less than 1×10^8 CFU/cm^3, and additional substances (water, milk serum, glucose, and yeast extract). Lactobacillus strains are able to succeed in birth canals of cows and have antagonistic effect on opportunistic pathogenic microbial flora penetrating to womb. Multiprobiotic Simbiter-2 — is a multicomponent medicine for normalization of vaginal microflora in female organism. Medicine is based on the key protective microorganisms of urogenital tract, i.e. bacteria *Lactobacillus acidophilus*, *L. casei*, *L. plantarum*, *L. gasseri*, *L. brevis*, *Bifidobacterium bifidum*, *B. longum*, *B. breve*, *B. infantis*, *B. adolescentis*, *Propionibacterium freudenreichii* ssp. *shermanii*, *P. acidipropionici*. They actively ferment glycogen to organic acids, synthesize hydrogen peroxide, bacteriocine and lysozyme having high adhesive ability regarding epiteliocytes, produce vitamins and polysaccharides [38].

1. Microbial flora (lg CFU/cm^3) **of birth canals in red and pied cows at treatment by Giprolam and Simbiter-2 probiotics** ($M\pm m$)

Bacteria	Group I	Group II	Group III
Lactobacillus spp.	7.53±0.59*	7.64±0.83*	6.55±0.71*
	7.64±0.61*	7.58±0.10*	5.64±0.94*
Bifidobacterium spp.	7.64±0.72*	7.78±0.69*	6.50±0.46*
	7.75±0.86*	7.54±0.73*	5.47±0.68*
Corynebacterium spp.	2.25±0.02*	2.49±0.17*	2.32±0.19*
	2.20±0.01*	2.66±0.23*	3.51±0.41*
Staphylococcus saprophyticus	5.52±0.49*	4.61±0.16*	5.86±0.12*
	5.76±0.17*	4.55±0.21*	5.61±0.81*
Staphylococcus epidermidis	3.64±0.02*	4.62±0.61*	5.37±0.25*
	4.54±0.74*	5.59±0.32*	4.32±0.17*
Staphylococcus aureus	not isolated	not isolated	not isolated
	not isolated	not isolated	4.32±0.03 (25.0)
Streptococcus group C	4.50±0.16 (50.0)	4.57±0.69 (25.0)	5.70±0.45 (75.0)
	4.43±0.58 (50.0)	4.51±0.65 (33.3)	6.64±0.81 (75.0)
Enterococcus faecalis	not isolated	3.95±0.01 (16.7)	3.67±0.22 (25.0)
	2.63±0.01 (25.0)	3.49±0.01 (25.0)	4.35±0.85 (75.0)
Streptococcus spp. hemolytic	not isolated	2.56±0.86 (8.3)	5.68±0.38 (25.0)
	3.91±0.01 (25.0)	3.64±0.73 (8.3)	5.39±0.52 (75.0)
Enterobacter spp.	not isolated	2.77±0.85 (8.3)	not isolated
	not isolated	not isolated	4.49±0.02 (25.0)
Escherichia coli	2.51±0.89 (25.0)	3.46±0.25 (16.7)	4.52±0.74 (25.0)
	not isolated	not isolated	5.70±0.65 (50.0)

N o t e. See description of groups in section "Methodology". Figures in parenthesis show frequency of isolation, %; microorganisms marked by star were identified in 100 % of animals. Values prior calving and after calving are above and under bar, respectively.

Microflora of birth canals before administration of medicines (Table 1) and in control group within 5-7 days before calving did not significantly differ. *Lactobacillus*, bifidobacteria, *Corynebacterium* spp., *Staphylococcus saprophyticus* and *S. epidermidis* were found in all specimens, while *Streptococcus* group C and *Escherichia coli* were rare. Besides, hemolytic streptococci *Enterococcus faecalis* were found prior to administration of Simbiter-2 in group II and in control animals, and *Enterobacter* spp. — prior to treatment with the probiotic (see Table 1).

During the period before calving, due to Giprolam, positive dynamics in lactobacillus (growth by 1.5 %), bifidobacteria (by 1.4 %), *Staphylococcus saprophyti-*

cus (by 4.2 %), and epidermal staphylococcus (by 19.9 %) counts occurs in birth canal, with notable trend towards reduction of the number of streptococcus group C (by 1.6 %). In 25.0 % cases, *Enterococcus faecalis* and hemolytic streptococci but no *Escherichia* were isolated (see Table 1). Prior to administration of Simbiter-2, number of lactobacillus and bifidobacteria was optimal, number of epidermal staphylococci grew by 17.4 %, hemolytic streptococci grew by 29.7 %, and number of *Enterococcus faecalis* decreased by 11.7 %. *Escherichia* and *Enterobacter* spp. were not found (see Table 1).

After calving, abundance of lactobacillus (by 13.9%), bifidobacterium (by 15.9%) was reduced in the control group with increase of the number of opportunistic pathogenic microflora: streptococci group C — by 14.2 %, *Enterococcus faecalis* — by 15.7 %, Escherichia — by 20.7 %. Frequency of isolation of *Enterococcus faecalis*, hemolytic streptococci and *Escherichia* increased by 50.0; 50.0 and 25.0 %, receptively, besides, at frequency of 25.0 %, staphylococci aureus and *Enterobacter* spp. were isolated (see Table 1). In group I, number of lactobacilli and bifidobacteria was 26.2 and 29.4 % higher than in control, while abundance and frequency of streptococci group C was 33.3 and 25.0 % lower than in control, *Enterococcus faecalis* — by 39.6 and 50.0 %, hemolytic streptococci — by 27.5 and 50.0 % lower. *Staphylococci aureus*, *Escherichia* and *Enterobacter* spp. were not found. Number of lactobacilli in group II as compared to control was 25.6 % higher, of bifidobacteria —27.5 % higher, count of streptococci group C was 32.1 % lower, of *Enterococcus faecalis* — 32.5 % lower, hemolytic streptococci — 19.8 % lower. *Staphylococci aureus*, *Escherichia* and *Enterobacter* spp. were not found. In animals treated by Simbiter-2 frequency of hemolytic streptococci and streptococci group C was 16.7 % lower than in animals treated with Giprolam.

2. Preventative effectiveness of use of Giprolam and Simbiter-2 probiotics in red and pied cows

Indicator	Group I	Group II	Group III
Number of animals	12	12	12
Delivery, min ($M\pm m$)	60±10	30±10	120±30
Postpartum endometritis, number (%)	2 (16.7)	1 (8.3)	7 (58.3)
Preventative effectiveness, %	71.4	85.8	

N o t e. See description of groups in section “Methodology”.

Calving in cows treated with probiotics was without obstetric aid, while frequency of purulent-catarrhal endometritis did not exceed 16.7 % (Table 2). In control in 50.0 % cases, cows were rendered with obstetric aid, fetal removal was 2 and 4 times longer than in groups I and II, one cow and its calf died, and acute purulent-catarrhal endometritis was in over half of animals (see Table 2).

Intravaginal injection of Giprolam and Simbiter-2 in down-calving cows promotes postpartum indigenous microflora at physiological level, prevents colonization of birth canals by opportunistic pathogenic microflora and occurrence of postpartum endometritis in 71.4 and 85.8 % cases.

Correction of birth canal biocenosis in cows was accompanied by optimization of quantitative and qualitative composition of colostrum microflora. In day 1 postpartum, in colostrum of animals treated with Giprolam the number of lactobacilli and bifidobacteria was 9.8 and 29.2 times higher than in control, *Staphylococcus epidermidis* was 6.6 times less; *Staphylococci aureus*, streptococci group D and *Escherichia* were not found. Colostrum of cows from group II contained 6.7 and 17.5 times more lactobacilli and bifidobacteria, 1.5 times less *Staphylococcus epidermidis. Staphylococcus aureus* was 2.3 times less frequent at 11 times lower count; streptococci group D and *Escherichia* found in the control with frequencies of 25.0 and 8.3 % were not isolated. Comparison of Giprolam

and Simbiter-2 effects shows that Giprolam increases number of lactobacilli and bifidobacteria 1.5- and 1.7-fold, *Staphylococcus epidermidis* was 4.3 times less, and *Staphylococcus aureus* was not found.

Intravaginal administration of probiotics had positive effect on formation of gastrointestinal normoflora in calves, formation of which starts from fetal movement through the mother's birth canals and directly depends of the sanitary quality and timely production of colostrum (milk), being the lactobacillus and bifidobacterium source. In the day 1 of life, quantity of lactobacillus in the large intestines of calves from cows treated with Giprolam, as compared to control, was 237.4 times higher, bifidobacteria —38.9 times higher, and lactose positive *Escherichia* — 2.2 times higher than in control, ratio of the latter and lactose negative *E. coli* increased 17.7 times; quantity of *Enterobacter* and *Citrobacter* genera was 3.5 and 10.7 times less; staphylococci and protei were not found. On day 7, quantity of lactobacilli and bifidobacteria was 165.5 and 131.3 times higher, saprophyte staphylococci was 8.3 times higher; opportunistic pathogenic microorganisms *Enterococcus faecalis* was 21.7 times less, *Enterococcus faecium* — 25.3 times less, lactose negative *Escherichia* — 8.7 times less, *Citrobacter* and *Enterobacter* genera — 10.9 and 18.5 times less. On day 1, quantity of lactobacilli was 19.7 times higher than in the control, bifidobacteria — 15.6 times higher, lactose positive *Escherichia* — 16.3 times higher; opportunistic pathogenic *Citrobacter* and *Enterobacter* bacteria were 29.7 and 7.4 times less; *Staphylococcus aureus* and protei were not found on day 1 in the large intestines of calves from cows treated with Simbiter-2. Optimization of normaflora in calves during colostral period due to microecologic effects of Giprolam and Simbiter-2 prevents gastrointestinal diseases in calves in 50.0 and 41.7 % cases, respectively.

Therefore, correction of vaginal biocenosis in down-calving cows by Giprolam and Simbiter-2 to a significant degree prevents development of acute postpartum endometritis. High effectiveness of use of such probiotics is due to ability of lactobacilli to colonize birth canals of mother-cows and maintain the optimal composition of indigenous microflora to prevent infection by pathogenic microorganisms and excessive colonization of birth canals by opportunistic pathogenic bacteria. Such treatment also optimizes quantitative and qualitative microflora composition of colostrum and intestines of calves that prevents their gastrointestinal diseases.

REFERENCES

1. Nezhdanov A.G., Shakhov A.G. *Veterinarnaya patologiya*, 2005, 3: 61-64 (in Russ.).
2. Le Blanc S.J., Duffield T.F., Leslie K.E., Bateman K.G., Keefe G.P., Walton J.S., Johnson W.H. Defining and diagnosing postpartum clinical endometritis and its impact on reproductive performance in dairy cows. *American Dairy Science Association*, 2002, 85: 2223-2236 (doi: 10.3168/jds.S0022-0302(02)74302-6).
3. Gilbert R.O., Shin S.T., Guard C.L., Erb H.N., Marcel F. Prevalence of endometritis and its effects on reproductive performance of dairy cows. *Theriogenology*, 2005, 64(9): 1879-1888 (doi: 10.1016/j.theriogenology.2005.04.022).
4. Gautam G., Nakao T., Yusuf M., Koike K. Prevalence of endometritis during the postpartum period and its impact on subsequent reproductive performance in two Japanese dairy herds. *Anim. Reprod. Sci.*, 2009, 116(3-4): 175-187 (doi: 10.1016/j.anireprosci.2009.02.001).
5. Dubuc J., Duffield T.F., Leslie K.E., Walton J.S., Leblanc S.J. Effects of postpartum uterine diseases on milk production and culling in dairy cows. *J. Dairy Sci.*, 2011, 94(3): 1339-1346 (doi: 10.3168/jds.2010-3758).
6. Donofrio G., Herath S., Sartori C., Cavirani S., Flammini F., Sheldon I.M. Bovine herpesvirus 4 is tropic for bovine endometrial cells and modulates endocrine function. *Reproduction*, 2007, 134(1): 183-197 (doi: 10.1530/REP-07-0065).
7. Donofrio G., Ravanetti L., Cavirani S., Herath S., Capocefalo A., Sheldon I.M. Bacterial infection of endometrial stromal cells influences bovine herpesvirus 4 immediate early gene activa-

tion: a new insight into bacterial and viral interaction for uterine disease. *Reproduction*, 2008, 136(3): 361-366 (doi: 10.1530/REP-08-0171).
8. Fábián K., Makrai L., Sachse K., Szeredi L., Egyed L. An investigation of the aetiological role of bovine herpesvirus 4 in bovine endometritis. *Vet. J.*, 2008, 177(2): 289-292 (doi: 10.1016/j.tvjl.2007.04.010).
9. Liu M.C., Wu C.M., Liu Y.C., Zhao J.C., Yang Y.L, Shen J.Z. Identification, susceptibility, and detection of integrongene cassettes of *Arcanobacterium pyogenes* in bovine endometritis. *J. Dairy Sci.*, 2009, 92(8): 3659-3666 (doi: 10.3168/jds.2008-1756).
10. Petit T., Spergser J., Rosengarten R., Aurich J. Prevalence of potentially pathogenic bacteria as genital pathogens in dairy cattle. *Reprod. Domest. Anim.*, 2009, 44(1): 88-91 (doi: 10.1111/j.1439-0531.2007.01002.x).
11. Bicalho R.C., Machado V.S., Bicalho M.L., Gilbert R.O., Teixeira A.G., Caixeta L.S., Pereira R.V. Molecular and epidemiological characterization of bovine intrauterine *Escherichia coli*. *J. Dairy Sci.*, 2010, 93(12): 5818-5830 (doi: 10.3168/jds.2010-3550).
12. Samokhin V.T., Shakhov A.G., Shegidevich E.I, Fedorov Yu.N., Yurov K.P., Zhidkov S.A., Voronin E.S., Arkhipov A.V., Burlakov A.V., Subbotin V.V., Ivkin N.S., Donchenko A.S., Shkil' N.A., Volkov G.K., Sidorov M.A., Ovsyannikova T.O., Sisyagin P.N., Kavruk L.S., Nikitin V.F., Antipov V.A., Terekhov V.I., Shipitsyn A.G., Petrov Yu.F., Degtyarev V.P. *Nauchno obosnovannaya sistema polucheniya zdorovogo molodnyaka i profilaktika zheludochno-kishechnykh boleznei novorozhdennykh telyat* [A grounded system for producing healthy young animals and preventing gastrointestinal diseases of newborn calves]. Moscow, 2002 (in Russ.).
13. Cerri R.L., Rutigliano H.M., Lima F.S., Araújo D.B., Santos J.E. Effect of source of supplemental selenium on uterine health and embryo quality in high-producing dairy cows. *Theriogenology*, 2009, 71(7): 1127-1137 (doi: 10.1016/j.theriogenology.2008.12.005).
14. Brozos C.N., Kiossis E., Georgiadis M.P., Piperelis S., Boscos C. The effect of chloride ammonium, vitamin E and Se supplementation throughout the dry period on the prevention of retained fetal membranes, reproductive performance and milk yield of dairy cows. *Livestock Science*, 2009, 124(1-3): 210-215 (doi: 10.1016/j.livsci.2009.01.018).
15. Lobodin K.A. *Veterinariya*, 2006, 7: 38-42. Available https://elibrary.ru/item.asp?id=9242726. No date (in Russ.).
16. Kornienko V.S. *Lekarstvennyi preparat Ban. A.S. 2140275 (RF) MKIZ A61K35/12, A61K35/50. Filial № 5 GNTS — Institut biofiziki FU «Medbioekstrem» pri MZ Rossii № 98110825/13. Zayavl. 04.06.98. Opubl. 27.10.99* [Medication Ban. A.C. 2140275 (RF) МКИЗ A61K35/12, A61K35/50. Appl. 04.06.98. Publ. 27.10.99] (in Russ.).
17. Arlt S., Padberg W., Drillich M., Heuwieser W. Efficacy of homeopathic remedies as prophylaxis of bovine endometritis. *J. Dairy Sci.*, 2009, 92(10): 4945-4953 (doi: 10.3168/jds.2009-2142).
18. Barrett A.J., Murray R.D., Christley R.M., Dobson H., Smith R.F. Effects of the administration of oxytocin or carbetocin to dairy cows at parturition on their subsequent fertility. *Vet. Rec.*, 2009, 165(21): 623-626 (doi: 10.1136/vr.165.21.623).
19. Zidane K., Niar A., Tainturier D. Comparative effect on clinical use of PGF2 and REPROCINE in the treatment of retained placenta in dairy cows at Tiaret region (Algeria). *Asian Journal of Animals and Veterinary Advances*, 2011, 6(6): 593-598 (doi: 10.3923/ajava.2011.593.598).
20. Runciman D.J., Anderson G.A., Malmo J., Davis G.M. Effect of intrauterine treatment with cephapirin on the reproductive performance of seasonally calving dairy cows at risk of endometritis following periparturient disease. *Aust. Vet. J.*, 2008, 86(7): 250-258 (doi: 10.1111/j.1751-0813.2008.00302.x).
21. Galvão K.N., Greco L.F., Vilela J.M., SáFilho M.F., Santos J.E. Effect of intrauterine infusion of ceftiofur on uterine health and fertility in dairy cows. *J. Dairy Sci.*, 2009, 92(4): 1532-1542 (doi: 10.3168/jds.2008-1615).
22. Kaufmann T.B., Westermann S., Drillich M., Plöntzke J., Heuwieser W. Systemic antibiotic treatment of clinical endometritis in dairy cows with ceftiofur or two doses of cloprostenol in a 14-d interval. *Anim. Reprod. Sci.*, 2010, 121: 55-62 (doi: 10.1016/j.anireprosci.2010.04.190).
23. Machado V.S., Bicalho M.L.S., Pereira R.V., Caixeta L.S., Bittar J.H.J., Oikonomou G., Gilbert R.O., Bicalho R.C. The effect of intrauterine administration of mannose or bacteriophage on uterine health and fertility of dairy cows with special focus on *Escherichia coli* and *Arcanobacterium pyogenes*. *J. Dairy Sci.*, 2012, 95: 3100-3109 (10.3168/jds.2011-5063)
24. Changjun X., Minglei H., Daqing G., Jinghua G., Guizhen X., Jianwei L., Hongbo N. Therapeutic efficacy experiments of Xuyanning in treating endometritis of dairy cows. *Journal of Heilongjiang Bayi Agricultural University*, 2012, 2. Available https://en.cnki.com.cn/Article_en/CJFDTOTAL-HLJK201202016.htm. No date.
25. Kira E.F. *Bakterial'nyi vaginoz* [Bacterial vaginosis]. St. Petersburg, 2001 (in Russ.).
26. Kolesaeva Zh.Yu., Martikainen Z.M., Savicheva A.M. Tarasova M.A. *Zhurnal akusherstva i zhenskikh boleznei*, 2009, 3: 25-31 (in Russ.).
27. Dobrokhotova Yu.Z., Zatikyan N.G. *Akusherstvo, ginekologiya, reproduktsiya*, 2008, 1: 7-9. Available http://www.gyn.su/article.php?what=21. No date (in Russ.).
28. Garoussi M.T., Khosrave A.R., Havareshti P. Mycoflora of cervicovaginal fluids in dairy

cows with or without reproductive disorders. *Mycopathologia*, 2007, 164(2): 97-100 (doi: 10.1007/s11046-007-9031-x).
29. Ankirskaya A.S. *Akusherstvo i ginekologiya*, 1995, 6: 13-16 (in Russ.).
30. Lincke A., Drillich M., Heuwieser W. Die subklinische Endometritis des Rindes und ihr Einfluss auf die Fruchtbarkeit eine Übersicht neuerer Untersuchungen. *Berl. Münch. Tierärztl. Wschr*, 2007, 120(5-6): 245-250 (doi: 10.2376/0005-9366-120-245).
31. LeBlans SJ. Postpartum uterine disease and dairy herd reproductive performance: a review. *Vet. J.*, 2008, 176(1): 102-114 (doi: 10.1016/j.tvjl.2007.12.019).
32. Dolezel R., Vecera M., Palenik T., Cech S., Vyskocil M. Systematic clinical examination of early postpartum cows and treatment of puerperal metritis did not have any beneficial effect on subsequent reproductive performance. *Veterinarni Medicina*, 2008, 53(2): 59-69. Available http://www.vri.cz/docs/vetmed/53-2-59.pdf. No date (in Russ.).
33. Santos T.M., Caixeta L.S., Machado V.S., Rauf A.K., Gilbert R.O., Bicalho R.C. Antimicrobial resistance and presence of virulence factor genes in *Arcanobacterium pyogenes* isolated from the uterus of postpartum dairy cows. *Vet. Microbiol.*, 2010, 145(1-2): 84-89 (doi: 10.1016/j.vetmic.2010.03.001).
34. Turchenko A.N., Koba I.S., Novikova E.N., Reshetka M.B., Petenko A.I., Gorpichenko E.A. *Veterinariya Kubani*, 2012, 3: 11-13. Available https://elibrary.ru/item.asp?id=17785427. No date (in Russ.).
35. Otero M.C., Morelli L., Nader-Macias M.E. Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle. *Letters in Applied Microbiology*, 2006, 43: 91-97 (doi: 10.1111/j.1472-765X.2006.01914.x).
36. Fátima M., Nader-Macías E., Claudia O.M., Carolina E.M., Natalia M.C. Advances in the design of probiotic products for the prevention of major diseases in dairy cattle. *Journal of Industrial Microbiology & Biotechnology*, 2008, 35(11): 1387-1395 (doi: 10.1007/s10295-008-0438-2).
37. Ametaj B.N., Iqbal S., Selami F., Odhiambo J.F., Wang Y., Gänzle M.G., Dunn S.M., Zebeli Q. Intravaginal administration of lactic acid bacteria modulated the incidence of purulent vaginal discharges, plasma haptoglobin concentrations, and milk production in dairy cows. *Research in Veterinary Science*, 2014, 96(2): 365-370 (doi: 10.1016/j.rvsc.2014.02.007).
38. Yankovskii D.S. *Mikrobnaya ekologiya cheloveka: sovremennye vozmozhnosti ee podderzhaniya i vosstanovleniya* [Microbial human ecology maintenance and restoration: modern approaches]. Kiev, 2005. Available https://lad.mosuzi.ru/antropologiya/d7f4f70236136854768 6f389025d7bda. No date (in Russ.).
39. Sidorov M.A., Skorodumov D.I., Fedotov V.B. *Opredelitel' zoopatogennykh mikroorganizmov* [Identification keys of zoopathogenic microorganisms]. Moscow, 1995 (in Russ.).
40. Belyakov V.D. *Immunoprofilaktika v immunologii* [Immunoprophylaxis in immunology]. Moscow, 1961 (in Russ.).

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REAL TIME PCR FOR THE DETECTION OF FIELD ISOLATES OF LUMPY SKIN DISEASE VIRUS IN CLINICAL SAMPLES FROM CATTLE

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Abstract

Lumpy skin disease caused by lumpy skin disease virus (LSDV, *Capripoxvirus*, *Poxviridae*) is a capripoxviral disease with significant morbidity in cattle, which necessitates the development of reliable diagnostic tools in the context of live vaccine administration. OIE-recommended PCR assays target not only LSDV but also sheep pox virus and goat pox virus. Conventional PCR is prone to carry-over contamination, whereas real-time PCR offers more advantages, including prevention of amplicon carryover contamination post amplification. In this paper we report the development of a PCR real time assay for the detection of field isolates of lumpy skin disease virus in clinical samples from cattle. The specificity was validated against a panel of homologous and heterologous viruses retrieved from the strain depository of FGBI ARRIAH. The PCR assay was shown to be highly specific toward field LSDV. When tested in the presence of vaccine strain DNA and related capripoxviruses, no false-positive results were obtained. Using a series of 10-fold dilutions the assay proved to be highly sensitive with a detection limit of 0.21 lg TCD_{50}/ml. The calculated efficiency of amplification was 98.6 %, with SD ranging from 0.11 to 0.33 over five orders of magnitude. The PCR assay was also validated on samples from experimentally inoculated bulls. The animals received a subcutaneous injection of a field LSDV and were tested for the presence of LSDV DNA in blood and nasal swab in comparison to PCR by D.C. Ireland и Y.S. Binepal (1998) (data not shown). Overall, the presented assay demonstrated high specificity and sensitivity and can be recommended as a diagnostic tool for the detection of field isolated of LSDV.

Keywords: lumpy skin disease, diagnostics, real-time PCR, genome, virus

Capripoxviruses of *Capripoxvirus* genus (infectious virus of nodular dermatitis in bovine animals, capripoxvirus in sheep and goats) are agents of cross-border zoonotic diseases in large and small cattle, which represent serious threat to cattle breeding industry, causing significant losses to farming and country economies [1, 2]. During the past few years, due to intensive trading and, possibly, natural factors, capripoxviruses had started massive dissemination northwards, including countries of Near East, Europe, Turkey, and Russia [3-7]. Today, lumpy skin disease virus (LSDV) in large cattle is deemed the most dangerous. Massive spread of LSDV in 2015-2016 at south of Europe, Balkan Peninsula, and in the Russian Federation, has reinforced the need to use highly sensitive methods for monitoring and accurate diagnosis of the disease as soon as possible for timely and adequate preventative and protective measures.

LSDV (*Capripoxvirus* genus, *Poxviridae* family) genome consists of double-stranded DNA [8]. LSDV transmission occurs mechanically through bites of insects or through contaminated feed, water, and sperm at fertilization [1, 8-11]. Recent studies established the role of mites in spreading LSDV [12, 13].

LSD is cross-border viral disease in large cattle that is accompanied by fever, reduction of live weight, injury of lymphatic system, swelling of internal organs, formation of nodes (nodules, bumps) on skin and internal organs [14]. Recently, disease is included in the list of World Organization for Animal Health (International epizootic bureau, OIE — IEB, Paris) [15]. Infection of large cattle by nodular dermatitis is subject to compulsory notification. Subject to Decree Nr. 62 of the Ministry of Agriculture of the Russian Federation dated March 09, 2011, infectious nodular dermatitis in large cattle is included in the List of infectious and other diseases in animals, but is not included in the List of infectious, including highly dangerous diseases, for quarantine, approved by the Ministry of Agriculture of the Russian Federation Nr. 476 dated December 19, 2011.

Earlier, LSD seasonally emerged in large cattle in African countries [16]. Unprecedented spread of LSD causative agent at south of Russia in 2015-2016 [5, 17] emphasizes the need for highly sensitive and specific diagnostic tests. IEB recommends detecting LSDV genome by PCR [15], with both classic PCR [18] and real-time PCR used [19]. The problem is that the IEB tests also detect DNA of sheep and goat capripoxviruses and not discriminate these viruses from cattle capripoxvirus. Besides, in classic PCR there is a risk of contamination of amplification products as electrophoretic detection is needed. C.E. Lamien et al. [20] suggest PCR amplification with high-resolution melting analysis of DNA fragments to detect of LSDV and capripoxvirus of sheep and goats. However, this method is unusable in routine diagnostics because of high dependence on DNA quality and concentration. Besides, use of live Neethling strain-based vaccines against LSD requires tests able to identify and differentiate field isolates.

We propose rapid, reliable, sensitive, and specific real-time PCR method to identify DNA of LSDV field isolates in tissues and organs of experimentally and naturally infected animals.

Purpose of this research was development and validation of real-time PCR test for identification of LSDV DNA in biomaterial.

Techniques. Total DNA was extracted from 100 μl analyzed specimen suspension with RIBO-sorb kit (Central Research Institute of Epidemiology, Moscow) subject to the producer's instruction.

Primers and probes were developed with Primer3 Engine Software (available at http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and synthesized (Beagle Biotechnology, Saint Petersburg). For detection of virus, we used primers f2 TAGAAAATGGATGTACCACAAATACAG and r33 TTGTTACAACTCAAATCGTTAGGTG, and probe Taqman ACCACCTAATGATAGTGTTTATGATTTAC 5′-end labeled with fluorescent dye carboxyfluorescein (6FAM), and 3′-end labeled with fluorescence quencher BHQ-1.

For PCR, we used reagent kit GoTaq Flexi DNA Polymerase, 5× Colorless GoTaq PCR Buffer, $MgCl_2$ (Promega Corp., USA) and 100 mM dNTPs (Invitrogen, USA). Ultimate 25 μl reaction mixture contained 5 μl 10× PCR buffer, 3 μl of 25 мМ $MgCl_2$, 0.5 μl 10 nmol dNTP, 12.5 pmol of each primer (forward and reverse), and 7.5 pmol probe. Real-time PCR (qPCR) was carried out with the use of a Rotor Gene instrument (Qiagen N.V., Germany). PCR protocol was as follows: 10 minute activation at 95 °C; 40 cycles including 15 seconds at 95 °C and 1 minute at 60 °C.

The results were interpreted based on intersection of fluorescence curve and threshold line that correlates to presence or absence of threshold cycle value Ct in relevant column in the table of results at machine-based analysis. Results were deemed valid provided positive ($Ct < 30$) and negative (Ct not identified) amplification controls. Specimen was positive (presence of LSDV DNA) if Ct did not exceed 35, and negative if Ct was absent or exceeded 37.

Primer specificity was checked with BLAST online resource software (https://blast.ncbi.nlm.nih.gov/) and by testing genetic material of homologous and heterologous viruses. Specificity was assessed for DNA of each virus individually and also for several viral DNAs in a single reaction mixture. qPCR analytic sensitivity was determined with 10-fold dilutions (up to 10^{-5}, initial titer of 5.21 lg TCD_{50}/ml) of reference strain NDV/Dagestan/2015 genome DNA. For statistical verification, 3 repeated tests with 10-fold dilutions of genome DNA were carried out to assess result linearity.

Six bulls aged 1.5 years were experimentally infected by intravenous injection of 2 ml bovine virus NDV/Dagestan/2015 field strain (5.21 lg TCD_{50}/ml) according to common recommendations. One animal (control) was not infected. To detect LSDV on days 4 and 14 post inoculation, blood samples stabilized with EDTA and nasal washes were collected. To confirm qPCR results, animals were slaughtered at the end of tests to collect biomaterial for virus isolation in continuous lamb testis cells (collection of cell cultures of the Federal Center for Animal Health Control) as per description [21].

Linear regression was plotted with Statistica 10 software (StatSoft, Inc., USA). At assessment of repeatability, standard errors (±SD) and determination coefficient r^2 were calculated.

Results. The primers and probe were developed for amplification and detection of LSDV EEV (extracellular enveloped virions, ORF126) gene fragment with a 27 bps deletion characteristic of other *Capripoxviridae* members and also of Neethling vaccine strains but absent in field isolates [22, 23].

Specificity. For testing and optimization of the developed method, we used DNA of reference strains of heterologous viruses (deposited in collection of microorganisms of Federal Center for Animal Health Control), as well as DNA of LSDV strains isolated in different regions of Russia in 2015-2016 (Table 1). These results indicate absence of false positives and specific identification of field isolates both separately and at presence of DNA of heterologous viruses (viruses of sheep and goat pox), as well as vaccine strain.

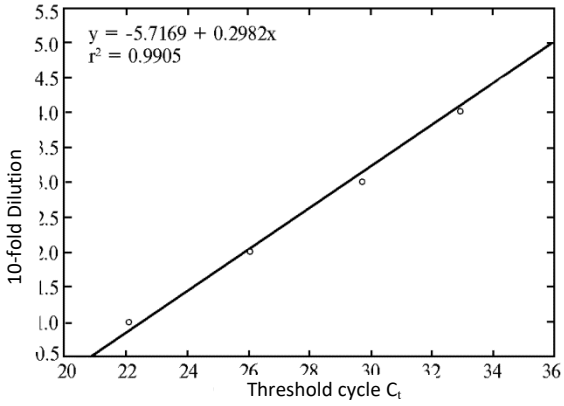
qPCR sensitivity. Sensitivity of the proposed method with diagnostic strain NDV/Dagestan/2015 is 0.21 lg TCD_{50}/ml at initial titer of 5.21 lg TCD_{50}/ml. To assess amplification effectiveness during repeated tests, Ct values were determined. Based on mean Ct values, we obtained linear regression with amplification effectiveness value (E) of 98.6 % (Fig.).

1. Specificity of the developed real-time PCR (qPCR) **method in detection of lumpy skin disease virus (LSDV) field isolates**

DNA source	Origin/collection	qPCR
NDV LC/Dagestan/2015 (reference strain)	Russia/FSBE VNIIZZH	
NDV LC E-95	Africa/ FSBE VNIIZZH	
Attenuated vaccine strain	SAR VRI Onderstepoort/ FSBE VNIIZZH	
Sheep pox virus, Afganian strain	Afganistan/ FSBE VNIIZZH	
Sheep pox virus, strain VNIIZZH	Russia/ FSBE VNIIZZH	
Sheep pox virus, field isolate	Russia (Yaroslavl Region)/–	
Goat pox virus, strain Primorskiy 2003	Russia / FSBE VNIIZZH	
Goat pox virus, strain VNIIZZH 2003	Russia / FSBE VNIIZZH	
Goat plague (PPR) virus plagues, strain VNIIZZH	Russia / FSBE VNIIZZH	
Vesicular stomatitis virus, strain VNIIZZH	Russia / FSBE VNIIZZH	
Sheep ecthyma virus, field isolate	Russia /–	
Cowpox viruss, strain VNIIZZH	Russia / FSBE VNIIZZH	

Note. FSBE VNIIZZH — Federal Center of Animal Health; dashes mean that sample was not included into the collection.

Repeatability was determined by standard deviation (±SD) for each series of dilutions, with the use of Ct values. SD for five 10-fold dilutions varied from 0.11 to 0.33. Determination coefficient r^2 is 0.9905.



Linearity of real-time PCR estimates at 10-fold DNA dilutions (reference strain NDV LC/Dagestan 2015 of lumpy skin disease virus; linear regression is plotted with Statistica 10 software, StatSoft, Inc., USA).

qPCR assay upon experimental infection of animals. Prior to virus inoculation and on days 4 and 14 post infection, we collected nasal washes from all animals using sterile prewetted cotton applicator. Blood samples were collected too.

Obtained results show (Table 2) that all animals prior to inoculation were LSDV negative. On day 4 blood samples of bulls No 1, 2 and 3 were positive in qPCR while in washes LSDV genome was found in bulls No 2, 5 and 6. On day 14, LSDV genome was found in all bulls, except for animal No 3, in washes — in all animals, except for bulls No 1 and No 3. All probes from control animal were negative in qPCR. In cell culture, virus was isolated from animals No 2, 3 and 4.

2. Identification of lumpy skin disease virus genome (LSDV) in experimentally infected bull by real-time PCR method (qPCR) and isolation in lamb testis cell culture

Animal No	qPCR (Ct values)					Virus isolation in cell culture
	before infection	day 4		day 14		
		blood	nasal wash	blood	nasal wash	
1	0/–	25.01	nf	24.07	nf	–
2	0/–	26.11	29.87	26.30	29.00	+
3	0/–	29.50	0	nf	nf	+
4	0/–	nf	nf	29.09	29.40	+
5	0/–	nf	29.21	21.00	25.54	–
6	0/–	nf	29.44	19.30	29.22	–
Control	0/–	nf	nf	nf	nf	–

N o t e. «–»/«+» mean absence/presence of virus at isolation in cell culture, nf — genome not found.

When compared two methods, i.e. the qPCR that we developed and PCR recommended by IEB [15], we failed to detect viral DNA by classical PCR if Ct exceeded 26.4 (data not shown).

Main purpose of our research is development of real-time PCR effective for practical identification of LSDV field isolate DNA in situation when live homological vaccines against infectious nodular dermatitis in cattle are widely used. Developed primers and probe were tested for specificity by BLAST and experimentally with DNA of all members of capripoxviruses of different origin deposited to strain collection of the Federal Center for Animal Health Control (see Table 1), as well as related sheep and goat capripoxviruses. Positive results obtained in all the cases indicate high analytical specificity of developed test-system. Importantly, that this test system allows identification of only field LSDV isolates with negatives for vaccine virus and other related capripoxviruses (see Table 1).

Analysis of scholarly publications shows that qPCR for LSDV field isolate identification described herein is of interest as a routine diagnostic tool. In Russia, papers on the topic are few and those dealing with diagnostic tests are practically absent. Validation of commercial test systems known at the Russian market are also not reported that makes objective comparison reasonable.

Recently, we know the only Russian report [22] on detection and differentiation of capripoxviruses by classic PCR method. Multiplex PCR described

therein has a number of advantages. However it is qPCR that avoids the risk of cross contamination at identification of LSDV genome due to lowering probability of false positives and shortening analysis time since amplification products are not studied in agarose gel. Also, qPCR method is more sensitive and allows quantitation. Assessment of viral particle copy number at experimental infection is outside the scope of this research and will be subject of our further study of LSDV biological properties.

We have not found any available information on experimental inoculation of sensitive animals with LSDV in Russian academic periodicals. So this paper is the first such report. However, data we obtained are insufficient to make statistically reliable conclusions because of small sample.

Proposed qPCR method had shown higher sensitivity compared to the classical method [18] as the latter did not identify virus DNA at threshold values of more than 26.4. Nevertheless, virus isolation resulted in three positives, of which two positives were in animals LSDV negative in classical PCR [18]. Interestingly, at the end of experiment virus commenced to appears in nasal washings [24, 25]. In identification of LSDV genome by proposed qPCR method, Ct did not practically differ in blood samples and nasal washings, whereas M. Sevik et al. [24] noted lesser Ct values for nasal washing specimens than for blood specimens. Possibly, the reason is that in our study the bulls were infected experimentally, whereas M. Sevik et al. [24] reported on naturally infected animals.

The qPCR test system had already been successfully validated on field specimens from cattle during LSD outbreaks in 2015-2016. These data will be a scope of our next report.

Therefore, we suggest rapid, reliable, sensitive, and specific real-time PCR method to identify genetic material of LSDV (lumpy skin disease virus) field isolates in cattle. This method can be recommended for diagnostic laboratories to control nodular dermatitis virus.

REFERENCES

1. Tuppurainen E.S., Venter E.H., Shisler J.L., Gari G., Mekonnen G.A., Juleff N., Lyons N.A., De Clercq K., Upton C., Bowden T.R., Babiuk S., Babiuk L.A. Capripoxvirus diseases: current status and opportunities for control. *Transbound. Emerg. Dis.*, 2017, 64: 729-745 (doi: 10.1111/tbed.12444).
2. Beard P.M. Lumpy skin disease: a direct threat to Europe. *Vet. Rec.*, 2016, 28: 557-558 (doi: 10.1136/vr.i2800).
3. Şevik M., Doğan M. Epidemiological and molecular studies on lumpy skin disease outbreaks in Turkey during 2014-2015. *Transbound. Emerg. Dis.*, 2016, 64(4): 1268-1279 (doi: 10.1111/tbed.12501).
4. Abutarbush S.M., Ababneh M.M., Al Zoubi I.G., Al Sheyab O.M., Al Zoubi M.G., Alekish M.O., Al Gharabat R.J. Lumpy skin disease in Jordan: disease emergence, clinical signs, complications and preliminary-associated economic losses. *Transbound. Emerg. Dis.*, 2015, 62(5): 549-554 (doi: 10.1111/tbed.12177).
5. Mishchenko A.V., Karaulov A.K. Mishchenko V.A. *Veterinariya*, 2016, 4: 3-6 (in Russ.).
6. Tasioudi K.E., Antoniou S.E., Iliadou P., Sachpatzidis A., Plevraki E., Agianniotaki E.I., Fouki C., Mangana-Vougiouka O., Chondrokouki E., Dile C. Emergence of lumpy skin disease in Greece, 2015. *Transbound. Emerg. Dis.*, 2016, 63(3): 260-265 (doi: 10.1111/tbed.12497).
7. Mercier A., Arsevska E., Bournez L., Bronner A., Calavas D., Cauchard J., Falala S., Caufour P., Tisseuil C., Lefrançois T., Lancelot R. Spread rate of lumpy skin disease in the Balkans, 2015-2016. *Transbound. Emerg. Dis.*, 2017, 00: 1-5 (doi: 10.1111/tbed.12624).
8. Tulman E.R., Afonso C.L., Lu Z., Zsak L., Kutish G.F., Rock D.L. Genome of lumpy skin disease virus. *J. Virol.*, 2001, 75(15): 7122-7130 (doi: 10.1128/JVI.75.15.7122-7130.2001).
9. Chihota C.M., Rennie L.F., Kitching R.P., Mellor P.S. Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (*Diptera*: *Culicidae*). *Epidemiol. Infect.*, 2001, 126: 317-321 (doi: 10.1017/S0950268801005179).
10. Irons P.C., Tuppurainen E.S., Venter E.H. Excretion of lumpy skin disease virus in bull semen. *Theriogenology*, 2005, 63: 1290-1297 (doi: 10.1016/j.theriogenology.2004.06.013).
11. Annandale C.H., Holm D.E., Ebersohn K., Venter E. H. Seminal transmission of lumpy skin disease virus in heifers. *Transbound. Emerg. Dis.*, 2013, 61: 443-448 (doi: 10.1111/tbed.12045).

12. Tuppurainen E.S., Lubinga J.C., Stoltsz W.H., Troskie M., Carpenter S.T., Coetzer J.A., Venter E.H., Oura C.A. Evidence of vertical transmission of lumpy skin disease virus in *Rhipicephalus decoloratus* ticks. *Ticks Tick Borne Dis.*, 2013, 4: 329-333 (doi: 10.1016/j.ttbdis.2013.01.006).
13. Lubinga J.C., Tuppurainen E.S., Mahlare R., Coetzer J.A., Stoltsz W.H., Venter E.H. Evidence of transstadial and mechanical transmission of lumpy skin disease virus *by Amblyomma hebraeum* ticks. *Transbound. Emerg. Dis.*, 2013, 62: 174-182 (doi: 10.1111/tbed.12102).
14. Makarov B.V., Grubyi V.A., Gruzdev K.N., Sukharev O.I. *Spisok MEB i transgranichnye infektsii zhivotnykh: monografiya* [OIE List and transboundary animal infections: monograph]. Moscow, 2012: 76-79 (in Russ.).
15. Beard P., Lubisi B.A. *Manual of diagnostic tests and vaccines for terrestrial animals 2017. Chapter 2.4.13. Lumpy skin disease.* Available http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.13_LSD.pdf. Accessed December 21, 2017.
16. Abera Z., Degefu H., Gari G., Ayana Z. Review on epidemiology and economic importance of lumpy skin disease. *International Journal of Basic and Applied Virology*, 2015, 4(1): 8-21 (doi: 10.5829/idosi.ijbav.2015.4.1.9117).
17. Mishchenko A.V., Mishchenko V.A., Kononov A.V., Shevkoplyas V.N., Dzhailidi G.A., Dresvyannikova S.G., Chernykh O.Yu. *Veterinariya Kubani*, 2015, 5: 3-6 (in Russ.).
18. Ireland D.C., Binepal Y.S. Improved detection of capripoxvirus in biopsy samples by PCR. *J. Virol. Methods*, 1998, 74: 1-7 (doi: 10.1016/S0166-0934(98)00035-4).
19. Bowden T.R., Babiuk S.L., Parkyn G.R., Copps J.S., Boyle D.B. Capripoxvirus tissue tropism and shedding: a quantitative study in experimentally infected sheep and goats. *Virology*, 2008, 371(2): 380-393 (doi: 10.1016/j.virol.2007.10.002).
20. Lamien C.E., Lelenta M., Goger W., Silber R., Tuppurainen E., Matijevic M., Luckins A.G., Diallo A. Real time PCR method for simultaneous detection, quantitation and differentiation of capripoxviruses. *J. Virol. Methods*, 2011, 171: 134-140 (doi: 10.1016/j.jviromet.2010.10.014).
21. Kononova S.V., Shumilova I.N., B'yadovskaya O.P. *Metodicheskie rekomendatsii po vydeleniyu virusa nodulyarnogo dermatita krupnogo rogatogo skota v kul'ture kletok: metodicheskii material* [Guidelines on the isolation of the nodular dermatitis virus in cattle cell culture]. Moscow, 2017: 5-17 (in Russ.).
22. Orlova E.S., Shcherbakov A.V., Diev V.I., Zakharov V.M. *Molekulyarnaya biologiya*, 2006, 40(1): 158-164 (in Russ.).
23. Menasherow S., Rubinstein-Giuni M., Kovtunenko A., Eyngor Y., Fridgut O., Rotenberg D., Khinich Y., Stram Y. Development of an assay to differentiate between virulent and vaccine strains of lumpy skin disease virus (LSDV). *J. Virol. Methods*, 2014, 199: 95-101 (doi: 10.1016/j.jviromet.2013.12.013).
24. Şevik M., Avci O., Doğan M., İnce Ö.B. Serum biochemistry of lumpy skin disease virus-infected cattle. *Biomed. Res. Int.*, 2016, 2016: 6257984 (doi: 10.1155/2016/6257984).
25. Balinsky C.A., Delhon G., Smoliga G., Prarat M., French R.A., Geary S.J., Rock D.L., Rodriguez L.L. Rapid preclinical detection of sheep pox virus by a real-time PCR assay. *J. Clin. Microbiol.*, 2008, 46: 438-442 (doi: 10.1128/JCM.01953-07).

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VALIDATION OF A TEST SYSTEM FOR AFRICAN SWINE FEVER SERODIAGNOSIS USING IMMUNOBLOTTING

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Abstract

Because of the lack of a vaccine, African swine fever (ASF, caused by African swine fever virus (ASFV) of *Asfivirus* genus, *Asfarviridae* family) control strategy is based on making a rapid and early diagnosis and taking strict veterinary and sanitary measures. In the Eastern Europe countries where the infection has currently spread, highly virulent isolates are usually detected (J.M. Sanchez-Vizcaino et al., 2013). In the laboratory diagnosis, polymerase chain reaction (PCR) and direct immunofluorescence method are predominantly used. However, since 2012, researchers have observed some alteration in biological and genetic properties of a number of ASFV isolates. Therefore, serological methods may become prevalent in the laboratory diagnosis as it was during an ASF epizooty in the Iberian Peninsula in 1960-1990. We have earlier reported the development of a test system for the disease immunoblotting serodiagnosis (Rec p30-IB) based on a recombinant structural ASFV protein p30 (A.S. Kazakova et al., 2014). In this paper, the Rec p30-IB test system validation is shown. The diagnostic sensitivity of the Rec p30-IB was of 99.3 %, and the specificity was 100 %. Antibodies against p30 were detected in blood serum and organ samples taken from domestic pigs or wild boars irrespective of the seroimmunotypic membership and the virulence levels of the ASFV strains. In the blood serum samples collected from domestic pigs infected with heterologous viruses, no false-positive results were seen. In the serum of domestic pigs which were survived after intramuscular injection of attenuated strains LK-111, KK-262/C, MK-200, FK-135, PSA-1-NH and SCA 2015 VNIIVViM at 10^3 to 10^4 HAU_{50}/CPE_{50}, antibodies to p30 were detected on day 7 to 10. For organ samples from domestic pigs that had died from ASF 5 to 10 days post intramuscular infection with highly virulent strains Lisbon-57, Mozambique-78 or Stavropol 01/08 at a dose of 10^3 HAE_{50}, the antibodies to p30 were detected in 30 % of the animals. The validation results indicate that the Immunoblotting Test System for African Swine Fever Serodiagnosis (Rec p30-IB) can be used for laboratory practice and monitoring of blood sera and organ samples collected from ASFV-infected domestic pigs or wild boars.

Keywords: African swine fever, protein p30 ASFV, serodiagnosis, immunoblotting, validation

African swine fever (ASF) is a contagious septic disease characterized by fever, hemorrhagic diathesis, and high mortality rate. In acute form, 100 % of animals die within 5-10 days after manifestation of clinical traits. The disease is caused by coated large African swine fever virus (ASFV) with double-stranded DNA, the only representative of *Asfarviridae* family, which is characterized by

significant variability of biological and genetic properties [1-3]. ASFV has extremely high potential to cross-border spread. In 2007, it penetrated from Africa and Georgia, followed by Asian and Eastern-European countries (Armenia, Iran, Azerbaijan, Russia, Belarus, Ukraine, Latvia, Lithuania, Estonia, Poland) [4, 5].

Due to lack of vaccine in strategy of combat against the disease, the accent is made on rapid and early diagnostic and strict veterinary and sanitary measures. However, ASFV diagnostic is challenged by numerous pathogenesis forms and epizootic scenarios, as well as similarity of clinical and pathomorphological signs of this disease and other hemorrhagic infectious diseases, e.g. classical swine fever (CSF), acute pasteurellosis, swine erysipelas, and salmonellosis. In East-European countries, where CSF is recently spread, high-virulent ASFV isolates are usually identified [6]. For laboratory diagnostics, different variants of polymerase chain reaction (PCR) and direct immune-fluorescence method are mainly used. However, since 2012 variability of biological and genetic properties were sighted in several isolates extracted at the territory of Russia, Poland, and Baltic States [7-10]. It provides the basis to assume that pathogenicity of ASFV circulating in Eastern-Europe varied towards decrease of virulence. Thus, serologic methods could become dominant in the laboratory diagnostics as it was during CSF epizooty in 1960-1990 at Iberian Peninsula. Antibodies in blood serum and immune organs at CSF are identified 7-10 days after infection and in furtherance during a long time [11]. Their presence serves convincing testimony for diagnosing. Today, the inventory of diagnostic means for identification of antibodies is mainly presented by kits for Indirect Immune Fluorescence Reaction (IIFR), immune-enzyme analysis (ELISA) and immunoblotting assay (IB). International Epizootic Bureau (IEB) (IEB — World Organization for Animal Health, OIE, France) recommends confirming $ELISA^+$ specimens by IB method since samples of field blood serums lose their reactivity in ELISA earlier than in IB, because the latter can identify linear epitops [12]. Amongst IB advantages are simplicity and objectivity of interpretation, as nitrogen-cellulose strips with absorbed virus-specific polypeptides could be kept up to 6 months at room temperature in dry atmosphere [13, 14].

IEBB recommends for ELISA and IB tests cytoplasmic soluble antigen produced in ASFV infected cell line of monkey's kidney raised in presence of swine serum [14, 15]. To secure production of diagnostic test systems, virus-specific antigens based on recombinant (chimer) immune dominant proteins of ASFV are developed [16-22]. Such antigens increase sensitivity and specificity of diagnostics by decreasing the frequency of false positives caused by substances of cell culture which inevitably contaminate antigens prepared on its basis [15].

Earlier, we have reported on development of a test system for serological diagnostic of African swine fever by immune blotting, prepared based on recombinant ASFV protein p30 [23]. In present paper, we have for the first time presented data on its validation.

Our objective was estimate of validity of immune blotting test for ASFV detection based on use recombinant protein p30.

Techniques. Specimens were collected from Large White pigs weighting 30-50 kg of Animal Sector of Federal Research Center of Virology and Immunology, and from wild boars of hunting farm Pokrovskoye, Vladimir Region. ASFV virulente strains were Lisbon-57 (L-57, seroimmunotype I), Congo-49 (C-49, seroimmunotype II), Mozambique-78 (M-78, seroimmunotype III), Stavropol 01/08 (seroimmunotype VIII), ASFV attenuated strains were KK-262/C (seroimmunotype II), MK-200 (seroimmunotype III), FK-135 (seroimmunotype IV), PSA-1-NH (seroimmunotype IV), CKA 2015 VNIIVViM (seroimmunotype VIII) (all strains) (State Collection of Microorganisms of the Federal Research

Center of Virology and Immunology) [24, 25; Patent RF No 2439152, 2012].

Swine antisera against causative agents of CSF, transmissible gastroenteritis (TGE), and Aujeszky's disease were produced in Diagnostics and Monitoring Laboratory of the Federal Research Center of Virology and Microbiology. Reference negative blood serum sample included 28 specimens from domestic pigs and wild boars (Smolensk, Voronezh, Tver, Pskov, Rostov, and Volgograd regions), 25 specimens from healthy domestic pigs and wild boards (vivarium of the Federal Research Center of Virology and Microbiology), 480 specimens from domestic pigs from ASF-free pig breeding farms (Voronezh Region). Reference positive blood serum sample included 42 specimens from domestic pigs (farming units of Volgograd and Tver regions), 59 specimens from experimentally infected domestic pigs (Federal Research Center of Virology and Microbiology, collected at different times after intramuscular inoculation with virulent or attenuated ASFV strains, seroimmunetypes I-IV, VIII) and from wild boars. $ASFV^+$ blood sera were also received from the reference laboratory of CISA-INIA (Centro de Investigaciyn en Sanidad Animal, Instituto Nacional de Investigaciyn y Tecnologна Agraria y Alimentaria, Spain). Total 18 spleen specimens from wild boars of Smolensk Region were reference $ASFV^-$; 11 spleen specimens from wild boars of Smolensk Region, 18 spleen specimens from domestic pigs of Tula Region, and 10 specimens from experimentally infected pigs (Federal Research Center of Virology and Microbiology) were reference $ASFV^+$. Absence or presence of anti-ASFV antibodies in each reference specimen were confirmed by indirect immune fluorescence assay (IIFA), the reference test recommended by IEB. Blood sera and 10 % tissue suspension processing, hemadsorption, detection of antibodies to ASFV by IIFA and ELISA were performed as per GOST 28573-90 (Moscow, 2005). If required, blood serum was incubated during 30 minutes at 56 °C prior to tests to inactivate infectious virus.

In IIFA, commercial Kit for Differential Immune Fluorescent Diagnostic of African swine fever, classical swine fever, and Aujeszky's disease (Federal Research Center of Virology and Microbiology) and cell culture of African green monkey's kidney CV-1 (Collection of cell cultures of the Federal Research Center of Virology and Microbiology) infected by avirulent non hemadsorping ASFV (strain 691/88, State Collection of Microorganisms of the Federal Research Center of Virology and Microbiology) were used with luminescent microscopy (Eclipse E200, Nikon Co., Japan) to read the results.

ELISA test was performed with commercial ASF-IFA Ab/Ag kit (Federal Research Center of Virology and Microbiology) [26]. For comparison, we used ELISA tests for antibodies to ASFV — Ingezim PPA Compac 1.1.PPA.K.3 based on virus protein vp73 and monoclonal antibodies (Inmunologia y Genetica Aplicada S.A., Spain), and also ID Screen® African Swine Fever Indirect based on mixture of recombinant proteins p32, p62, and p72 (IDvet Genetics, France).

Immunoblotting assay (IB) was performed with Test System for African Swine Fever Serodiagnosis by Immunoblotting Method (Rec p30-IB) (five experimental series, Federal Research Center of Virology and Microbiology).

Mean (M) and standard errors of the mean (±SEM) were calculated. Statistical indicators of test effectiveness (sensitivity and specificity) were determined as described [26] at 95-99 % confidence interval.

Results. Rec p30-IB test kit includes immunostrips, positive and negative control sera, protein A conjugate with horseradish peroxidase, chromogenic substrate and stock solutions required for analysis.

The rate of accurately identified truly positive cases (as sensitivity estimate) was assessed for Rec p30-IB using a panel of blood serum and organ specimens positive for anti-ASFV antibodies as shown by IIFA ("golden standard"

test). The rate of truly negative reads accurately identified by Rec p30-IB for blood serum and organ specimens which were ASFV$^-$ in IIFA were considered as specificity estimate. Reproducibility we assessed by comparison of data obtained by two researchers working independently with two experimental series of Rec p30-IB. During tests, we used blood serum and organ specimens from domestic pigs and wild boars sampled both in field and under laboratory conditions.

1. Immunoblotting assay (IB) **and indirect immunofluorescent assay** (IIFA) **of specimens from healthy and ASFV experimentally infected**

Specimens	IIFA		Total
	positive	negative	
Infected (IB positive):			
blood serum	100	0	139
organs	39	0	
Healthy (IB negative):			
blood serum	1	53	72
organs	0	18	
Total	140	71	211

A total of 100 blood specimen out of 101 ones positive in IIFA (including 42 field specimens and 58 specimens from experimental animals) were also positive in IB. All 39 out of 39 IIFA$^+$ specimens of organs were IB$^+$. Hence, diagnostic sensitivity of Rec p30-IB makes 99.3 % at confidence interval of 95 to 99 %. Antibodies to p30 were detected in blood serum of domestic pigs and wild boars regardless of seroimmunotype and virulence of ASFV strains. Out of 53 blood specimens negative in IIFA, 53 were IB$^-$, and out of 18 IIFA$^-$ organ specimens all were IB$^-$. Hence, diagnostic specificity of Rec p30-IB is 100 % (Table 1). Out of 480 blood samples from domestic pigs which were negative in test with validated commercial ASF-IEA Ab/Ag kit, all were IB$^-$ with Rec p30-IB. In 12 blood serum specimens from domestic pigs infected by heterologous viruses (DIC syndrome, CSF, and Aujeszky's disease), antibodies were not detected by IB method, i.e. no false positives were registered. For comparison: reported sensitivity and specificity of ELISA and immunoblotting with baculovirus recombinant p30 and field sera of European domestic pigs are within 96-99 % [18, 27]. Estimates of Rec p30-IB testify on highly reliable results of anti-ASFV antibody identification in blood serum and organs of domestic pigs and wild boars.

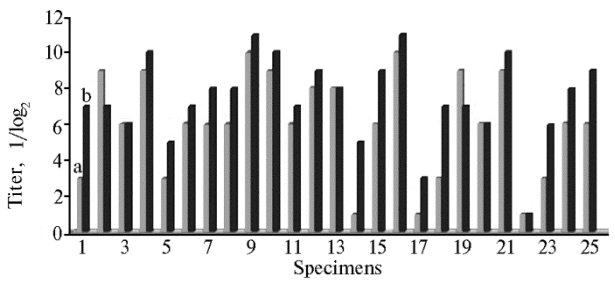

Fig. 1. Analytic sensitivity of indirect immunofluorescence assay (IIFA, a) **and immunoblotting test** (IB, b) **in identification of blood antibodies to ASFV after inoculation:** specimens NoNo 1-7, 10, and 17-25 — inoculated with SKA 2015 VNIIVViM; 8, 9 — with MK-200; 11, 12 — with KK-262/C; 13 — with FK-135 (domestic pigs); 14-16 — with MK-200 (a wild boar) (laboratory test).

We compared effectiveness of Rec p30-IB and ASFV-specific commercial tests offered on the market (Inmunologia y Genetica Aplicada S.A., Spain; IDvet Genetics, France) for 10 ASFV$^-$ specimens (9 of intact non-infected and clinically healthy domestic pigs and 1 of a wild boar) and 10 ASFV$^+$ specimens (9 of domestic pigs and 1 of a wild boar experimentally inoculated with attenuated and virulent ASFV strains). Additionally, we tested 4 positive blood sera received from the reference laboratory CISA-INIA (Madrid). The obtained results show 100 % sensitivity and specificity of Rec p30-IB.

For analytical sensitivity evaluation, we compared IIFA and Rec p30-IB performance for detection of anti-ASFV antibodies in 2-fold dilutions of positive blood serum specimens from domestic pigs and a wild boar inoculated with attenuated ASFV strains of seroimmunotypes II-IV and VIII (Fig. 1). Among 25 exam-

ined positive specimens, the antibody titers with IB exceeded those with IIFA in 19 specimens, were the same in 4 specimens and lower in 2 ones. In 10 organ specimens from domestic pigs inoculated with strain MK-200, the antibody titers determined with Rec p30-IB were 4-8 times higher compared to IIFA. This testifies on higher analytic sensitivity of IB as compared to IIFA.

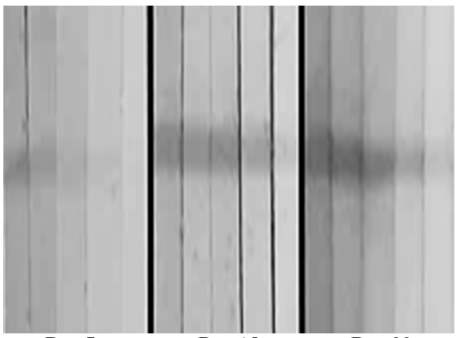

Fig. 2. Titration of blood anti-ASFV antibodies (1:8-1:128) **7 to 28 days after intramuscular inoculation of Large White domestic pigs with strain CKA 2015 VNIIVViM** (10^6 HAU_{50}, immunoblotting test).

For reproducibility estimate, Rec p30-IB should be evaluated either by independent researchers or with different series of the kit. In this experiment, two experts got the same reads for 4 negative and 4 positive specimens. The same results were for 8 negative and 19 positive blood sera of domestic pigs and wild boars tested with two Rec p30-IB series.

Antibodies to p30 are detected from day 7 to day 10 in blood serum of domestic pigs survived after inoculation with attenuated strains LK-111, KK-262/C, MK-200, FK-135, PSA-1-NH, CKA 2015 at 10^3-10^4 HAU_{50}/$TCID_{50}$. On days 15 and 28, the antibody titers rise and intensity of IB-bands increases (Fig. 2).

IB test revealed specific antibodies in 30 % of organ specimens from domestic pigs which died from ASF on days 7-10 after intramuscular inoculation with high virulent strains L-57, M-78 and Stavropol 01/08 at 10^3 HAU_{50}.

As relapses and remissions in chronic ASF occur periodically, it is important whether the serodiagnosis is always correct. In our experiment, we first inoculated pigs with attenuated strain KK-262/C (106 HAU_{50}), and then, 28 days after, infected these animals with virulent strain K-49 (103 HAU_{50}) for simulation of ASF relapse. Specific antibodies induced by strain KK-262/C were detected on days 3 and 5 after K-49 inoculation and not revealed on day 7 (Table 2). From days 12 to 17 of surveillance antibody titers of 1:16 to 1:256 were detected by IB assay. These results testify that in chronic ASF, antibodies may not be detected during remissions. This leads to false negatives in serodiagnosis. Therefore, due to occurrence of low virulent isolates, both serologic and PCR methods are needed for laboratory diagnosis.

2. Blood antibodies in Large White domestic pigs after inoculation with attenuated ASFV strain KK-262/C followed homologous virulent strain K-49 (n = 3, M±SEM, immunoblotting assay)

Days after infection with K-49	Antibody titer, log_2			
	animal No 1	animal No 2	animal No 3	animal No 4
3	6.7±0.6	5.7±0.3	6.3±0.3	6.3±0.6
5	6.3±0.3	5.7±0.6	5.7±0.3	6.3±0.3
7	0.0	0.0	0.0	0.0
12	5.3±0.6	6.3±0.6	5.3±0.3	7.3±0.3
17	6.7±0.3	6.7±0.6	3.7±0.6	7.7±0.6

Note, the collection of the Federal Research Center of Virology and Microbiology involves over 100 ASFV accession from Africa, Latin America, Europe, while abroad, the total number of deposited samples are 500. In our studies of blood of domestic pigs and wild boars infected by virulent, low virulent, attenuated, hemadsorbing and non-hemadsorbing ASFV strains and isolates referred to 5 seroimmunotypes out of known 9, there were no cases when positive serums did not react with recombinant p30.

Therefore, the proposed immunoblot-based test for African swine fever serological diagnosis in blood and biopsy material is effective for laboratory practice and ASF monitoring of domestic pigs and wild boars. Immunoblotting (IB) assay is reliable and does not need complex technical equipment. Given recommendations of the International Epizootic Bureau (OIE — World Organization for Animal Health, Paris, France), IB method should be approved in ASF diagnosis scheme adopted in the Russian Federation.

REFERENCES

1. Dixon L.K., Costa J.V., Escribano J.M., Rock D.L., Vinuela E., Wilkinson P.J. *Family Asfarviridae.* M.H.V.V. Regenmortel (ed.). London Academic Press, 2000: 159-165.
2. Sereda A.D., Balyshev V.M. *Voprosy virusologii*, 2011, 4: 38-42.
3. Malogolovkin A., Burmakina G., Titov I., Sereda A., Gogin A., Baryshnikova E., Kolbasov D. Comparative analysis of African swine fever virus genotypes and serogroups. *Emerg. Infect. Dis.*, 2015, 21(2): 312-315 (doi: 10.3201/eid2102.140649).
4. Gogin A., Gerasimov V., Malogolovkin A., Kolbasov D. African swine fever in the North Caucasus region and the Russian Federation in years 2007-2012. *Virus Res.*, 2013, 173: 198-203 (doi: 10.1016/j.virusres.2012.12.007).
5. Abrahantes J.C., Gogin A., Richardson J., Gervelmeyer A. Epidemiological analyses on African swine fever in the Baltic countries and Poland. *EFSA Journal*, 2017, 15(3): 4732 (doi: 10.2903/j.efsa.2017.4732).
6. Sanchez-Vizcaino J.M., Mur L., Martinez-Lopez B. African swine fever (ASF): five years around Europe. *Vet. Microbiol.*, 2013, 165: 45-50 (doi: 10.1016/j.vetmic.2012.11.030).
7. Gallardo C., Fernández-Pinero J., Pelayo V., Gazaev I., Markowska-Daniel I., Pridotkas G., Nieto R, Fernández-Pacheco P., Bokhan S., Nevolko O., Drozhzhe Z., Pérez C., Soler A., Kolvasov D., Arias M. Genetic variation among African swine fever genotype II viruses, Eastern and Central Europe. *Emerg. Infect. Dis.*, 2014, 20(9): 1544-1547 (doi: 10.3209/eid2009.140554).
8. Vlasova N.N., Varentsova A.A., Shevchenko I.V., Zhukov I.Yu., Remyga S.G., Gavrilova V.L., Puzankova O.S., Shevtsov A.A, Zinyakova N.G., Gruzdev K.N. Comparative analysis of clinical and biological characteristics of African swine fever virus isolates from 2013 year Russian Federation. *British Microbiology Research Journal*. 2015, 5(3): 203-215 (doi: 10.9734/BMRJ/2015/12941).
9. Goller K.V., Malogolovkin A.S., Katorkin S., Kolbasov D., Titov I., Hoper D., Beer M., Keil G.M., Portugal R., Blome S. Tandem repeat insertion in African swine fever virus. Russia, 2012. *Emerg. Infect. Dis.*, 2015, 21(4): 731-732 (doi: 10.3201/eid2104.141792).
10. Frączyk M., Woźniakowski G., Kowalczyk A., Bocian Ł., Kozak E., Niemczuk K., Pejsak Z. Evolution of African swine fever virus genes related to evasion of host immune response. *Vet. Microbiol.*, 2016, 25(193): 133-44 (doi: 10.1016/j.vetmic.2016.08.018).
11. Reis A.L., Parkhouse R.M.E., Penedos A.R., Martins C., Leitro A. Systematic analysis of longitudinal serological responses of pigs infected experimentally with African swine fever virus. *J. Gen. Virol.*, 2007, 88(9): 2426-2434 (doi: 10.1099/vir.0.82857-0).
12. Arias M., Escribano J.M., Sánchez-Vizcaíno J.M. Persistence of African swine fever antibody reactivity on ELISA and immunoblotting assays. *Vet. Rec.*, 1993, 133(8): 189-190.
13. Pastor M.J., Laviada M.D., Sanchez-Vizcaino J.M., Escribano J.M. Detection of African swine fever virus antibodies by immunoblotting assay. *Can. J. Vet. Res.*, 1989, 53(1): 105-107.
14. World Organization for Animal Health (OIE), 2012. African swine fever. Chapter 2.8.1. In: *Manual of diagnostic tests and vaccines for terrestrial animals* (mammals, birds and bees). Available http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.01_ASF.pdf. No date.
15. Escribano J.M., Pastor M.J., Sánchez-Vizcaino J.M. Antibodies to bovine serum albumin in swine sera: implications for false-positive reactions in the serodiagnosis of African swine fever. *Am. J. Vet. Res.*, 1989, 50: 1118-1122.
16. Alcaraz C., Rodriguez F., Oviedo J.M., Eiras A., De Diego M., Alonso C., Escribano J.M. Highly specific confirmatory western blot test of African swine fever virus antibody detection using the recombinant virus protein p54. *J. Virol. Methods*, 1995, 52(1-2): 111-119 (doi: 10.1016/0166-0934(94)00150-F).
17. Kollnberger S.D., Gutierrez-Castaneda B., Foster-Cuevas M., Corteyn A., Parkhouse R.M. Identification of the principal serological immunodeterminants of African swine fever virus by screening a virus cDNA library with antibody. *J. Gen. Virol.*, 2002, 83(6): 1331-1342 (doi: 10.1099/0022-1317-83-6-1331).
18. Gallardo C., Reis A.L., Kalema-Zikusoka G., Malta J., Soler A., Blanco E., Parkhouse R.M., Leitro A. Recombinant antigen targets for serodiagnosis of African swine fever. *Clin. Vaccine Immunol.*, 2009, 16(7): 1012-1020 (doi: 10.1128/CVI.00408-08).

19. Gallardo C., Dufton M.M., Macharia J.M., Arias M., Taracha E.A., Soler A., Okoth E., Martin E., Kasiti J., Bishop R.P. Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes. *Virus Genes*, 2009, 38: 85-95 (doi: 10.1007/s11262-008-0293-2).
20. Oviedo J.M., Rodríguez F., Gymez-Puertas P., Brun A., Gymez N., Alonso C., Escribano J.M. High level expression of the major antigenic African swine fever virus proteins p54 and p30 in baculovirus and their potential use as diagnostic reagents. *J. Virol. Methods*, 1997, 64(1): 27-35 (doi: 10.1016/S0166-0934(96)02140-4).
21. Pérez-Filgueira D.M., González-Camacho F., Gallardo C., Resino-Talaván P., Blanco E., Gymez-Casado E., Alonso C., Escribano J.M. Optimization and validation of recombinant serological tests for African swine fever diagnosis based on detection of the p30 protein produced in *Trichoplusia ni* larvae. *J. Clin. Microbiol.*, 2006, 44 (9): 3114-3121 (doi: 10.1128/JCM.00406-06).
22. Kazakova A.S., Imatdinov I.R., Dubrovskaya O.A., Imatdinov A. R., Sidlik M.V., Balyshev V.M., Krasochko P.A., Sereda A.D. Recombinant protein p30 for serological diagnosis of African swine fever by immunoblotting assay. *Transbound. Emerg. Dis.*, 2017, 64: 1479-1492 (doi: 10.1111/tbed.12539).
23. Sereda A.D., Dubrovskaya O.A., Imatdinov A.R., Strizhakova O.M., Vasil'ev A.P., Sindryakova I.P., Lunitsin A.V. Laboratory diagnostics of chronic and asymptomatic forms of African swine fever. *Agricultural Biology*, 2016, 51(4): 459-466 (doi: 10.15389/agrobiology.2016.4.459eng).
24. Prudnikova E.Yu. *Adaptatsiya virusa afrikanskoi chumy svinei, vydelennogo na territorii Rossiiskoi Federatsii, k perevivaemym kul'turam kletok i izuchenie ego biologicheskikh svoistv. Kandidatskaya dissertatsiya* [Adaptation of the African swine fever virus Russian isolate to transplantable cell cultures and the study of its biological properties. PhD Thesis]. Pokrov, 2013. Available http://search.rsl.ru/ru/record/01005543218. No date (in Russ.).
25. Balyshev V.M., Kalantaenko Yu.F., Bolgova M.V., Prudnikova E.Yu. *Doklady RASKHN*, 2011, 5: 52-53 (in Russ.).
26. Strizhakova O.M., Lyska V.M., Malogolovkin A.S., Novikova M.B., Sidlik M.V., Nogina I.V., Shkaev A.E., Balashova E.A., Kurinnov V.V., Vasil'ev A.P. Validation of an ELISA kit for detection of antibodies against ASF virus in blood or spleen of domestic pigs and wild boars. *Aricultural Biology*, 2016, 51(6): 845-852 (doi: 10.15389/agrobiology.2016.6.845eng).
27. Cubillos C., Gymez-Sebastian S., Moreno N., Nucez M.C., Mulumba-Mfumu L.K., Quembode C.J., Heath L., Etter E.M.C., Jori F., Escribano J.M., Blanco E. African swine fever virus serodiagnosis: a general review with a focus on the analyses of African serum samples. *Virus Res.*, 2013, 173: 159-167 (doi: 10.1016/j.virusres.2012.10.021).

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LUMPY SKIN DISEASES VIRUS ISOLATED IN 2015 IN RUSSIA FROM CATTLE IS PATHOGENIC FOR SHEEP AT EXPERIMENTAL INFECTION

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Abstract

Lumpy skin disease is an economically significant transmissible infectious disease with mortatity rate from 4 to 95 %. Purebred animals are more susceptible to this infection, most seriously the disease occurs in young animals, not enough well-fed individuals, lactating cows. In Russia, the disease is registered since 2015. To eradicate this infection, it is necessary to study all components of the epizootic process. Currently, the studies on the pathogenicity of lumpy skin disease virus for sheep and goats and wild ruminants are insufficient to assess the role of such animals in the transmission of the virus. We estimated for the first time that lumpy skin disease virus isolated from cattle in the Republic of North Ossetia-Alania in 2015 is pathogenic for sheep. The causative agent was identified by sequencing the GPCR gene. In the experiment with 1.5-month-old lambs (n = 4), intravenous and intradermal administration of the suspension of the biopsy samples from sick cows caused the formation of nodules on the skin at the sites of virus inoculation. Nodules were benign in nature, after two weeks it formed the scabs and separated from the skin. On the skin in places of formation nodules there were small scars. The genome of lumpy skin disease virus was detected by real-time PCR in blood samples collected from 9 to 17 days post infection, and in the oral swabs collected from 17 to 27 days post infection. The duration of viremia in lambs ranged from 3 to 8 days. The presence of infectious virus was confirmed by isolation of virus on continuous cell culture of sheep kidney. The clinical signs of the disease corresponded to 2 points calculated in accordance with clinical scoring system within the range from 0 («no visible response») to 10 points («severe generalization, requiring slaughter»). After euthanasia the samples of the liver, popliteal lymph node, lungs and spleen were collected to test for the presence of the viral genome. The genome of the virus was detected only in the lung and lymph nodes. So, our results confirm literature data about pathogenicity of lumpy skin disease virus for sheep. Potentially, sheep can be involved in the epizootic process of lumpy skin disease as source of virus transmitted by blood feeding arthropods.

Keywords: lumpy skin disease, lumpy skin disease virus, sheep, experimental infection, virulence, viremia, PCR, genome, cell culture

Nodular dermatitis in cattle (infectious nodular dermatitis, malignant nodular dermatitis, lumpy skin disease, Dermatitis nodularis bovum) is a transmissible viral disease in cattle manifested by numerous nodules (nods) in skin, epithelium of mouth and nasal musoca, esophagus, trachea, and bronchus. Disease is characteristic of cattle, African buffalos (*Syncerus caffer*), springbucks (*Antidorcas marsupialis*), and gemsbucks (*Oryx leucoryx*, *O. gazelle*) [1, 2].

Nodular dermatitis causative agent is DNA-containing virus of *Poxviridae* family (*Capripoxvirus* genus) [3, 4]. Source of infection is ill animals, as well

as asymptomatic carriers [5]. Nodular dermatitis virus is mainly transmitted by inoculation. C.M. Chihota [6] had shown that nodular dermatitis virus may be transmitted by mosquitoes *Aedes aegypti* within 2-6 days after the agent enters the insect organism with blood of the infected animal. Ability to mechanically transmit virus to sensitive animals was also established in ixodid ticks of *Rhipicephalus*, *Amblyomma*, and *Hyalomma* genera from South Africa [7, 8]. There are data on a relationship between the nodular dermatitis outbreaks and activity of *Stomoxys calcitrans* fly [9]. Contact mode of transmission of nodular dermatitis virus is not proved. As per V.M. Carn and R.P. Kitching [10], intact animals located within 1 month in one box with infected ones remained clinically healthy during the entire surveillance period.

Protection measures at first drifts of the disease to healthy regions are stamping out and radical quarantine [11].

Along with skin nodes, nodular dermatitis virus causes fever, lymphadenopathy, swellings of subcutaneous tissue and organs, conjunctivitis, reduction of milk yields, sexual malfunctions and sterility in cattle. Purebred animals are more sensitive to the infection, most hardly the disease flows in young animals, animals deficient in weight, and lactating cows. Mortality varies from 4 to 95 % [12-14].

Incubation period at experimental infection of the cattle by nodular dermatitis virus is 2-5 days. Viremia is registered in infected animals during 1-2 weeks. Virus dissemination results in injury of mucosa, udder, salivary glands, testis, and other organs. Within 6-9 days following the experimental infection of cattle, nodes of nearly 1 cm in diameter appear in virus inoculation points, and in 12-14 days body temperature goes high up to 40.5 °C. Generalized form of disease is characterized by appearance of nodules on the skin in all parts of the animal body. Formation of nodules is accompanied by inflammation of subcutaneous tissue, and sometimes muscular tissue. Secondary bacterial infection leads to inflammation of lymphatic nodes and skin sores [15, 16].

At pathoanatomical examination, nodules are found in mucosa of intestines, trachea, ventricle, and in udder tissues of lactating cows [17]. Appearance of nodules is accompanied by skin swelling. In generalized form, nodules appear in mucosa of mouth and nasal cavities, vulva and preputial skin with further necrosis and purulent inflammation. In respiratory tract, pathogen causes heavy swelling with possible death from asphyxia or lung swell [18-20].

First cases of nodular dermatitis in Russia were registered in 2015 in cattle in settlements of Tlyaratinsk Region of the Republic of Dagestan bordering with Azerbaijan and Georgia. Later, the diseases occurred in cattle of Naursk Region of Chechen Republic and Kirov Region of the Republic of North Ossetia-Alania [21-23]. According to data of the Information Analytic Department of the Russian Service for Veterinary and Phytosanitary Surveillance, outbreaks of nodular dermatitis in cattle were registered in 2016-2017 in the Russian Federation in the Republic of Dagestan, Bashkortostan, Volgograd, Saratov, Samara, and Orenburg Regions [24].

Effective protection against nodular dermatitis requires deep studies of all components of epizootic process. However, the pathogenicity of the lumpy skin disease virus (LSDV) in small domestic and wild ruminants is still poorly understood, which hampers comprehension of their role in LSDV transmission. There are only few publications on the issue. M.S Kukushkina. et al. [25] report on low pathogenicity of LSDV strain 95 manifested as nodules in the injection point and fever of experimentally infected adult sheep. LSDV pathogenicity for wild impalas (*Aepyceros melampus*) and giraffe (*Giraffe camelopardalis*) is experimentally shown (animals of both sexes died 6-15 day post infection) [26].

This paper reports on the first estimate of pathogenicity of cattle LSDV field isolate (Republic of North Ossetia-Alania, Russia, 2015) for sheep. Our findings show 2-point severity score in infected animals as per a 10-point scale of V.M. Carn et al. [10]. Virus was detected in blood by PCR analysis and direct isolation in sheep kidneys cell culture (PO-VNIIVViM), as well as in lungs and spleen. These data indicate that sheep, despite weak pathogenicity of LSDV at infection, may be involved in LSDV transmission.

Purpose of this study was to estimate pathogenicity of cattle isolate of LSDV for experimentally infected sheep.

Techniques. Bioptates of skin nodes from cows with nodular dermatitis (Republic of North Ossetia-Alania) were collected by employees of the Republican Veterinary Service in 2015 and kept in thermal container (+4...+10 °C). For analysis, bioptates were grinded in porcelain jar with phosphate buffer. After centrifugation, the prepared 10 % suspensions were used to infect experimental animals and to extract viral DNA for identification.

DNA for sequencing and PCR analysis was extracted with RIBO-sorb kit (ILS CJSC, Moscow). After experimental infection, viral genome DNA was detected as per T.R. Bowden et. al. [27] method with oligonucleotide primers CaPV 074 F_1 (5′-AAAACGGTATATGGAATAGAGTTGGAA-3′), CaPV 074 R_1 (5′-AAATGAAACCAATGGATGGGATA-3′′) and hybridization probe CaPV-074P_1 (5′-FAM-TGGCTCATAGATTTCCT-MGB-NFQ-3′). PCR mixture contained 10 pM of each primer, 3 pM fluorescent probe (Sintol CJSC, Russia), 2,5 μl 10× DNA buffer, 10 mM dNTPs and 1.5 IU recombinant Taq DNA polymerase (Thermo Fisher Scientific, USA). Real-time PCR was protocol was as follows: initial denaturation at 95 °C for 10 minutes; amplification at 95 °C for 15 sec, 60 °C for 1 min (45 cycles) (Rotor Gene 6000 amplification detection system, Corbette Research, Australia).

GPCR gene fragment PCR amplification (Gradient Palm Cycler, Corbett Research, Australia) and Sanger sequencing (3130xl Genetic Analyzer, Applied Biosystems, USA) were performed. Amplification was performed as per C. Le Goff et al. [28] with olygonucleotide primers (5′-TTAAGTAAAGCATAACT-CCAACAAAAATG-3′ and 5′-TTTTTTTATTTTTTATCCAATGCTAATACT-3′) and Encyclo Plus PCR kit (Eurogen CJSC, Russia) according to following protocol: initial denaturation at 94 °C for 3 minutes; 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 60 sec (35 cycles).

Nucleotide sequences were analyzed with MEGA 7.0 software (https://www.mega-software.net/) and Neighbor-Joining tree clustering.

Infection activity of 10 % bioptate suspension was determined in 2 clinically healthy Kalmyk calves aged 6 months (210-220 kg weight) by subcutaneous injection of 0.25 cm^3 aliquots of 10^{-1}-10^{-5} dilutions in four points along lines vertical to spinal column at 5-6 cm distance between dilutions. Skin injuries in 14-20 days post injection were and lumps were inspected visually.

To assess pathogenicity of LSDV for sheep, four Romanov lambs aged 1.5 months (7-8 kg weight) were inoculated with virus containing 10 % bioptate suspension (1.0 cm^3 in jugular vein and 0.25 cm^3 subcutaneously in each of four points in axilla). Prior to infecting, the control lamb was places in separate housing and inoculated with physiological solution in the same mode. Animals were clinically inspected daily. Each 3 days, blood samples, mouth and nasal washes were collected for viral genome analysis.

Positive blood samples from infected lambs were used for LSDV identification in sheep kidney cell culture (PO VNIIBBiM) as per protocol of World Organization for Animal Health (International Epizootic Bureau, Paris, OIE — IEB) [29-31]. LSDV infection activity in specimens was determined by titration in

sheep kidney cell culture (48-well plates, Corning-Costar, USA) with Eagle MEM (Biolot LLC, Russia). Titers were calculated by Kerber's method.

After blood-free euthanasia (Adilin-super medicine, Federal Center of Toxicology, Radiation, and Biological Safety, Kazan), samples of hepatic tissue, popliteal lymph nodes, lungs, and spleen were collected to detect viral genome.

Tests on animals were carried out according to approval of Federal Agency for Scientific Organizations of Russia (No 33-11-0132/16.06.2016) under supervision of Bioethics Commission of Federal Research Center of Virology and Microbiology.

At calculation of virus titers, mean (*M*) and standard errors of the mean (±SEM) were determined.

Results. Pathogen which caused clinical signs of nodular dermatitis in cows in 2015 in the Republic of North Ossetia-Alania was identified by GPCR (G-protein-coupled chemokine receptor) gene sequencing. *GPCR* is a host-range gene suitable for discrimination of capripoxviruses [28]. Analysis of the sequence we obtained and deposited in GenBank database (accession No KY595106) with other LSDV full-size *GPCR* gene sequences from GenBank identifies this Russian isolate as nodular dermatitis virus (Fig. 1).

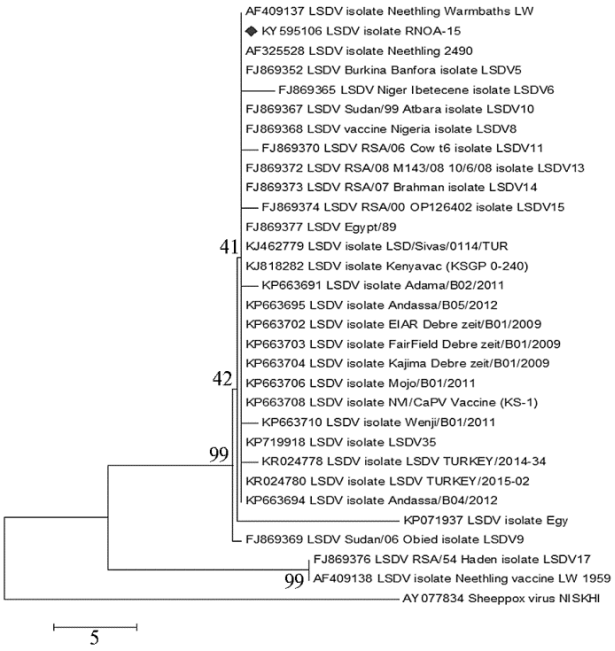

Fig. 1. *GPCR* gene-based phylogenetic tree of LSDV isolates. *GPCR* gene sequence of sheep pox virus strain NISHI is external comparison group. Rhomb labels the studied isolate. Tree branches are scaled in number of nucleotide replacements for sequence.

In preliminary test, the bioptate suspension diluted up to 10^{-4} causes local skin nodes in calves on day 20. Hence, the LSDV titer is $10^{5.1}$ ID_{50}/cm^3 (given the used volume of 0.25 cm^3) and in experimental infection, total (intravenous and subcutaneous) infectious dose is $10^{5.4}$ ID_{50}. Nodules in the points of virus entering (Fig. 2) occurred in lambs on day 10 to day 13 post inoculation. Skin indurations were constantly growing in diameter from 2 to 5 cm during 7 days. The injured skin healed and scabs appeared since week 2 after infection with full healing on week 3. Temperature reaction in all infected lambs during 25 day surveillance was within the norm (38.8-40.5 °C).

In this experiment we used 10-point scale proposed by V.M. Carn et al. [10], with 0 for absence of visual reactions, 1 for transitive local response, 2 for local moderate response (nodules of less than 5 cm in diameter, no lymphadenopatia), 3 for moderate local response with nodules of less than 5 cm in diameter and moderate lymphdenopatia, and 10 for generalized infection with numerous secondary nodules of 0.5-5.0 cm in diameter, swelling, hyperemia, severe lymphadenopatia, conjunctivitis, rhinitis, apathy, loss of appetite and deaths, which requires slaughter. As per the scale, clinical severity in lambs infected with LSDV from cattle scores 2 points that means low pathogenicity for sheep. Skin of a control

lamb had no visual changes. Body temperature was 38.5-39.0 °C during the entire surveillance period.

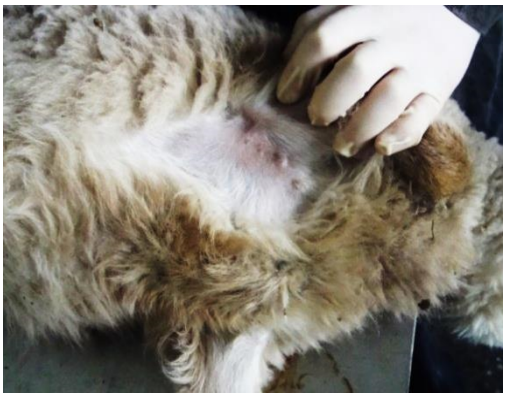

Fig. 2. Skin nodules in lamb at inoculation with LSDV containing biobtate collected in 2015 in the Republic of North Ossetia-Alania (day 13 post injection).

On day 15, the lamb No 2 was subjected to euthanasia for autopsy of internal organs. No pathologic changes typical for bovine nodular dermatitis were visually found. Of liver, popliteal lymph node, lungs and spleen specimens, only in lungs and lymph nodes LSDV genome was detected by qPCR method (Ct values of 36.48 and 32.94, respectively).

qPCR test revealed LSDV genome in blood of experimentally infected lambs on days 9-17 and in mouth washes on days 20-27 post inoculation (Table 1), unlike the control lamb.

1. qPCR identification of cattle LSDV genome (Ct) **in experimentally infected lambs**

Days post experimental inoculation	Lamb No 1		Lamb No 2		Lamb No 1		Lamb No 4	
	blood	washes	blood	washes	blood	washes	blood	washes
3	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	37.45	–
13	–	–	–	–	–	–	36.23	–
15	35.99	–			-	–	–	-
17	38.38	–			39.08	–	37.23	-
20	–	36. 65			–	–	–	35.71
23	–	35.51			–	–	–	36.43
27	–	38.34			–	–	–	33.19
29	–	–			–	–	–	–

N o t e. Virus containing bioptate for inoculation was collected in 2015 in the Republic of North Ossetia-Alania. Samples with Ct ≤ 40 are positive; «–» means negative samples with no Ct. Lamb No 2 was subjected to euthanasia on day 15 for pathoanatomic and PCR study.

2. LSDV genome isolation from blood of experimentally infected lambs in sheep kidneys cell culture PO VNIIVViM

Days post experimental inoculation	Virus titer, lg TCD_{50}/cm^3			
	control lamb	lamb No 1	lamb No 3	lamb No 4
3	Not tested	Not tested	Not tested	Not tested
6	Not tested	Not tested	Not tested	Not tested
9	–	–	–	–
13	–	–	–	–
15	–	1,0±0,12 (+)	–	1,5±0,14 (+)
17	–	1,5±0,24 (+)	1,6±0,15 (+)	1,7±0,18 (+)
20	–	–	–	–
23	–	–	–	–
27	Not tested	Not tested	Not tested	Not tested
29	Not tested	Not tested	Not tested	Not tested

N o t e. «–» — virus not found (no cytopathic effect for 5 consecutive passages); «+» — presence of specific cytopathic action of virus on the 2nd passage.

Isolation from blood of the experimentally infected lambs in sheep cell culture confirms viremia after day 13 till day 23 post inoculation. Note that the LSDV-specific cytopathic action occurs only in passage 2 in 5-7 days after culture inoculation at viral titer of 1.5-1.7 lg TCD_{50}/cm^3 (Table 2).

Thus, our studies confirm the available data on pathogenicity of nodular dermatitis virus for sheep during experimental infection [25].

Therefore, field LSDV isolate from cattle (the Republic of North Ossetia-Alania, 2015) injected to sheep subcutaneously and intravenously causes skin nod-

ules only the points of injections. Observed clinical signs correspond to 2 points score of 10-point scale of severity. qPCR test detects LSDV genome in blood, mouth washes, lungs and popliteal lymph nodes of experimentally infected lambs. LSDV isolation from qPCR positive blood samples in sheep cell culture also confirms presence of the pathogen. These facts bring to assumption that sheep can serve a source of nodular dermatitis virus. More studies, including those on the carrier role, are required to ultimately understand whether LSDV transmission by sheep may naturally occur.

REFERENCES

1. Ryabikina O.A., Diev V.I., Kukushkina M.S.). *Voprosy veterinarnoi biologii*, 2015, 4(28): 45-52 (in Russ.).
2. European Food Safety Authority. Scientific opinion on lumpy skin disease. *EFSA Journal*, 2015, 13(1): 3986.
3. Tulman E.R., Afonso C.L., Lu Z., Zsak L., Kutish G.F., Rock D.L. Genome of lumpy skin disease virus. *J. Virol.*, 2001, 75(15): 7122-7130 (doi: 10.1128/JVI.75.15.7122-130.2001).
4. *Virus Taxonomy: classification and nomenclature of viruses. Ninht report of the International Committee on Taxonomy of Viruses.* A.M.Q. King (ed.). Elsevier Academic Press, 2012.
5. Zakutskii N.I., Balyshev V.M., Yurkov S.G., Guzalova A.G., Lunitsin A.V. *Veterinarnyi vrach*, 2016, 4: 3-12 (in Russ.).
6. Chihota C.M., Rennie L.F., Kitching R.P., Mellor P.S. Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (*Diptera*: *Culicidae*). *Epidemiology and Infection*, 2001, 126(2): 317-321.
7. Lubinga J.C., Tuppurainen E.S.M., Mahlare R., Coetzer J.A.W., Stoltsz W.H., Venter E.H. Evidence of transstadial and mechanical transmission of lumpy skin disease virus by *Amblyomma hebraeum* ticks. *Transbound. Emerg. Dis.*, 2015, 62(2): 174-182 (doi: 10.1111/tbed.12102)
8. Tuppurainen E.S.M., Stoltsz W.H., Troskie M., Wallace D.B., Oura C.A.L., Mellor P.S., Coetzer J.A.W., Venter E.H. A potential role for ixodid (hard) tick vectors in the transmission of lumpy skin disease virus in cattle. *Transbound. Emerg. Dis.*, 2011, 58(2): 93-104 (doi: 10.1111/j.1865-1682.2010.01184.x).
9. Chihota C.M., Rennie L.F., Kitching R.P., Mellor P.S. Attempted mechanical transmission of lumpy skin disease virus by biting insects. *Med. Vet. Entomol.*, 2003, 17(3): 294-300 (doi: 10.1046/j.1365-2915.2003.00445.x).
10. Carn V.M., Kitching R.P. An investigation of possible routes of transmission of lumpy skin disease virus (Neethling). *Epidemiol. Infect.*, 1995, 114(1): 219-226.
11. Mishchenko A.V., Mishchenko V.A., Kononov A.V., Shevkoplyas V.N., Dzhailidi G.A., Dresvyannikova S.G., Chernykh O.Yu. *Veterinariya Kubani*, 2015, 5: 3-6 (in Russ.).
12. Chernykh O.Yu., Mishchenko A.V., Mishchenko V.A., Gubeeva E.G., Papunidi K.Kh., Chernov A.N., Lysenko A.A., Shevchenko A.A., Shevkoplyas V.N., Vatsaev Sh.V. *Veterinariya Kubani*, 2017, 3: 1-3 (in Russ.).
13. Samuilenko A.Ya., Solov'ev B.V., Nepoklonov E.A., Voronin E.S., Fomina N.V., Grin' S.A., Belousov V.I., Mel'nik N.V., Guban E.A., Eremets V.I., Sapegina E.P., Yamnikova S.S., Tsybanov S.Zh. *Nodulyarnyi dermatit. Infektsionnaya patologiya zhivotnykh. Tom 1* /Pod redaktsiei A.Ya. Samuilenko, B.V. Solov'eva, E.A. Nepoklonova, E.S. Voronina [Nodular dermatitis. Infectious pathology of animals. V. 1. A.Ya. Samuilenko, B.V. Solov'ev, E.A. Nepoklonov, E.S. Voronin (eds.)]. Moscow, 2006 (in Russ.).
14. Kosareva, O.A., Kukushkina M.S., Konstantinov A.V., Diev V.I, Starov S.K., Yasneva E.A., Basova D.K. *Trudy Federal'nogo tsentra okhrany zdorov'ya zhivotnykh* (Vladimir), 2010, 8: 73-84 (in Russ.).
15. Body M., Singh K.P., Hussain M.H., Al-Rawahi A., Al-Maawali M., Al-Lamki K., Al-Habsy S. Clinico-histopathological findings and PCR based diagnosis of lumpy skin disease in the Sultanate of Oman. *Pak. Vet. J.*, 2012, 32(2): 206-210.
16. Iros P.S., Tupprainen E.S.M., Venter E.H. Excretion of lumpy skin disease virus in bull semen. *Theriogenology*, 2005, 63: 1290-1297 (doi: 10.1016/j.theriogenology.2004.06.013).
17. Babiuk S.L., Bowden T.R., Parkyn G., Dalman B., Manning L., Neufeld J., Embury-Hyatt C., Copps J.S., Boyle D.B. Quantification of lumpy skin disease virus following experimental infection in cattle. *Transbound. Emerg. Dis.*, 2008, 55(7): 299-307 (doi: 10.1111/j.1865-1682.2008.01024).
18. Brenner J., Haimovitz M., Oron E., Stram Y., Fridgut O., Bumbarov V., Kuznetzova L., Oved Z., Waserman A., Garazzi S., Perl S., Lahav D., Edery N., Yadin H. Lumpy skin disease (LSD) in a large dairy herd in Israel. *Isr. J. Vet. Med.*, 2006, 61: 73-77.

19. Tuppurainen E., Alexandrov T., Beltrán-Alcrudo D. *Lumpy skin disease field manual for veterinarians.* FAO Animal Production and Health Manual No. 20. Food and Agriculture Organization of the United Nations (FAO), Rome, 2017: 7-15.
20. Tuppurainem E., Oura C. Lumpy skin disease: an African cattle disease getting closer to the EU. *Vet. Rec.*, 2014, 175(12): 27 (doi: 10.1136/vr.g5808).
21. Mishchenko A.V., Mishchenko V.A. *Tezisy konferentsii «X Baltiiskii forum veterinarnoi meditsiny i prodovol'stvennoi bezopasnosti 2014»* [Proc. X Baltic Forum of Veterinary Medicine and Food Safety 2014]. St. Petersburg, 2014: 165-167 (in Russ.).
22. Krivonos R.A., Dzhailidi G.A., Mishchenko A.V., Mishchenko V.A, Chernykh O.Yu., Shevkoplyas V.N., Dresvyannikova S.G., Kolomiets D.V., Tikhonov S.V. *Veterinariya segodnya*, 2017, 1(20): 38-44 (in Russ.).
23. Gerasimov V.N., Lunitsin A.V., Sal'nikov N.I., Gogin A.E., Eremeev N.A., Kolbasov D.V. *Veterinariya*, 2016, 3: 11- 14 (in Russ.).
24. Rossel'khoznadzor. *Neblagopoluchnye regiony RF po nodulyarnomu dermatitu v 2017 godu* [Federal Service for Veterinary and Phytosanitary Surveillance. Territories of nodular dermatitis risk in Russia, 2017]. Available http://fsvps.ru. No date (in Russ.).
25. Kukushkina M.S., Ryabikina O.A., Kononov A.V., Diev V.I. *Veterinariya segodnya*, 2016, 4(19): 46-48 (in Russ.).
26. Young E., Basson P.A., Weiss K.E. Experimental infection of game animals with lumpy skin disease virus prototype strain Neethling. *Onderstepoort J. Vet.*, 1970, 37(2): 79-87.
27. Bowden T.R., Babiuk S.L., Parkyn G.R., Copps J.S., Boyle D.B. Capripoxvirus tissue tropism and shedding: a quantitative study in experimentally infected sheep and goats. *Virology*, 2008, 371: 380-393 (doi: 10.1016/j.virol.2007.10.002).
28. Le Goff S., Lamien Ch.E., Fakhfakh E., Chadeyras A., Abu-Adulugba E., Libeau G., Tuppurainen E., Wallace D.B., Adam T.,Silber R., Gulyaz V., Madani H., Caufour P., Hammami S., Diallo A., Albina E. Capripoxvirus G-protein-coupled chemokine receptor: a host-range gene suitable for virus animal origin discrimination. *J. Gen. Virol.*, 2009, 90: 1967-1977 (doi: 10.1099/vir.0.010686-0).
29. Yurkov S.G., Kolbasova O.L., Zuev V.V., Kushnir S.D., Neverovskaya N.S., Anisimova L.I., Smyslova N.Yu., Prilepskaya E.P., Zhdanova N.A., Filatov A.V. *Katalog kollektsii kletochnykh kul'tur GNU VNIIVViM* [Catalog of GNU VNIIVViM cell culture collection]. Pokrov, 2010 (in Russ.).
30. Office International des Epizooties (OIE). *Manual of diagnostic tests and vaccines for terrestrial animals 2017. Chapter 2.4.13. Lumpy skin disease.* Available http://www.oie.int/fileadmin/Home/eng/. No date.
31. Syurin V.N., Belousova R.V., Solov'ev B.V., Fomina N.V. *Metody laboratornoi diagnostiki virusnykh boleznei zhivotnykh* [Methods for laboratory diagnosis of viral diseases in animals]. Moscow, 1986 (in Russ.).

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