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D-AMINO ACIDS AND THEIR OXIDASE IN FARM ANIMALS — THE ROLE AND OPPORTUNITIES

(review)

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Abstract

In the article, the basic physiological and biochemical functions and the importance of D-amino acids and D-amino acid oxidase in mammalians are described. Serious attention is paid to metabolic role of D-amino acid oxidase in health and disease. D-amino acids and D-amino acid metabolizing enzyme has been discovered by Krebs 1935 (H. Krebs, 1972). Nowadays, most attention is given to the signal, communication and regulatory role of peroxisomes in metabolism. The peroxisomes are considered as candidate agents able to provide a relationship between the nervous and endocrine systems and participate in metabolism regulation in cells, organs and the body. Significant amounts of D-amino acids, the L-amino acids stereoisomers, are found in the peroxisomes of various organs and tissues: in the nervous system (J. Sasabe et al., 2014.), endocrine glands (A. D'Aniello et al., 2000), liver, kidney, breast, etc. (S.V. Khoronenkova, V.I. Tishkov, 2008). These active molecules provide communications in the neuronal network via the synapses of nerve endings (C.W. Morgans et al., 2013) and are involved in cell aging and apoptosis (A.V. Worms, 2010), biosynthesis and secretion of hormones, blood pressure regulation, maintenance of cell osmotic pressure (Y. Nishina, 2008), anti-inflammatory reactions and anti-carcinogenesis (G.H. Fisher, 1998). D-amino acid oxidase affects the activity of body as a whole in very diverse ways due to simultaneous engagement in diametrically opposite multi-parametric processes, such as regulation of cell level of D-amino acids and amino acid D-amines; the activity of central and peripheral nervous system; the maintenance of cell ecology; biosynthesis and secretion of epiphyseal melatonin (H.K. Park et al., 2007), hypothalamic releasing factors, pituitary, thyroid and steroid hormones (A. Santillo et al., 2014); defense against xenobiotics, microorganisms, viruses, stresses, and malignant tumors (R. Rana et al., 2012). Special attention the authors of this paper pay to the role of D-amino asides and D-amino aside oxidase in farm animals. World publications are mostly devoted to physical and chemical properties and the involvement of these agents in a number of neurodegenerative diseases and human clinical pathology of the central and peripheral parts of brain. As to farm animals, the data are almost completely absent. Recently, a growing body of in-deep examinations appear of the impact of D-amino acid oxidase and D-amino acids on the entire hierarchy of the endocrine system from the pineal gland, the hypothalamus and the pituitary gland to the ovaries and testes (S. Yasuaki et al., 2012). The studies are mostly focused on the regulation mechanisms of reproductive function in humans and animals. Elucidation of the involvement of D-amino acids and D-amino acid metabolising enzyme in excitation and inhibition processes in the central nervous system is of a particular interest for farm animal biology. Cognitive function in farm animals is of a separate interest as it is tightly associated with animal adaptability to commercial production, formation of the nervous system type and a reduced aggressiveness. Eventually, these will result in control of farm animal behavior and performance.

Keywords: D-amino acids, D-amino acid oxidase, peroxisomes, nervous and endocrine regulation of metabolism

The phenomenon of optical isomerism was discovered in the XIX century by Louis Pasteur, who discovered the left and right rotations of polarization in culture media. Pasteur associated it with the ability of individual molecules to exist in the form of two mirror-antipodal "dissymmetric" spatial configurations, and

called them chiral (Gr. “hand”). Pasteur defined this property of molecules as fundamental and universal, by this attracting close attention to stereoisomerism (tautomerism). There still are undiscovered spots in this field. Discussions continue; the hypotheses are put forward and require theoretical and experimental justification [1]. Since the middle XX century it is known that the use of a small amount of D-amino acids instead of L-amino acids in feeding laboratory and farm animals leads to negative consequences. There are also forecasts that chiral pollutants may become a global environmental problem [1]. Interest in D-amino acids and their oxidases has grown into an interdisciplinary issue [1]. The majority of scientific works deal with these substances' functions in the central and peripheral nervous system in humans. However, the studies on the issue with special reference to agricultural animals are extremely few and have not been conducted in Russia.

The aim of this review is to systematize all available data on the role of D-amino acids and their oxidases in metabolism in agricultural animals in normal and pathological ways, describing their specific functions in metabolic pathways in various systems.

Special interest is drawn to studying D-amino acids and their oxidase (D-amino-acid oxidase, DAAO) due to the processes of excitation and inhibition in the central nervous system, adaptability to industrial conditions, the formation of the type of the nervous system, the reduction of aggressiveness, the establishment of the social rank of an animal and, finally, with the knowledge and management of behavioral responses in agricultural animals [2]. This field should expect the emergence of brainstorm and the consequent development of ways to increase the quantity and quality of livestock products. Scientists still cannot count the amino acids existing in living nature in the form of D-isomers, and how many oxidases of D-amino acids are found in the body. All known amino acids undergo racemization reactions, but their rates vary greatly. As for the oxidase, the answer is also ambiguous. In all the world literature, D-amino acid oxidase was called a singular, and it had the classification number EC 1.4.3.3. However, the official nomenclature has another enzyme, D-asparaginic acid oxidase (EC 1.4.3.1). It is mentioned in a much smaller number of papers [3]. The first enzyme has broad substrate specificity with the corresponding imino acid, ammonia and hydrogen peroxide as the final products of the catalyzed reactions. The byproducts of the second enzyme (also being a deaminating one) are oxaloacetate, ammonia and hydrogen peroxide. To some extent, the D-amino acids oxidase is quantitatively capable of exhibiting activity on D-aspartate and vice versa. But this fact still needs to be specified. Scientific literature does not have clear physiological and biochemical justification for these two enzymes functioning in the body. Perhaps it is predetermined by the exclusive part of D-aspartate in the N-methyl-D-aspartic receptor, the specific value of oxaloacetate in the metabolism and the functions of D-aspartic acid in the endocrine system. This work refers to the oxidase of D-amino acids in the singular, as it is generally accepted in the world scientific literature.

D-amino acids play a key role in the regulation of a number of processes in a cell. DAAO is one of the main enzymes responsible for maintaining the required amount of D-amino acids in the body. The mechanism of this regulation is multidirectional and has multiple parameters. The same enzyme simultaneously affects the content of all D-amino acids and D-derivatives of biologically active amines, which can initiate various metabolic pathways and physiological effects [4]. As a rule, with advancing age of animals and in case of pathological conditions of different nature, the concentrations of D-serine in blood and cerebrospinal fluid decrease. However, at the same time there always is a simultane-

ous decrease in the number of D-amino acids and an increase in the activity and expression of D-amino acid oxidase [5].

For quite a long time, they believed that D-amino acids were not synthesized in the mammalian body, and their presence was explained by the hydrolysis carried out by commensal microorganisms. However, due to modern analytical methods, significant amounts of D-amino acids were found in mammals. D-serine and D-aspartate are the most common. D-serine, which is synthesized by serine racemase and degraded by DAAO, is found in the brain and is considered as one of the two leading neurotransmitters. D-aspartate, synthesized by the aspartate racemase and degraded by oxidase D-aspartate, is present in significant amounts in neuroendocrine structures and organs of the endocrine system. It is known as a significant regulator of synthesis and secretion of hormones and spermatogenesis. D-serine and D-aspartate join the glycine site of the corresponding subtype of the N-methyl-D-aspartate receptor (NMDA) and operate as two major agonists and co-agonists to accelerate signal transfer between glia and neurons [6].

D-amino acid oxidase is a flavoenzyme that contains flavin adenine dinucleotide (FAD) in the active site. D-amino acid oxidase mainly catalyzes the oxidative deamination of the basic and neutral D-amino acids to the corresponding imino acids responsible for optical inversion. The reaction also results in forming hydrogen peroxide, the FAD is reduced, and the imino acid is non-enzymatically decomposed to the α-keto acid and the ammonium ion. The active center has SH-groups and ferrum atom. The optimal pH for both oxidases ranges from 8.5 to 11; the medium should be oxygenated. D-amino acids oxidase was found in all eukaryotic cells, except for the cells of higher plants. In mammals, it is found in brain, kidneys, liver, lungs, etc. It is remarkable that DAAO concentration in liver and kidneys of pigs is particularly high [7]. Multiple enzyme functions indicate the relation between changes in DAAO activity in the cell with different regulatory processes in the body [8, 9]. A characteristic feature of DAAO is the almost absolute specificity to D-isomers with different specificity to various D-amino acids, while catalytic activity on L-forms is almost not detectable [10].

DAAO performs a variety of physiological functions which rank from anabolic one in yeast cells, that allows them to grow only on D-amino acids as sources of carbon, nitrogen and energy, to the regulatory one in brain of higher animals, where DAAO controls the amount of D-serine neuromodulator (11). The enzyme that oxidizes D-amino acids was discovered by H.A. Krebs in 1935 and has become the object of numerous studies [11]. This enzyme is considered as a general model to describe the functioning flavoproteins of the dehydrogenase-oxidase class. Structural and functional research suggests that a specific physiological function is performed through various structural elements that control access to the active center and substrate or byproducts [12]. The kinetic characteristics of pig DAAO indicate that the enzyme binds FAD more rapidly than L-amino acid oxidase and exists as a stable homodimer even in apoprotein form. The chemical properties of oxidase are determined by the specificity of hydrophobic bonds of short fragments of amino acid sequences. These fragments are associated with the surface of the flavin nucleous, forming a unique stable conformation with specific kinetic characteristics [13].

The interest of scientists in DAAO has been rapidly growing since the mid-1990s. Firstly, this was due to numerous experimental data that justified the extremely important role of D-amino acids in the vital activity of an organism. Secondly, genetic engineering create recombinant producers of D-amino acid oxidase and allows obtaining the enzyme in quantities sufficient for studying.

Thirdly, the sequencing of entire genomes of prokaryotes and eukaryotes has provided the background for the search and cloning of the new DAAO genes. Fourth, the methods of enzymological engineering allow constructing an enzyme capable of easy and strong fixation on insoluble carriers, for use as sorbents, disinfectants, enzyme electrodes, etc. [14].

A number of metabolic conditions are associated with increased expression of DAAO and its specific activator, the G72 protein, discovered in 2002. It is still discussed, by what mechanisms G72 protein plays a regulatory role in the activity of D-amino acid oxidase. It was found that amino acid residues at positions 123-153 and 138-153 in the long G72 isoform bind to DAAO and increase its activity by 22 and 32 %, respectively. More detailed studies [15] showed that these regions of G72 interact with special loops in the DAAO molecule and create a tunnel that facilitates the substrate and cofactor's penetration into the active center of the enzyme. Molecular mechanism involving the interaction between the C-terminal fragment of G72 and DAAO that can affect the properties of the N-methyl-D-aspartate receptor potentiating neurotransmission, is also considered. Similar channels, or tunnels, are also described for hydrogen peroxide and oxygen [15]. Protein G72, activating DAAO, promotes oxidative deamination of D-3,4-dihydroxy phenylalanine and D-serine. D-3,4-dihydroxy phenyl alanine is converted to L-3,4-dihydroxyphenylalanine, a precursor of dopamine, while D-serine is involved in the glutamatergic system. These results show that variations in the D-amino acid oxidase gene affect the turnover of dopamine in healthy adult individuals. Perhaps the dopamine metabolism disorder serves as a mechanism responsible for the relationship between the genetic variations of D-amino acid oxidase and the behavioral phenotype.

Since the discovery in mammals, D-serine has become the most studied “unnatural amino acid”. This transmitter-like amino acid, found in brain in a significant amount, plays a major role in the central nervous system in higher animals by modeling the activity of the subtype of the glutamine N-methyl-D-aspartate receptor. Normal brain development and functioning requires the physiologically optimal content of D-serine. Any changes in the amount of this neuromodulator may cause pathologies [16]. About a half of research works on D-amino acids published in the last few years deals with the role of D-serine as a high-grade neurotransmitter, the neuromodulator and allosteric agonist of glycine-binding site at the glutamine receptor of N-methyl-D-aspartate, which plays an important role in many physiological and pathophysiological processes [17].

A decrease in D-serine content is dangerous because of high affinity of D-serine interaction with the glycine-binding site of NMDA receptors [18]. The decrease in the amount of D-serine reduces the functional activity of NMDA receptors, which is considered to be one of the main causes of abnormalities development in the central and peripheral nervous system. Recently, this hypothesis has been accepted as the main one, since the long-term potentiation of the synaptic transmission between the two neurons can maintain a prolonged activation of the synapses activity. By acting on the synaptic path, D-serine participates in mechanisms that provide synaptic plasticity, allowing the nervous system to adapt to changing environment [19].

In the brain of mutant mice and rats with DAAO-associated enzyme defect, the amount of D-amino acids, particularly D-serine, significantly increases. DAAO catabolizes D-serine and thus modulates neurotransmission. Mutant mice behave like animals with altered activity of the glutamate N-methyl-D-aspartate receptor subtype due to the increased content of D-serine. D-serine and DAAO play an important role in cerebral activity and synaptic mobility. They are involved in various physiological and pathological responses of the central nervous

system [20]. D-serine performs the NMDA functions and is known as an enhancer of the negative impact and cognitive symptoms in nervous disorders, unlike the weak effects of standard D-2 antagonists. The inhibition of DAAO is of significant interest as an effective way to increase the amount of D-serine in brain. Over the past few years, a large number of DAAO inhibitors have been discovered. Some of them have significantly higher activity than that of conventional D-amino acid oxidase inhibitors. Many of the recently described DAAO inhibitors show activity in increasing the amount of D-serine. They are efficient in laboratory animals when modeling behavioral responses, aggressiveness and adaptability [21].

Mice with partial defects in serine racemase and D-aspartate oxidase are capable of reproduction and growth. They have mutations in D-amino acid oxidase gene which is found in rats and mice. Behavioral responses in mutant animals differ from those of normal animals due to increased or decreased activity of the NMDA receptor [22].

Oxidase of D-amino acids catalyzes oxidative deamination of D-amino acids including the D-serine, the agonist of glycine modulatory site in the receptor for N-methyl-D-aspartate. It is shown that DAAO is important in the D-serine clearance during blood circulation. Therefore, DAAO inhibitors with a longer biological half-life can maintain a higher D-serine blood level for a long time, which can have a significant effect in the treatment of systemic hypertension [23].

According to the recent fidings, D-3,4-dihydroxy-phenylalanine (D-DHPA) is the best DAAO substrate in laboratory animals compared to D-serine [24]. The catalytic efficiency denoted as k_{cat}/K_M (the catalytic constant to the Michaelis constant ratio) for DHPA was 14 times higher than that for D-serine. That is, DAAO provides an efficient alternative metabolic pathway for the conversion of D-DHP to dopamine, which may be interesting for controlling behavioral responses and reducing animal aggressiveness.

G.H. Fisher [25], while studying the physiological role of high D-alanine concentrations in the intracellular space of tissues and organs, suggested that this amino acid is involved in the regulation of intracellular osmotic pressure.

According to the concentration in the cells of higher animals, D-proline and D-leucine are on the fourth place after D-serine, D-asparagine and D-alanine. Currently, the physiological role of D-proline is being discussed. In the organism, unidirectional optical inversion of amino acids and analogues of D-amino acids is carried out. Exogenous NG-Nitro-D-arginine and endogenous D-phenylalanine and D-methionine are inverted much faster than other D-amino acids. In the cells of long-lived tissues (dentin, tooth enamel, crystalline lens, etc.), there is a clear correlation between the age of the animal and the D-hydroxyproline and D-aspartate concentrations [25]. Data on neuro-, hepato- and renal toxicity of D-proline for rats are actively discussed [26]. The study of the content of D-proline in various tissues in normal and mutant (absence of the gene responsible for the synthesis of DAAO) in mice showed that in the latter, D-proline accumulates in larger amounts in the kidneys, and its excess is naturally excreted [26].

D-aspartate oxidase found in peroxisomes of the liver, kidneys and many other tissues and organs in humans, cattle, pigs, and sheep serves as a peroxisome marker enzyme together with catalase and DAAO.

D-aspartic acid is recognized as one of the most important regulators of the synthesis and secretion of steroid hormones and releasing factors of protein hormones [27]. High content of D-aspartic amino acid is observed in the glands of internal secretion, in the nervous and endocrine tissues. For this amino acid,

the highest concentration is characteristic of embryonic period. With advancing age, D-aspartic acid level gradually decreases in the nervous tissue, but increases in the endocrine glands, especially in the pineal gland, pituitary gland, adrenal glands and testes. In the pineal gland, D-aspartate reduces the secretion of melatonin, in the testes it stimulates Leydig cells, as well as the biosynthesis and secretion of testosterone that occurs through the activation of steroidogenesis by expression of a specific regulatory protein. This discovery shows that D-aspartic acid is a new type of messenger in the mammalian body [28].

Regulation of the formation and secretion of the epiphyseal hormone melatonin is a specific physiological function of D-aspartate. Various mechanisms for the response to noradrenaline stimulation of pinealocytes which were previously treated with D-asparagine [29] are discussed. Significant amounts of D-aspartate are found in the mammary gland. L-aspartic acid demonstrates an inversely proportional dose-dependent effect on prolactin secretion. Testosterone, cortisone and thyroid hormones increase the activity of oxidases. In the kidneys of rats fed with a diet limited in Na, oxidase activity also increases. Hypolipidemic drugs reduce the formation of peroxisomes in liver and the DAAO activity (the latter is more noticeable than expected). In the neuroendocrine system, decreasing D-aspartate concentrations are found as follows: NMDA > anterior pituitary gland > hypothalamus > pineal gland. In intraperitoneal injections of amino acids, the greatest accumulation occurs in these glands. It is accompanied by a dose-dependent increase in the concentration of prolactin and somatotropin [30-32].

The dependence was found of hormonal activity and the onset of maturity on the D-aspartate level in the pituitary gland and testicles. Immunocytochemical studies have shown that this enantiomer is localized in Leydig and Sertolly cells, and have confirmed its important role in steroidogenesis [33]. In various animals, the authors demonstrated the regulating effect of D-aspartic acid on the biosynthesis and secretion of testosterone and luteinizing hormone, but not in estradiol secretion. The mechanism underlying the effect of D-aspartate injections on testicular androgen receptor proteins is described in detail. The content of these proteins increases substantially, while the amount of estrogen α-receptors and P-450 aromatase receptors decreased [33].

In studying the molecular mechanisms of the evolutionary transition from asexual to sexual reproduction, a hypothesis was put forward that DAAO inhibits the formation of immature ovaries by degradation of D-amino acids [34]. As a result of sexual induction, the oxidase expression in ovaries increases. Absent oxidase slows the maturation of all reproductive organs besides the ovaries. This circumstance allowed admitting that oxidase boosts the maturation of the ovaries that determine the complete sexual induction. The authors also suggested that oxidase which is formed in somatic cells prevents the reduction of sexual induction in asexual status. After sexual induction, female reproductive cells specifically produce DAAO to induce complete maturation

A combination of accurate and sensitive methods of fluorimetry and high-performance liquid chromatography not only confirmed the presence of D-aspartate in the nervous tissue and endocrine glands, but also showed the induction of the secretion of growth hormone and luteinizing hormone in the anterior pituitary gland. It is interesting that the induction of luteinizing hormone was recorded only in the case of a joint incubation of the anterior pituitary gland with the hypotalamus. It could occur due to the induction of the gonadotropin hypothalamic releasing, which is responsible for the secretion of the luteinizing hormone of the pituitary gland, by D-aspartic acid. Compared to NMDA, D-aspartate concentration to release the hormone is a 100 times less [34].

As early as in 1939, it was shown that tumor proteins contain significant amounts of D-amino acids, especially D-glutamic acid, D-valine, D-leucine, and D-lysine. Later, the D-serine content was found to be extremely high. In precancerous cells and malignant tumors, DAAO activity is extremely low, which points at their important role in metabolic control, including possible participation in the control of cell growth. It was assumed that the initiation and autonomic nature of the uncontrolled growth of tumors depends on D-amino acid contents in cellular proteins. This postulate remains controversial for many years. The published scientific papers were both confirming and refuting this hypothesis. The discussion was heating among scientists from the Netherlands, England and Germany. However, many years later, with the development of analytical methods for the detection of amino acids enantiomers, more convincing evidence has appeared. It claimed that D-amino acids do not serve as a common indicator for tumors and, apparently, are not responsible for malignant transformation processes (at least if yes, then not for all tissues) [35]. It is known that tumor cells grow very fast, so many researchers believe that L-amino acids do not have enough time to be racemized into D-isomers, as this process is rather slow. However, the use of the DAAO test to diagnose cancer continues to be one of the interesting and promising areas, as experiments in laboratory animals show almost complete absence of the DAAO activity in cancer cells. Some D-amino acids may come in small amounts with food intake, but it is not known which enzyme systems are capable of incorporating D-amino acids into tumor tissues. However, significant amounts of D-amino acids are found in tumor proteins. There is no reliable argument for their emergence here, but studying this phenomenon is certainly important for understanding the mechanisms of autogenesis and supporting the development of tumors [36].

Prostaglandin D-synthetase is a bifunctional protein acting as both an enzyme producing PGD_2 (prostaglandin D2), and as a lipid transporter. This enzyme is a member of the lipocalin superfamily of proteins that can bind to a large number of lipophily molecules. Expression of the enzyme is increased in neuronal cells treated with H_2O_2. The content of the prostaglandin-producing enzyme is closely interrelated with apoptosis induced by hydrogen peroxide. This enzyme protects neuronal cells from death from hydrogen peroxide. The cell viability test shows a dose-dependent effect of the enzyme [37]. Moreover, the titration of free thiols in the prostaglandin synthetase treated with hydrogen peroxide proves that in case of exposure to H_2O_2 per cell, the thiol is oxidized to sulfinic acid. The affinity of the oxidized form of the enzyme for lipophilic molecules is comparable to that of the untreated form. These results show that the enzyme protects neuronal cells from death by neutralizing reactive oxygen species without lowering the ligand-binding function. This property of prostaglandin synthetase may be useful for suppressing stress-induced neurodegenerative oxidative pathologies [38, 39].

Since D-amino acid oxidase generates H_2O_2 and uses D-amino acid as a substrate, it has been suggested that it can prevent bacterial infections. Indeed, D-amino acid oxidase inhibits the growth of *Staphylococcus aureus* in vitro in a dose-dependent concentration. Adding catalase to the incubation medium prevented the bacteriostatic effect. However, the presence of myeloperoxidase in the same system increased bactericidal activity. This paradoxical observation needs clarification, since both enzymes are capable of decomposing H_2O_2, but the physiological effect of their combined action is antipodal. Nevertheless, it is reliably known that an increase in the D-serine level and a decrease in DAAO activity are efficient in treating various diseases, including mental disorders and oncological ones [40-45].

Thus, this work systematized and analyzed a wide range of functions of D-amino acids and their oxidase, with an emphasis on complex competitive physiological and biochemical relationships in different systems (from the epiphysis, hypothalamus and pituitary gland to the ovaries and testes). The peroxisomes contain D-amino acids and their oxidase which are involved in providing the communication between the nervous, endocrine and immune systems in the regulation of metabolism in cell, organ and organism. The signal, communicative and regulatory functions of the peroxisome is discussed. As it comes from the publications in the world academic and medical literature, researchers prefer to clarify the role of D-amino acids and their oxidase in the central and peripheral parts of the human nervous system. In our opinion, it is necessary to raise interest in this problem in biological livestock science. The confidence in the prospects of such works is based on the experimental findings on the kinetic characteristics of DAAO which indicate the ability to provide an efficient alternative metabolic pathway for the conversion of D-dihydrophenylalanine to dopamine. It may be useful for finding mechanisms to manage behavioral responses and reducing the aggressiveness of animals.

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CONTENT OF β-GLUCANS IN OAT GRAIN AS A PERSPECTIVE DIRECTION OF BREEDING FOR HEALTH PRODUCTS AND FODDER

(review)

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Abstract

The paper offers a review of the published data on the structure of β-glucan molecules in the oat kernel, their influence on the lipoprotein content and glycemic index, on the digestive system operation and cancer cells, as well as on other human health indicators. It is noted that water-soluble food fibers have both dietary, prophylactic and healing effects on the human organism (official reports of the US Food and Drug Administration and European Food Safety Association, EFSA). It is discussed that physicochemical properties, chemical modifications and possibilities of industrial application of -glucans define clear perspectives for their potential use in foods, medicinal and cosmetic products. Besides, the results of analyzing the diversity of oat cultivars and species for the β-glucans content in the kernel are discussed. It is stated that the forms of naked oat have a higher total content of this polysaccharide than the hulled oats, while the latter contain more insoluble β-glucans in the oat kernel. It should be noted that the content of fibers depends on the meteorological conditions and agricultural methods of oat cultivation. The content of β-glucans in the kernel is related to the accumulation of protein and fat in the kernel, to the grain volume weight and to grain productivity. The problems of creating new productive oat cultivars with the maximum content and optimal structure of the said polysaccharide combined with other qualitative characters of the kernel, as well as possibilities of producing functional foods from the processed grain of such cultivars are considered. It is concluded that β-glucans will have an increasing importance in the global food and pharmaceutical industries.

Keywords: oat, naked, hulled, *Avena*, β-glucans, polysaccharides, food fibers, lipoproteins, glycemic index, cholesterol, cancer cells, breeding, processing

In recent years, in some Western governments, work had been initiated to study barley and oat grains containing β-glucans, the substances that assist in preventing a number of human diseases. In Russia, only single works are devoted to the study of these chemical compounds in oat grain today. *Avena* L. genus belongs to the *Poacea* family and includes more than 20 species, four of which (*Avena sativa* L., *A. byzantina* C. Koch, *A. strigosa* Schreb. and *A. abyssinica* Hochst.) are cultivated by humans. World collections of *Avena* species count more than 130 thousand samples, preserved in 63 countries [1]. Common oat (*Avena sativa*) is the main economically significant cultivated species. According to the world grain harvest (24 million tons annually) oats are on the 6th place after wheat, rice, corn, barley and sorghum [2]. Oats are mostly cultivated in Europe, Asia, South America, Australia, and less cultivated in Africa. It occupies the largest areas in Russia, Canada and the USA. This mesophyte agricultural crop is well adapted to a wide range of soils and temperature conditions.

Oat grain has a high nutritional value, contains unsaturated fatty acids, basic mineral elements, globular proteins and β-glucans (the highest values

among cereals), characterized by the presence of a variety of chemical substances exhibiting antioxidant properties [3]. Oats are traditionally regarded as a nutritious grain crop, and there is evidence that products derived from it can help in the prevention of certain chronic diseases [4, 5]. Besides, by the EU Regulation 41/2009 oats has recently been included in the number of gluten-free ingredients safe for celiac disease (i.e. chronic intolerance to gluten-forming proteins, which are mainly contained in wheat, rye and barley weevil), provided that the gluten content in the grain should not exceed 20 ppm. When studying the biochemical and immunochemical characteristics of 36 cultivars of oats, it was found out that in most samples the content of such proteins was below 20 ppm and only in some cultivars exceeded 80 ppm [6].

Structure of β-D-glucans. In cereals (unlike most crops), the cell walls of the endosperm of the weevil contain very little count of cellulose and consist mainly of arabinoxylans and (1,3;1,4)-β-D-glucans, the ratio of which varies significantly in different species: arabinoxylans predominate in rye and wheat, while (1,3; 1,4)-β-D-glucans predominate in barley and oats [7]. The latter are typical for all members of *Poacea* family. The level of (1,3;1,4)-β-D-glucans varies from 3 to 11 % in barley, riches 1-2 % in rye and is < 1 % in wheat, with only traces manifested in other grains [8]. (1,3;1,4)-β-D-glucans are water-soluble linear homopolysaccharides whose molecules consist of approximately 2500 residues of β-(1,3)- and β-(1,4)-D-glucopyranose. Most segments in polymer chains are trimers and tetramers [9], usually in the oat grain their molar ratio is 1.5:2.3 [10, 11]. Trisaccharides predominate in soluble β-glucans [12]. The molar weight of oat β-glucans is about 500,000 g/mole for soluble ones, and less than 200,000 g/mole for insoluble ones [13]. Recently, a computer 3D model of the β-glucan molecule has been created. In form, the β-glucan molecule is an elongated sinuous chain with a pitch of 41.35 E. It is estimated that the rigidity of the chain increases with the increment the proportion of trisaccharides to tetrasaccharides [14].

When dissolved in water, arabinoxylans and β-glucans, due to their large molecular weight, form hydrocolloids with a high viscosity [15, 16]. In β-glucans of oats with a higher tetrasaccharide:trisaccharide ratio, the viscosity of the solutions is higher [17].

The concentration of β-glucans is measured by chemical and physical methods. According to the international standard, two-stage hydrolysis with lichenase and β-glucosidase to glucose is used to determine the content of β-glucans in the oat fraction and groats (Megazyme Inc. test system, USA), the products of which are then recorded spectrophotometrically in the visible spectrum [18]. Recently, a cheaper, modified express micromethod for grain analysis has been developed. By efficiency, when wheat bread, barley flour and oat bran were examined, it was comparable with the standard method [19]. Another physical method is based on measuring the reflection from the ground grain in the near infrared spectrum [20].

Effect of β-glucans on blood lipoprotein contents. β-Glucans are dietary fibers, the high-molecular carbohydrates of plant origin, which have a beneficial effect on gastrointestinal tract and systemic processes in human body [21]. β-Glucans assist in reducing risk of cardiovascular disease [22], maintain or reduce blood cholesterol and the risk of hyperglycemic syndrome [23]. Thus, the hypocholesterolemic effect of β-glucans of cereals was noted when comparing two diets. The first diet included whole-grain wheat bread, and the second one was enriched with monounsaturated fatty acids and included bread with oat β-glucan. As a result, total cholesterol was reduced in testers, i.e. blood low density cholesterol decreased in the first group by 16.8 %, and in the second group by

27.3 %. Both diets helped to reduce the overweight, in the second group to a greater extent [24]. In another experiment, the oat diet resulted in a statistically significant decrease in total and low density blood cholesterol, while these indices did not decrease under the corn diet [25].

Glycemia and β-glucans. Dietary fibers are capable of lowering the glycemic index of food [26]. In glycemia, the dose of an intake of dietary fibers is important for controlling the intake of glucose into the blood and insulin secretion in response to glucose, since in patients with obese, insulin secretion decreased significantly in response to the intake of 3.8 g of oat β-glucans [27]. Oat β-glucans, interacting with other carbohydrates, affect their accessibility and, as a consequence, reduce the glycemic reaction. Thus, for certain ratios of β-glucans and starch in the diet, the accessibility of starch decreases and the intake of glucose into the blood decreases. What is more, the effect manifested itself more strongly with a higher ratio of these components in the diet [28]. In experiments on mice and in vitro culture, it has been shown that oat β-glucans are able to regulate glucose metabolism, acting as a potential inhibitor of β-glucosidase, which can effectively improve the gastrointestinal tract state [29].

A meta-analysis of the articles describing 126 clinical cases demonstrated a significant inverse relationship between the content of total cholesterol, low density lipoproteins, increased high density cholesterol on the one hand, and the use of β-glucans on the other hand. At the same time, the influence of β-glucans on the decrease in glucose concentration is not strictly proven and requires longer clinical studies [30]. The ability to reduce the glycemic index is associated with the viscosity of β-glucans, which determines their functional and physiological effects [31]. As to the normalization of cholesterol, the role of viscosity of β-glucans is often confirmed no more than indirectly, and the result is not always statistically significant. It is suggested that the different efficacy of β-glucans may be partly explained by a change in their properties, depending on the diet and dose [32].

The effect of β-glucans on other indicators of human health. The addition of dietary fibers prolongs the feeling of satiety [33]. The possibility of using β-glucans in oncology is being intensively studied. The antitumor activity of low-molecular β-glucans from oat grain was studied on cancerous cells Me45 and A431. The polysaccharide significantly reduced their viability without being toxic to normal HaCaT cells. Increasing the duration of incubation and concentration of β-glucans significantly enhanced the effect. Immunocytochemically, it has been shown that β-glucans induce the expression of a specific protein in both cancer cell lines, whereas in normal cells this reaction is significantly lower [34]. β-Glucans from oat grain had a cytotoxic effect on human melanoma HTB-140 cells. A detailed mechanism for such activity needs further study, but preliminary results are certainly of interest [35].

Low molecular β-glucans assisted in a decrement of the activity of superoxide dismutase in rats with experimentally induced inflammation of the intestine which received oat β-glucans of different molecular weights. Under oxidative stress, the inclusion of β-glucans in the diet improved the indices in the spleen. The obtained data allow us to hope for the use of β-glucan hydrocolloids to create a composition with antioxidant properties [36].

Cereal dietary fibers, that are not digested in the small intestine and function either as a substrate for further intestinal fermentation or as a filler, serve as a means of preventing certain intestinal diseases and possibly to some extent act as a therapeutic agent [37].

Thus, the consumption of oat products helps reduce blood cholesterol level, including low density cholesterol [38, 39], the risk of cardiovascular diseases, improve liver function, and reduce overweight [40]. Water-soluble dietary

fibers (β-glucans) and phenolic alkaloids (avenanthramides) can be included in the daily diet as functional dietary ingredients [41]. However, the oat β-glucans have also negative properties. It is suggested that by binding iron ions, β-glucans may decrease its bioavailability [42]. In addition, the nutritional value of oats for non-ruminant animals is negatively correlated with the content of dietary fibers in the grain [43].

Screening of the cultivars and varieties of oats according to the content of β-glucans. For many decades, selection for high productivity and plant resistance to stress factors has led to a certain decrease in genetic diversity on quality parameters. Thus, an increase in the number of arabinoxylans and β-glucans in grain is currently very important. The content and composition of dietary fibers are genetically determined. This means [44] that it is possible to create plant lines with different ratios of β-glucans and arabinoxylans for the intended use. Thus, a change in the ratio of these components to increase the viscosity index let to get the cultivars for the production of whole grain foods, and its reduction in forage cultivars will prevent negative consequences when feeding poultry and pigs with grain [44]. The study of the content of β-glucans in oats is associated with its use not only for dietary purposes, but also for the medical industry [45].

A relatively recent identification of genes involved in the biosynthesis of β–glucans in cereals [15] and the first created genetic map [46] are important steps in the genetic improvement of grain quality and the grain-based food products. The research strategy includes the use of natural genetic diversity and its widening by mutagenesis and transgenesis [47]. The use of mutagenesis to change the content of β-glucans in the oat grain is described [48, 49]. In the population of 1700 lines of the Belinda cultivar (Sweden), obtained by mutagenesis, forms with high (> 6.7 %) and low (< 3.6 %) content of β-glucans in the grains were found with a maximum variation of the index from 1.8 to 7.5 %, with 4.9 % for the parent [48].

The genetic diversity of oats on grain β–glucan content was assessed in the framework of two European programs. In the HE-ALTHGRAIN Diversity Screen project (started in 2005 and performed mainly on wheat) in 5 cultivars of oats under the same conditions, the content of β-glucans and antioxidants in the grains varied significantly [3]. In the next project (the European Project on Avena Genetic Resources for Quality in Human Consumption), the study of 658 cultivars of oats confirmed the contribution of both the genetic and ecological components to the trait expression [50].

It is interesting that many wild species of oats have high grain content of β-glucans [50], although these studies are very few. The study of a limited set of wild samples, including *A. sterilis* L., revealed an increased (up to 6 %) amount of β-glucans in grain of all hexaploid species (*A. fatua* L., *A. occidentalis* Durie, *A. byzantina*) compared to di- and tetraploid species [51-56].

Comparison of the oat collection samples on the content of important biochemical components made it possible to identify the source material of interest for breeding [57]. In the American oat collection, the content of β-glucans varied from 2.6 to 8.5 % [58, 59]. In different reports the values were indicated from 1.9 to 7.5 % [8, 60]. In hexaploid oat cultivars from the VIR collection (Russia), the amount of water-soluble polysaccharides varies from 2.58 to 3.52 % [61]. In the Russian-Swedish project (VIR and Nordic Gene Bank, NordGen), the assessment of oat cultivars on the content of β-glucans revealed the variation from 3.3 to 6.2 % [62, 63].

In four oat cultivars grown in 11 different conditions (place and year), the genotype influence on the content of polysaccharides (23 %) exceeded the

impact on their molecular weight (4 %). The external conditions significantly affected the molecular weight of β-glucans (71 %) than their content in grain (42 %). A negative correlation was found between the amount of precipitation and the molecular weight of the β-glucans. At the same time, there is a significant positive relationship between the content of β-glucans and their molecular weight. Since both these indexes play an important physiological role in digestion, in the authors' opinion, selection for an increased content of β-glucans is likely to be accompanied by an increase in their molecular weight [64]. Cultivation of 15 samples of *Avena sativa* and 3 samples of *A. byzantia* revealed significant varietal differences in protein and β–glucan content, as well as a positive correlation between the grain yield and the content of β-glucans. The maximum accumulation of these polysaccharides for 2 years of study was noted in the cultivars Bw 103, Maxima and Rocio [65].

When crossing high-glucan and high-yielding oat lines, a positive correlation was found in F_4 hybrids grown at two points in Iowa between the content and viscosity of β-glucans, and the lines with high level of β-glucans, as a rule, were low-yielding. As among families, and among the lines within families, there were significant differences in most of the indicated traits. Consequently, targeted selection can increase the amount and viscosity of β-glucans in the grain in the cultivars being created [66], and the existing genotypic diversity of oats by β-glucan levels is sufficient for the progress in selection [67].

In intraspecific crossing, the content of grain β-glucans is inherited polygenically with the additive effect [68], does not depend so much on the place and growing conditions as the protein and oil content in grains, and does not correlate with the size of the grain itself [69-72]. The increase in the amount of β-glucans, on the one hand, is negatively associated with the amount of protein in gain [53], on the other hand, the increase is directly proportional to the rise in protein content and inversely proportional to fat level [73]. Apparently, the dynamics of accumulation of β-glucans in grains differs from that for other biochemical components [74].

Comparison of the β-glucan contents and other agronomical indicators in 9 barley and oat cultivars in the contrasting climatic conditions of Europe (at latitudes from Norway to Germany) revealed varietal differences in the range of 3.7-5.1 and 4.3-5.3 %, respectively. The influence of the ecological factor was 3.8-5.5 and 3.8-5.3 %, respectively. Warm and dry weather during the grain-filling period significantly increased the amount of β-glucan in oats [75].

The β-glucan content in grain of naked and hulled oats. In general, the average content of β-glucans varies from 3.1 to 4.5 % for hulled grains and from 3.8 to 4.9 % for naked grains [76]. During growing 10 oat cultivars for 3 years in the same place, the total content of dietary fiber, β-glucans and proteins was increased in the hulled genotypes compared to the naked genotypes [77]. The content of soluble β-glucans decreases from naked oats (3.91-7.47 %) to hulled oats (1.97-4.09 %), while insoluble β-glucans decreases from 13.79-33.73 to 5.15-10.80 % in reverse order. Histological examination of the grain showed that the amount of insoluble β-glucans falls in the direction from the outer covers to the endosperm [78].

In field examination of 11 samples of a naked oats (cv. Polar, the standard, and 5 lines) and hulled oats (cv. Bohun and 4 lines), it was noted that in naked forms the amount of grain fiber was lower than that in hulled ones, with a significantly higher protein and fat contents [79]. Naked and hulled cultivars grown in Italy for 2 years after a cereal precursor accumulated 9 % more β-glucans than those cultivated after legumes. Bikini, Nave (Italy) and Abel (Czech Republic) cultivars were characterized by a high content of β-glucans

among the naked genotypes, and Konradin cultivar accumulated the largest amount of dietary fiber. Naked samples are considered in connection with the production of dietary foods [80].

By computer modeling (the Monte Carlo method), the factors influencing the content of β-glucans in the hulled and naked oat cultivars were ranked. It turned out that the amount of β-glucans is mainly determined by the cultivar. The conditions of cultivation and the shelf life time adversely affected the analyzed indicator. The used model is proposed to be applied for evaluation of various agricultural technologies [81].

The study of the oat flakes, produced from 37 naked (China) and 44 hulled oats in the USA, Canada, New Zealand, Sweden, Denmark and the United Kingdom showed that in the first case the content of β-glucans and iron was significantly lower, and the water absorption rate at room temperature was higher. At the same time, an expert evaluation of the oat flakes from the naked samples was lower [82].

Quality and physiological role of β-glucans. It has already been noted that not only the content, but also the quality of the β-glucans determined, for example, by their viscosity and the functional activity is an important selection parameter. When comparing 1 % water extracts of β-glucan in 5 lines of oats with an increased content of this polysaccharide (up to 7.8 % against 4.4 % in the usual Paul cultivar), significant varietal differences were revealed in rheological properties. These facts, as well as the difference in the viscosity as a function of the concentration, are explained by the unequal molecular weight [83]. In another study, 5 varieties of oats cultivated for 2 years in Norway were evaluated for the chemical composition and nutritional properties of the grain when fed to broiler chickens within 2 weeks. The studied samples differed significantly in nutritional value which negatively correlated with the content of dietary fibers [43].

In oats, the growth conditions significantly influenced on the viscosity of the flour suspension evaluated by a rotational viscometer, as well as on the extractability of β-glucans. At the same time, there was no significant dependence of extractability on the genotype. As for the chemical structure of the β-glucans, significant differences in polymerization of the molecules, due to both the genotype and cultivation conditions, were found. In the HiFi cultivar (USA) with a higher percentage of β-glucans, the molecules of this polymer were lower in the content of trisaccharides and higher in the proportion of tetrasaccharides compared to varieties that have an average content of β-glucans [12].

Relationship between the grain β-glucan content and other agronomic traits. The selection programs include creation of both high- and low-glucan oat lines. To separate the hybrid population into these contrasting groups, clear markers are needed. Thus, possible links of the content of β-glucans with various physical, morphological, physiological, agronomic and genetic-molecular signs of genotypes are studied.

As a non-destructive indirect method, a rapid assessment of the barley and oat grain for hulled or naked types [84] is proposed, which may be useful for predicting the content of dietary fiber.

In Scotland, when studying 33 cultivars of oats, it was shown that the correlation between the accumulation of β-glucans in grains and agronomic traits is usually absent or the obtained results are contradictory depending on the year or the cultivation conditions. In one of the breeding nurseries, a significant positive relationship was observed between the amount of β-glucans in grains and their full-scale weight, the protein content, the milling yield, and also the negative relationship of β-glucans and the date of tasseling. The authors optimis-

tically concluded that simultaneous changes of these traits should not hamper the success of selection work, since the observed correlations are unstable [67]. A negative correlation between the content of β-glucans in oat grains and the total content of dietary fiber and crude fiber, as well as a positive correlation with the protein content (77) are described.

In Finland, comparison of β-glucans in 12 oat cultivars in eight points during 2 years revealed a significant positive correlation between the β-glucan content and grain yield, the vegetation period, grain weigh per volume (test weight), and the 1000 grain weight, as well as the significant negative correlation of the chemical index in question with the protein content and grain hull formation [76].

In the study of the 431 oat genotypes of the American Gene Bank by the genome-wide association study (GWAS) with determination of QTL for the content of β-glucans in grain, three independent markers closely associated with the target trait were found. Comparison of these data with the obtained results on rice showed that one of the markers located on the chromosome 7 of rice is associated with a family of *CslF* genes that are responsible for the synthesis of β-glucans in grain. In the future, such a study of the oat grsin can be successful in determining QTL for the markers of higher density [85].

Thus, the available data confirms the importance of β-glucans, the water-soluble dietary fibers, which have dietary, preventive and curative effects on the human body. Physicochemical properties, chemical modifications, and technological suitability open up clear prospects for the use of β-glucans in dietary food, medicines and cosmetics, and in these global industries β-glucans will play an ever-increasing role [86]. At the same time, the problem of reducing the amount of dietary fiber and the anti-nutritive properties of forage grain is topical in feed production. Under the joint Russian and international programs, the Vavilov All-Russian Institute of Plant Genetic Resources Currently conducts a comprehensive study of a large and diverse set of oat samples in order to reveal forms contrasting in β-glucan content for use in the dietary and feed sectors.

So, the development of functional food products from oat grain assumes the creation of highly productive cultivars with the maximum content and optimal chemical structure of β-glucans in combination with other quality parameters whereas the requirements are opposite for fodder cultivars. Insufficient knowledge and inconsistency of the available data hamper progress in these areas so far. Therefore, complex studies of the whole diversity of oat varieties are necessary to found contrasting parental plants and create cultivars for dietary and feed use.

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DOMAIN DISTRIBUTION OF MOBILE GENETIC ELEMENTS IN THE BOVINE GENOME

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Abstract

Genetic landscape of bovine genome attracts a lot of attention in recent years. This is due to the complexity of genomic selection task solution, i.e. the use of multilocus genotypes in order to simplify and hasten breeding. Accumulated data show that there is high evolutionary speed of different genetic elements and also they have structure functional polymorphism intensity (L. Chen et al., 2017). It was shown that interspersed repeats account for about 50 % of nucleotide sequence of the bovine genome (R.L. Tellam et al., 2009). Also it was found that some of the interspersed repeats cluster into conservative domains along the bovine genome due to joint localization (D.L. Adelson et al., 2009). The characteristics of domain distribution are still not fully studied despite the fact that it is very important to identify conservative and variable domains throughout the bovine genome to solve traditional tasks of their genetic resources management and controlling. In this work domain distribution of mobile genetic elements and their products of recombination in nucleotide sequences of 13,436,028 nucleotides of bovine chromosome 1 were analyzed by means of Repeat Masker mobile genetic elements database and Integrated Genome Browser software. It was revealed that the most prevalent types throughout analyzed region are SINE/tRNA-Core-RTE, LINE/RTE-BovB, LINE/L1 and LTR/ERV. Their joint localization in bovine genome has complicated structure. The most common pairwise clusters are SINE and LINE, SINE/tRNA-Core-RTE and LTR EVR, (LTR/ERVK)/(LINE/RTE-BovB), (LTR/ERVK)/(LINE/L1). Two last pairs are the bases for such triple clusters as (LINE/RTE-BovB)/(BTLTR1)/(LINE/RTE-BovB) and (LINE/L1)/(BTLTR1J)/(LINE/L1). It should be mentioned that there is no such clustering with other retrotransposons. It was revealed that there is some certain bias of these triple clusters high density to the distal end of studied region of chromosome 1. By the means of Integrated Genome Browser software the localization of obtained triple products of recombination between LINE and LTR ERV to structural genes was analyzed. It was found that only 34 clusters are localized in 12 structural genes (other are located in intergenic space). Besides, 10 and 12 copies are located in two genes that are closely connected with the function of central nervous system in mammals, *grik1* and *app*. The fact that 9 copies of triple gene construct (LINE/RTE-BovB)/(BTLTR1)/(LINE/RTE-BovB) are found in each of two genes and (LINE/L1)/(BTLTR1J)/(LINE/L1) had only 1 copy in *grik1* and 3 copies in *app*, suggests that these genes are ancestral targets for such insertions and their conservations. It also should be mentioned that (LINE/L1)/(BTLTR1J)/(LINE/L1) construct was found only in these two genes but not in other 10 genes where (LINE/RTE-BovB)/(BTLTR1)/(LINE/RTE-BovB) is also located. Specific features of distribution of products of recombination between LINE and LTR ERV throughout the studied chromosome 1 area and their localization in structural genes suggest the possible presence of structure functional elements there. Revealing of such elements is the subject of our further study.

Keywords: mobile genetic elements, retrotransposons, DNA transposons, products of recombination, domain distribution, genomic landscape, cattle

Observations of mobile genetic elements (MGE) in the genomes of mammals have a rather long history. The largest part (from 40 to 50 % of the total length) in the mammalian genomes is occupied by interspersed repeats. In most

mammalian species, including bovine cattle, retrotransposons (RTs) dominate in the genomes among mobile genetic elements, which for their movement use the mechanisms of reproduction of exogenous retroviruses [1]. One of the reptile units as *Squamata* could be the source of a number of mobile genetic elements for ruminants [2-4]. It is assumed that the horizontal transfer of RTs between taxonomically removed forms is promoted by the common habitat [5].

Many of the identified RTs were common to all mammals, which probably indicates the ancientry of their origin. Most RTs have lost their activity, but the increased polymorphism of some RTs may reflect their relatively recent origin and involvement in genomic reorganization processes, usually of functional and evolutionary significance [6]. Thus, insertions of some RTs into the promoter regions of structural genes substantially change the expression of the latter, and in the amino acid sequence they lead to the occurring of new proteins [7]. In bovine cattle, a number of structural gene mutations associated with RT insertions lead to lethal effects in the homozygous state [8].

In mammals, the main RT is a Long Interspersed Nuclear Element 1 (LINE1). They carry active and other LINE in the genome, such as the LINE RTE family. Non-autonomous Short Interspersed Nuclear Element (SINE) for transpositions requires LINE. Primates for transposition of SINE Alu require LINE L1. The ancient RT LINE2 (L2) encodes the proteins necessary for the distribution of the SINE MIR, widely represented in the genomes of mammals. In ruminants and marsupials, LINE RTE encodes proteins required for transpositions of SINE BovA, the (BOV-A2, Bov-tA1, 2, 3)/SINE ART2A or SINE RTE, respectively. RTE LINE contains BovB repeats, suggesting that they are horizontally transferred from reptiles to ruminants and marsupials [4, 9-11]. Ancient clusters of L2/MIR repeats form domains that are conserved in human and bovine genomes, and there are no younger repetitions, such as RTE/ART2A, in such domains. Since the ancient repetitions are clustered into evolutionarily conserved domains, this allows us to assume that there are special mechanisms providing such conservativeness that can be associated with blocks of expressed genes, with the specificity of localization in the interphase nucleus space, differences in the methylation pattern, or features of genomic sites different in protection from re-integration of retrotransposons [2]. A large amount of data has been accumulated, indicating that retrotranspositions in mammals are involved in the occurring of new genes and functional evolution [12], as well as in gene duplications [13]. Endogenous retroviruses (ERV) containing long terminal repeats (LTR) are another variant of RTs which is widespread in mammalian genomes (in particular, in bovine cattle) and characterized by high polymorphism; interbreed differences are described by the presence of some of them [14].

Detection of the conserved and variable domains of RT localization is of significant practical importance for the selection of the most polymorphic genomic elements suitable for use as anchors in genomic scanning (poly-locus genotyping) in controlling the genetic structure and its dynamics at the species and intra-specific differentiation level [15]. These studies acquired particular significance after the revealing zonality of the different RTs distribution and the short fragment copy number variability (CNV) in the cells of the germinal series and somatic cells, identified in human genomes [16].

Despite the importance of studying conserved and variable domains in the genomes of farm animals for solving traditional problems of control and management of genetic resources, such works still remain rare. Moreover, as a rule, these papers consider colocalization of full-size RTs, while in genomes there is a huge number of RT fragments marking the sites of transpositions and recombinations between them [17, 18].

In the present study, the patterns of colocalization and clustering of homology sections to the most frequently encountered retrotransposons of different families and their recombination products (binomial and trinomial associations) in chromosome 1, the longest autosome of bovine cattle, are revealed.

The aim of the work was to analyze the distribution and positioning of mobile genetic elements to clarify the possible patterns of their structural organization in the genome.

Techniques. Information on the genomic location of mobile genetic elements with the nucleotide coordinates in bovine cattle given in the RepeatMasker software [1] was used as the initial data; the archive bosTau7.fa.out.gz [20] created using the RepeatMasker version open-4.0.5 software in October 2011. Information on the distribution of mobile genetic elements within the primary sequence of chromosome 1 (161,428,367 bp), obtained from the archive, was analyzed with respect to the number and frequency of different mobile elements, using the Microsoft Office Word program for bio-informative purposes. For the subsequent study of the most common mobile genetic elements, the 13436028 bp nucleotide sequence of chromosome 1 was chosen. In this sequence, the number of domains and the frequency of their occurrence were determined, identifying the nearest neighborhoods for homology plots between different families of mobile genetic elements. Based on the obtained data on the number of mobile genetic elements that most frequently are found in the isolated site and the domains formed by them, a table was constructed to evaluate the patterns of distribution of mobile genetic elements in alternative DNA strands. Binomial domains with a frequency of more than 60 %, containing the same mobile genetic element in different chains, were studied in order to find more interesting regularities of distribution and structural organization of RTs in the genome. The functional characteristics of the detected clusters were judged on the basis of their positioning within the structural genes using the Integrated Genome Browser software [21]. Information on selected structural genes was obtained from the international GenBank database [22].

Results. The approach that we used made it possible to identify not only the pairwise colocalization of different elements, but also clusters consisting of three mobile elements. In the 161428367 bp fragment of bovine cattle chromosome 1 analyzed, among all interspersed repeats, SINE (38.27 7%) and LINE (34.002 %) were the most frequent (Table 1). It should be noted that the retrotransposon LINE1 is widely distributed in all genomes of eukaryotes, including mammals. It is known that there are full-size variants of RTs of this family in bovine cattle, which retain retrotransposition activity and participate in genomic transformations [23]. The number of microsatellites (simple repeat) and DNA transposons was 9.549 % and 6.105 %, respectively, and the number of microsatellites with low complexity did not exceed 1.452 %. The main families of SINE were tRNA-Core-RTE (20.989 %), Core-RTE (6.979%) and MIR (6.822 %). In the LINE family, L1 (15.380 %), RTE-BovB (11.631 %) and L2 (5.828 %) were most fully represented, ERVL-MaLR (3,133 %), ERV1 (2,218 %), ERVL (2,091 %), ERVK (2,350 %) and Gypsy (0,212 %) were more common in LTR ERV, and more than half of the DNA transposons were numbered in hAT-Charlie (3.361 %).

In the primary 13,436,028 bp sequence, the domains formation with pairwise localization was studied for the indicated families (Table 2, the percentage see in annexes 2.1 and 2.2 in the electronic version of the article at http://www.agrobiology.ru). The comparison showed that the difference in the frequency of occurrence of domains in both chains did not exceed 2.35 % for

1. Distribution of mobile genetic elements along 161,428,367 bp fragment of bovine cattle chromosome 1

Family	Number	Frequency, %
SINE	122589	38.277
/tRNA-Core-RTE	67222	20.989
/Core-RTE	22351	6.979
/MIR	21850	6.822
/tRNA	10781	3.366
/tRNA-RTE	221	0.069
/5S-Deu-L2	126	0.039
/tRNA-Deu	36	0.011
SINE?/tRNA	2	0.001
LINE	108898	34.002
/L1	49,258	15.380
/RTE-BovB	37251	11.631
/L2	18665	5.828
/CR1	2954	0.922
/RTE-X	669	0.209
/Penelope	61	0.019
/Dong-R4	27	0.008
/Jockey	9	0.003
/L1-Tx1	4	0.001
LTR ERV	33222	10.373
/ERVL-MaLR	10035	3.133
/ERV1	7105	2.218
/ERVL	6698	2.091
/ERVK	7526	2.350
/Gypsy	680	0.212
LTR?	336	0.105
LTR	341	0.106
/Gypsy?	302	0.094
/ERVL?	154	0.048
/ERV1?	45	0.014
Simple_repeat	30581	9.549
DNA	19553	6.105
/hAT-Charlie	10763	3.361
/TcMar-Tigger	3214	1.004
/hAT-Tip100	2114	0.660
/TcMar-Mariner	634	0.198
/hAT-Blackjack	881	0.275
/hAT	377	0.118
/hAT-Ac	256	0.080
/hAT-Tip100?	79	0.025
DNA	283	0.088
/TcMar-Tc2	315	0.098
DNA?	184	0.057
/hAT?	101	0.032
/TcMar	80	0.025
/hAT-Tag1	136	0.042
/PIF-Harbinger	16	0.005
DNA?/hAT-Tip100?	34	0.011
/PiggyBac	31	0.010
/TcMar-Tc1	25	0.008
DNA?/PiggyBac?	11	0.003
/TcMar?	6	0.002
/Kolobok	10	0.003
/TcMar-Pogo	3	0.001
Low_complexity	4650	1.452
Other	773	0.241
Unknown	315	0.098
snRNA	100	0.031
tRNA	116	0.036
RC/Helitron	95	0.030
rRNA	66	0.021
Satellite/centr	33	0.010
RNA	29	0.009
srpRNA	2	0.001
scRNA	1	0.000
RC?/Helitron?	16	0.005
Total	320266	

the indicated SINE families (377 MIR/tRNA-Core-RTE и 346 tRNA-Core-RTE/MIR per 1320 elements of MIR), 1.35 % for LINE (280 L2/tRNA-Core-RTE and 296 tRNA-Core-RTE/L2 per 1183 L2), 4.76 % for LTR ERV (for example, 3 Gypsy/Core-RTE and 0 Core-RTE/Gypsy per 63 Gypsy elements, although in the case of Gypsy/L1 and L1/Gypsy the difference was 7.94 %, i.e. 14.29 versus 6.35 %), and 2.58 % for DNA transposons. So further we considered pairs in the same strand.

Colocalization analysis of SINE with other mobile genetic elements showed that SINE, with the highest frequency, forms domains with elements of its own family. The tRNA-Core-RTE family is particularly active: the frequency of core-RTE/tRNA-Core-RTE domains for Core-RTE is 27.33 % (568 pairs per 2078 elements), of tRNA/tRNA-Core-RTE domains for tRNA is 21.48 % (290 pairs per 1350 elements), and MIR is adjacent to tRNA-Core-RTE in 28.56 % of cases (377 pairs per 1320 elements). In this case, the tRNA-Core-RTE family itself most actively interacts with RTE-BovB (21.06 %, or 1611 pairs per 7651 elements).

Core-RTE is clustered with RTE-BovB and L1 at a frequencies of 16.46 % (342 domains per 2078 Core-RTE) and 15.21 % (316 domains per 2078 Core-RTE), respectively. The tRNA family in 16.74 % of cases is adjacent to L1 (226 domains per 1350 tRNA). MIR/L1 domains occur twice as often as MIR/L2, i.e. 154 (11.67 %) and 75 (5.68 %), respectively, per 1320 MIR. Obtained data contradict the conclusions of D.L. Adelson et al. [2], who considered this option as forming conserved and most ancient binomial domains. Apparently, such disjunctions may be due to the fact that D.L. Adelson et al. considered combined localization of the full-size genes of MIR/L2 retrotransposons. In our study, we estimated the colocalization of homology sites to RTs, the sizes of which could be less than the total length of the identification genes of mobile elements. The data obtained by us shows that when analyzing the formation of retrotransposon domains in the genomic landscape in case of species-specific “young” mobile elements which are actively involved in transpositions, it is necessary to take into account not only full-size sequences

2. The number of pairs of mobile genetic elements (MGE) most often found along 13,436,028 bp fragment of bovine cattle chromosome 1

Family	SINE				LINE			LTR					DNA	Total MGE
	/tRNA-Core-RTE	/Core-RTE	/tRNA	/MIR	/L1	/RTE-BovB	/L2	/ERVL-MaLR	/ERV1	/ERVL	/ERVK	/Gypsy	/hAT-Charlie	number
SINE														
/tRNA-Core-RTE	1425	568	290	377	1038	1574	280	322	197	201	11	10	211	7651
/Core-RTE	584	107	73	77	307	373	81	64	42	40	6	3	56	2078
/tRNA	299	64	76	89	217	58	82	68	20	46	3	2	63	1350
/MIR	346	90	87	101	149	98	75	31	23	28	3	4	56	1320
LINE														
/L1	1055	316	226	154	925	318	118	129	67	72	127	9	88	4162
/RTE-BovB	1611	342	70	71	350	324	80	82	70	49	422	3	53	3756
/L2	296	78	83	75	125	65	142	46	19	30	5	1	45	1183
LTR														
/ERVL-MaLR	312	72	84	54	132	81	39	106	26	22	1	4	19	1071
/ERV1	189	43	41	26	70	71	20	34	134	26	3	2	15	743
/ERVL	191	45	34	24	78	59	25	32	32	69	2	3	16	709
/ERVK	10	2	1	4	135	416	2	2	4	3	7	0	3	599
/Gypsy	13	0	1	2	4	5	3	2	2	4	0	9	5	63
DNA														
/hAT-Charlie	190	55	67	46	97	41	57	28	15	15	2	2	79	813

but also products of MGE recombinations.

The LTR ERV and SINE families formed domains, in particular 84 tRNA/ERVL-MaLR domains, in no more than 6.22 % of cases. Nevertheless, the SINE families were closely associated with all families of LTR ERV, mostly tRNA-Core-RTE. Thus, the ERVL-MaLR/tRNA-Core-RTE domain occurred 322 times (with a frequency of 30.07 %), ERV1/tRNA-Core-RTE — 197 times (26.51 %), ERVL/tRNA-Core-RTE — 201 times (28.35 %), Gypsy/tRNA-Core-RTE — 10 times (15.87 %). Similarly, for DNA-transposons, tRNA was adjacent to hAT-Charlie 67 times with a frequency of 4.96 %, the rest formed binomial domains in less than 4 % of cases. At the same time hAT-Charlie was colocalized with tRNA 63 times with a frequency of 7.75 %, and with tRNA-Core-RTE 211 times (25.95 %). The LINE families were also adjacent to SINE families most often, namely to tRNA-Core-RTE. In particular, the RTE-BovB/tRNA-Core-RTE (1574 domains) accounted for 41.91 % of the sequences, L/tRNA-Core-RTE (1038 domains) for 24.94 %, and L2/tRNA-Core-RTE (280 domains) for 23.67 %. The L1/L1 pair was also common (925 cases, which were 22.22 %). In associations with the LTR ERV family, the RTE-BovB/ERVK domain was most often present (416 cases, or 11.08 %). The frequency of the adjacency of remaining LINE families with LTR ERV families did not exceed 3.30 %. Colocalization with DNA transposons occurred less frequently than in 4.82 % of cases, which was noted for L2/hAT-Charlie (57 domains per 1183 L2), and hAT-Charlie formed domains with the LINE family at a frequency of no more than 10.82 % which was revealed for hAT-Charlie/L1 (88 domains). DNA transposons also rarely coexisted with the LTR ERV family, the frequency of no more than 2.34 % was noted for hAT-Charlie/ERVL-MaLR (19 domains), and of no more than 3.17 % for Gypsy/hAT-Charlie (2 domains).

The most interesting is the colocalization of LTR/ERVK with LINE families. Thus, the number and frequency of domains were 422 cases and 70.45 %, respectively, for ERVK/RTE-BovB type, 127 cases and 21.20 % for ERVK/L1, and 5 cases and 0.83 % for ERVK/L2. In the alternative strand, the frequency for RTE-BovB/ERVK was 69.45 % (416 domains), for L1/ERVK was 22.54 % (135 domains), and for L2/ERVK was 0.33 % (5 domains).

The combined prevalence of the ERVK family with LINE was 92.49 % and 92.32 % in the forward and reverse chains, while the rest of the MGEs did not exceed 4 %. This fact assumes the presence of a trinomial domain of the type LINE/ERVK/LINE.

A closer analysis showed that the ERVK family actually existed in the LINE/ERVK/LINE domain in 85.31 % of cases (511 out of 599). Among the LINE/ERVK/LINE domains, there were two ones noted, the RTE-BovB/BTLTR1/RTE-BovB at a frequency of 74.74 % (382 out of 511), and L1/BTLTR1J/L1 at a frequency of 21.51 % (110 out of 511) (Table 3). Besides, 12 overlapping trinomial clusters were observed, which indicates the high variability of their localization.

Localization analysis of trinomial clusters showed that they cover 6.86 % of the 13,436,028 bp primary sequence of chromosome 1 and are unevenly distributed (Table 4). Moreover, an increase in the density of such clusters was observed closer to the distal end of the fragment. The uneven distribution of families of mobile genetic elements inside and between the chromosomes of bovine cattle was reported [24]. At the same time, analysis of such data is difficult because it is difficult to distinguish between the effects of new insertions and transpositions and the results of their deletion or natural selection against adverse variants (cleansing selection).

3. Types of LINE/ERVK/LINE mobile genetic element domains located along 13,436,028 bp fragment of bovine cattle chromosome 1

Domain type	Domains	
	number	frequency, %
RTE-BovB/BTLTR1/RTE-BovB	382	74.74
RTE-BovB/BTLTR1B/RTE-BovB	1	0.20
RTE-BovB/BTLTR1E2/RTE-BovB	1	0.20
RTE-BovB/BTLTR1J4/RTE-BovB	1	0.20
RTE-BovB/ERV2-1-LTR_BT/RTE-BovB	1	0.20
RTE-BovB/LTR2_BT/RTE-BovB	1	0.20
L1/BTLTR1J/L1	110	21.51
L1/BTLTR1/L1	3	0.59
L1/BTLTR1F/L1	1	0.20
L2/BTLTR1/L2	1	0.20
L1/BTLTR1/RTE-BovB	4	0.78
RTE-BovB/BTLTR1/L2	2	0.39
RTE-BovB/BTLTR1/L1	2	0.39
RTE-BovB/BTLTR1J/L1	1	0.20
Total	511	

4. Distribution of trinomial domains of mobile genetic elements along 13,436,028 bp fragment of bovine cattle chromosome 1

The number and per cent of domains on 12 equal segments of 1,119,669 bp fragments												
1	2	3	4	5	6	7	8	9	10	11	12	Total
LINE/ERVK/LINE												
33	21	29	40	38	33	41	39	34	56	62	85	511
6.46 %	4.11 %	5.68 %	7.83 %	7.44 %	6.46 %	8.02 %	7.63 %	6.65 %	10.96 %	12.13 %	16.63 %	100 %
В том числе												
RTE-BovB/BTLTR1/RTE-BovB												
26	15	21	29	31	25	28	30	29	40	44	64	382
6.81 %	3.93 %	5.50 %	7.59 %	8.12 %	6.54 %	7.33 %	7.85 %	7.59 %	10.47 %	11.52 %	16.75 %	100 %
L1/BTLTR1J/L1												
7	6	8	9	6	5	11	6	5	16	13	18	110
6.36 %	5.45 %	7.27 %	8.18 %	5.45 %	4.55 %	10.00 %	5.45 %	4.55 %	14.55 %	11.82 %	16.36 %	100 %

5. Positions of trinomial clusters of mobile elements as related to structural genes along 13,436,028 bp fragment of bovine cattle chromosome 1

Structural genes	Cluster number	
	1	2
Bos taurus potassium voltage-gated channel subfamily E regulatory subunit 2 (*kcne2*), mRNA (–)	1	0
Bos taurus phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase (*gart*), mRNA (+)	1	0
Bos taurus transmembrane protein 50B (*tmem50b*), mRNA (+)	1	0
Bos taurus interleukin 10 receptor subunit beta (*il10rb*), mRNA (–)	2	0
Bos taurus interferon alpha and beta receptor subunit 2 (*ifnar2*), mRNA (–)	1	0
Bos taurus URB1 ribosome biogenesis 1 homolog (S. cerevisiae) (*urb1*), mRNA (+)	1	0
Bos taurus glutamate ionotropic receptor kainate type subunit 1 (*grik1*), mRNA (+)	9	1
Bos taurus ubiquitin specific peptidase 16 (*usp16*), mRNA (–)	1	0
Bos taurus listerin E3 ubiquitin protein ligase 1 (*ltn1*), mRNA (+)	1	0
Bos taurus cysteine and tyrosine rich 1 (*cyyr1*), mRNA (+)	1	0
Bos taurus amyloid beta precursor protein (*app*), mRNA (+)	9	3
Bos taurus junctional adhesion molecule 2 (*jam2*), mRNA (–)	2	0

Note. 1 — RTE-BovB/BTLTR1/RTE-BovB, 2 — L1/BTLTR1J/L1; "–" and "+" mean that the gene is located in the reverse or the forward chain.

Using Integrated Genome Browser software, we evaluated the positioning of trinomial MGE clusters of the types RTE-BovB/BTLTR1/RTE-BovB and L1/BTLTR1J/L1 as related to structural genes of *Bos taurus*. It was found that these clusters are detected with high frequency within the structural genes which encode glutamate ionotropic receptor kainate type subunit 1 (*grik1*) and amyloid beta precursor protein (*app*). In the *grik1* gene sequences, there were 9 trinomial clusters RTE-BovB/BTLTR1/RTE-BovB out of 30 ones identified in structural genes in the studied chromosome fragment, and one out of 4 L1/BTLTR1J/L1 clusters found. In the *app* gene, 9 clusters RTE-BovB/BTLTR1/RTE-BovB were

found out of 30 clusters RTE-BovB/BTLTR1/RTE-BovB detected, and 3 clusters L1/BTLTR1J/L1 out of 4 clusters L1/BTLTR1J/L1 identified (Table 5). It should be noted that in the sequences of these structural genes, RTE-BovB/BTLTR1/RTE-BovB were encountered with the greatest frequency (see Table 5), despite the increased amount of L1 compared to RTE-BovB in the examined fragment of chromosome 1 (see Table 1). This is consistent with the conclusion that RTE-BovB is an older and more mutated bovine cattle genome element compared to L1 [2]. It is important to emphasize that L1 and RTE-BovB are historically quite distant from each other, although they belong to LINE; nevertheless, in both genes similar trinomial products, the RTE-BovB/BTLTR1/RTE-BovB and L1/BTLTR1J/L1, are present (see Table 5). It can be assumed that such colocalization is associated with the existing in these products recombinations of RT elements with structural and functional similarity, which will be the subject of our further research.

In the literature, there is data on the association of mutations of the subunit 1 of the ionotropic kainate receptor glutamate *grik1* with behavioral pathologies of man, e.g. schizophrenia, epilepsy, depression, bipolar disorder [25-27]. According to available data, the protein of the β-amyloid precursor APP is involved in neuroplasticity processes and is necessary for the survival of nerve cells [28]. The fragment of this protein, the so-called β-amyloid peptide (Aβ), is the main component of senile plaques whish formation is considered to be the main pathomorphological sign of Alzheimer's disease, and the Aβ peptide found in bovine cattle brain shows some similarity with similar human brain peptides in the early stages of aging [29]. High density of localization of trinomial recombination products of species-specific *Bos taurus* retrotransposons BTLINE and BTLTRERV with constant architectonics (i.e. direct repetitions of BTLINE on the flanks of a trinomial structure in one chain and a homology site to BTLTRERV in the center in the alternative chain) found in two genes, closely related to the functions of the central nervous system, let us to assume their certain connection with those signs (reduced aggressiveness toward human) which D.K. Belyaev identified in animals as leading in domestication [30]. Interestingly, the Bov-B insertions, previously identified in the structural gene which is associated with craniofacial peculiarities in bovine cattle, are absent in this gene in humans and mice [31].

In general, the revealed distribution of retrotransposons and their recombination products in the 13,436,028 bp nucleotide sequences of bovine cattle chromosome 1 allows the following conclusions. In the studied of fragment, tRNA-Core-RTE, RTE-BovB, L1 and LTR ERV are often found. Their mutual localization in the genome is complex. The binomial associations, i.e. SINE and LINE, tRNA-Core-RTE and LTR EVR, ERVK/RTE-BovB, ERVK/L1 are the most common. The last two variants serve as the basis for the trinomial clusters RTE-BovB/BTLTR1/RTE-BovB and L1/BTLTR1J/L1. Another RTs of these trinomial clusters are not actually formed. A certain shift was found in the relatively high density of localization of these trinomial clusters to the distal end of the studied fragment of chromosome 1. Localization analysis of the revealed trinomial recombination products between LINE and LTR ERV in relation to structural genes showed that 34 such constructs are detected in 12 structural genes (while the rest are found in intergenic spaces), with 10 and 12 copies in two genes (*grik1* and *app*), closely associated in mammals with central nervous system function. The fact that in each of these two genes there were 9 copies of the trinomial construction RTE-BovB/BTLTR1/RTE-BovB, and the construction L1/BTLTR1J/L1 was found only in one copy in *grik1* and in three copies in *app*, allows us to consider

these genes as ancient targets for insertions and conservation. Note that the construction of L1/BTLTR1J/L1 was found only in these two genes, but not in the remaining 10 in which the RTE-BovB/BTLTR1/RTE-BovB recombination product is present.

So, we obtained data on the existence of regularities in the distribution of retrotransposon fragments and their recombination products in bovine cattle genome. The specific features of the distribution of recombination products between LINE and LTR ERV in the studied fragment of chromosome 1, and their localization in structural genes allow us to assume the possible presence of conserved structural and functional elements that perform a regulatory role. The identification of such elements is the subject of our further research.

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POPULATION-GENETIC CHARACTERISTICS OF DOMESTIC REINDEER OF YAKUTIA BASED ON WHOLE-GENOME SNP ANALYSIS

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Abstract

The Republic of Sakha (Yakutia) is one of the main reindeer herding regions of the Russian Federation. The census population size of domesticated reindeer in the Sakha Republic amounts to more than 156 thousand individuals. Three of the four officially recognized breeds are being bred in Yakutia: the Even, Evenk and Chukotka (Khargin). The analysis of single nucleotide polymorphisms (SNPs) using DNA microarrays (DNA chips) is the useful tool to assess and preserve the biodiversity of this important agricultural species. In the present work, we have used the Bovine SNP50 BeadChip to determine the genotypes and population-genetic characteristics of three domestic reindeer originated from the territory of the Republic of Sakha (Yakutia). Tissue samples (ear skin samples) from reindeer of the breeds Even (EVN, $n = 8$), Evenk (EVK, $n = 11$) and Chukotka (CHU, $n = 7$) were used as biological material for the study. The PLINK 1.07 software was used to check the quality of genotyping. For data processing, we used the software PLINK 1.07, Admixture 1.3, and R packages diveRsity, VennDiagram with subsequent visualization in the R packages pophelper and ggplot2. According to the results of quality control, we selected 512 polymorphic SNPs for further analysis. Analysis of Venn-diagrams showed that the reindeer of Even and Evenk breeds have a maximal number of unique polymorphisms (14 SNPs). Eleven unique SNPs were detected in the Chukotka breed. The calculation of basic population parameters revealed that individuals of the Chukotka breed are characterized by higher levels of genetic diversity ($H_o = 0.180 \pm 0.011$, $H_e = 0.156 \pm 0.008$, $A_r = 1.488 \pm 0.022$) and a higher excess of heterozygotes ($F_{IS} = -0.124$), compared to Evenk ($H_o = 0.161 \pm 0.009$, $H_e = 0.153 \pm 0.008$, $A_r = 1.487 \pm 0.020$, and $F_{IS} = -0.047$) and Even ($H_o = 0.164 \pm 0.010$, $H_e = 0.149 \pm 0.008$, $A_r = 1.471 \pm 0.021$, and $F_{IS} = -0.089$) breeds. The results of multidimensional scaling and the calculation of pairwise genetic distances (F_{ST}) showed the greatest closeness of the breeds Even and Evenk. Admixture analysis revealed a high degree of genetic isolation of each of the studied breeds. However, among the domestic reindeer of Chukotka and Evenk breeds we identified individuals with a mixed genetic origin, which is close to Even genetics. The obtained data will be applied in the development of programs for the conservation and sustainable use of this important animal species.

Keywords: single nucleotide polymorphism, genetic diversity, reindeer breeds

Reindeer herding is an important component of the economy of the North, Siberia and Far East of Russia [1]. In the Far Eastern Federal district, Republic of Sakha—Yakutia is one of the largest reindeer breeding regions. For indigenous inhabitants of the North of Yakutia domestic reindeer herding is the original lifestyle, which ensures the maintaining traditional livelihoods of representatives of ethnic groups, national traditions and culture. The main competitive advantage of the Sakha Republic is that the whole of its territory belongs to the Far North with a high level of the natural resource economic potential. Yakutia includes taiga-tundra zoogeographical zone, subdivided on the seaside, subarctic and mountain tundra with an unusually rich fauna, suitable for reindeer herding [2]. Reindeer herding is practiced in 20 of the 36 districts and in 1 city district of the Sakha Republic. Out of 3103.2 thousand sq. km of the territory of Yakutia, 2456.5 thousand sq. km or 79.2% are owned by the reindeer farms [3].

Until 90-ies of the last century, Yakutia consistently held third place in the Russian Federation for the number of domesticated reindeer (361 thousand heads) after Chukotka (491 thousand heads) and Yamal-Nenets Autonomous districts (490.5 thousand heads) [3]. Since the early 1990s, the disruption of the large farms into small ones, reduction of industrial reindeer husbandry and the decline of production and economic indicators of the industry, led to a decrease in the number of reindeer [1]. However, due to the implementation of a number of Government Regulations [4], the Presidential Program [5] and State Programs [6, 7], long-term decline in the number of domestic reindeer was suspended, and by the end of 2016, their number in the Sakha Republic amounted to more than 156 thousand heads [3].

Three of the four officially recognized breeds of reindeer are bred in Yakutia — Even (bred in the twelve districts is mountain-taiga, tundra and forest-tundra zones), Evenk (bred in nine regions of the taiga and tundra zones) and Chukotka (Khargin, bred only on the territory of the Nizhnekolymsk ulus). As of January 1, 2015, the number of deer of the above-mentioned breeds was 89913, 41774 and 20750 animals, respectively, which is of 59.0, 27.4 and 13.6% of the total reindeer population in Yakutia [8, 9]. The deer of Even breed are animals of meat-transport body type with long and relatively elongated body. The body weight of adult males and females is 135-145 kg and 91-110 kg, respectively [9]. The animals of Evenk breed also have a meat-transport body type. Individuals of this breed are characterized by the largest sizes, the higher body weight and good loading capacity. Animals of this breed are long-legged and have elongated body, which provides the best working qualities. The average body weight of adult males is 140-170 kg and females at the age of 5.5 years are 108-120 kg [9]. Reindeer of Chukotka breed have meat body type and are not specially adapted for transport purposes [10]. Chukotka reindeer are characterized by early maturity and good growing capacity. The body weight of adult males is around 130-140 kg and females are 93-96 kg [9].

For further effective use and the maintenance of the domestic reindeer population, and for overcoming the negative effects of decreasing their number, it is necessary to apply modern approaches for assessing and preserving the biodiversity of this important agricultural species. The genetic diversity of reindeer was widely studied using different types of genetic markers: protein polymorphisms [11, 12], mitochondrial DNA [13, 14] and nuclear microsatellites [15-18]. Until recently, microsatellites were the most common type of genetic markers to study the genetic drift and evolutionary processes, and to evaluate the differentiation degree and genetic diversity of the populations [19]. However, over the last decade, the development and improvement of high-performance meth-

ods of genotyping single-nucleotide polymorphisms (SNPs) led to a revolution in their application as molecular markers, which can give a precise genetic characterization of populations [20]. A huge number of SNPs in the animal genome (according to various estimates, from three to ten million), allows to select about 100 thousand markers with an average distance between them of 30 kb [21]. Moreover, every known or expected gene has an average of two SNPs. No other type of genomic differences can provide such density of mapping. In addition to high-density, SNPs are characterized by a very low mutation rate per generation ($\sim 10^{-8}$), which makes them excellent markers for studying molecular evolution [22, 23].

Practical interest to SNPs has greatly increased in the process of implementing projects aimed to identify the complete nucleotide sequence of agricultural animal species. For example, the sequencing of several complete genomes of cattle revealed the presence of significant numbers of SNPs [24]. Now, their number in the dbSNP database of the National Center for Biotechnology Information (NCBI) of the United States is 73,439,641 [25]. Later, the genomes of several other farm animal species - horses [26], pigs [27], sheep [28] were successfully sequenced.

The demand of this type of molecular markers has led to the development and application of microarrays (DNA chips), which are solid supports of small size (no larger than a microscope slide) with anchored, in a certain manner, short oligonucleotides (in the range of 8-25 bp) or DNA fragments (larger than 100 bp) [29]. Development of DNA microarrays of different densities for main farm animals species allowed to find a solution for a variety of tasks, such as the evaluation of the variability within and between breeds; determinations of the geographic localization of populations and admixture patterns within populations with different genetic origin; obtaining information concerning evolutionary relationships (phylogenetic trees) and clarifying centers of origin and migration routes; the implementation of gene mapping; creation of DNA banks [30]. To date, the commercial DNA chips designed by Illumina are widely used for the genotyping of nearly all major farm animal species and to obtain information on the genetic diversity for dozens and hundreds of thousands of markers. For non-model species (including reindeer), due to the lack of information about the complete genomes, the development of such kind of an SNP matrix remains unachievable [31]. However, a number of authors have demonstrated the possibility of using such commercial DNA chip (designed for domestic animals) for obtaining information about the genetic diversity and population structure of non-model species [32-37]. In 2015, we published the first evidence of the successful application of DNA chips developed for cattle (Illumina BovineSNP50 BeadChip) and domestic sheep (Illumina OvineSNP50 BeadChip) to study the reindeer [38]. The obtained results served as the basis for further use of the BovineSNP50 BeadChip with the aim of genetic characterization of previously unstudied breeds and populations of this unique representative of the genus *Rangifer*.

In this paper, we report for the first time a more accurate genetic characterization of the three breeds of domestic reindeer in the Republic of Sakha (Yakutia). The obtained data can be used for improvement of reindeer breeding and husbandry in the region.

The aim of this work was the study of biodiversity of domestic reindeer *(Rangifer tarandus*) breeds – the Even, Evenk and Chukotka, which are bred on the territory of Yakutia, using a medium - density BovineSNP50K BeadChip.

Techniques. The study was carried out on the deer of three breeds: Even (EVN, FGUP Yukuhashi, $n = 8$), Evenk (EVK, NAOK Taba, $n = 11$) and

Chukotka-Khargin (CHU, JSC Khatystyr, n = 7). As comparison group, the samples of Yakut wild reindeer (WLD, n = 16) collected during field research in the taiga (TGA, n = 4) and tundra (TUN, n = 12) natural-climatic zones of Yakutia were included in the analysis.

The extraction of genomic DNA from tissue samples (ear samples) was performed using the Nexttec columns (Nexttec Biotechnologie GmbH, Germany) according to the recommendations of the manufacturer. The quality of the isolated DNA was checked by electrophoresis in agarose gel. The DNA concentration in the solution was determined by the measurement of absorption at wavelength 260 nm (OD_{260}). To check the purity of DNA we calculated the ratio of the absorption at wavelengths 260 nm and 280 nm (OD_{260}/OD_{280}). Screening of single nucleotide polymorphisms was performed using medium-density Bovine SNP50 BeadChip (Illumina, San Diego, CA, USA), including 54609 SNPs.

The PLINK v1.07 software [39] was used to perform quality control of genotyping. The following filters were applied: excluding SNPs genotyped in less than 90 % of individuals (--geno - 0.1); excluding individuals with more than 10% of missed SNPs of total number of SNPs (--mind - 0.1); excluding loci with minor allele frequency less than 5% (--maf 0.05); excluding markers not corresponding to the $\chi 2$ criterion for Hardy-Weinberg equilibrium in a population ($p \leq 1\times10^{-6}$); deleting SNP with the value of linkage disequilibrium (LD) between pair of single nucleotide polymorphisms equal to $r^2 > 0.05$ (we used a sliding window of 50 SNPs, sliding along in 5 SNP increments). Additionally, to assess the quality of SNP genotyping we used GC Score (quality of reading SNP) and GT Score (the level of clustering SNP) of at least 0.5 (50 %) [40].

To construct Venn diagram, indicating SNPs, which are unique and common to each breed, we used the R package VennDiagram. To assess genetic diversity in studied reindeer breeds we calculated the values of observed (H_o) and expected (H_e) heterozygosity, allelic diversity (A_r) using the rarefication procedure, the inbreeding coefficient (F_{IS}) [41]and visualized genetic diversity employing the R package diveRsity [42]. Multidimensional scaling (MDS) based on the matrix of distances of the identity-by-state (IBS) was performed in PLINK 1.07 (--cluster, --mds-plot 4). The degree of genetic differentiation of populations was assessed based on pairwise values of F_{ST} [43] calculated in the R package diveRsity [42]. Results of genetic structure of populations and relationships between populations were obtained using the Admixture 1.23 software [44] with subsequent visualization in the R package pophelper [45].

In data statistical processing, the arithmetic mean (M) and standard errors of mean ($\pm m$) were calculated.

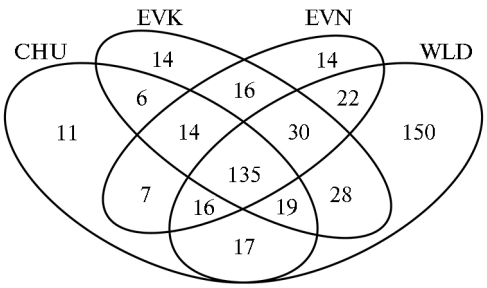

Fig. 1. Venn-diagram of the distribution of unique and common SNPs for studied groups of reindeer (*Rangifer tarandus*). Breeds of domesticated reindeer: CHU — Chukotka, EVK — Evenk, EVN — Even; WLD — population of wild reindeer (Sakha Republic, 2016-2017).

Results. In total, 22807 SNPs passed through the quality control and 512 of them were polymorphic and were used for further analysis. The small number of polymorphic markers is due to the fact that with increasing phylogenetic distances between *Bovidae* and *Cervidae* families, the number of polymorphic SNPs decreases, which was also revealed by a drop in the total number of genotyped SNPs (call rate) from 99.54 to 61.19% (37).

Analysis of genetic diversity in domesticated reindeer (Table 1)

showed a higher level of observed heterozygosity in the Chukotka reindeer breed (H_o = 0.180±0.011). Previously, it was shown that this breed was superior to the Even and Evenk breeds in terms of genetic diversity assessed using microsatellites [18]. Animals of the Evenk breed were characterized by the lowest values of observed heterozygosity (H_o = 0.161±0.009). Calculation of rarified allelic diversity (A_r) showed no significant differences between the breeds: the values of A_r ranged from 1.471 in EVN to 1.488 in CHU. All three breeds were characterized by a slight excess of heterozygotes, as evidenced by the negative values of the coefficient of inbreeding (F_{IS}), with a range from F_{IS} = –0.124 in CHU to F_{IS} = –0.047 in EVN.

1. Genetic diversity of three reindeer (*Rangifer tarandus*) breeds based on single nucleotide polymorphism genotyping (Sakha Republic, 2016-2017)

Breeds	*n*	H_o (*M*±*m*)	H_e (*M*±*m*)	F_{IS}	A_r (*M*±*m*)
CHU	7	0.180±0.011	0.156±0.008	–0.124	1.488±0.022
EVK	11	0.161±0.009	0.153±0.008	–0.047	1.487±0.020
EVN	8	0.164±0.010	0.149±0.008	–0.089	1.471±0.021

N o t e. Breeds of domesticated reindeer: CHU — Chukotka, EVK — Evenk, EVN — Even; H_o —observed heterozygosity, H_e — expected heterozygosity, F_{IS} — coefficient of inbreeding, A_r — allelic diversity, calculated using the rarefication procedure.

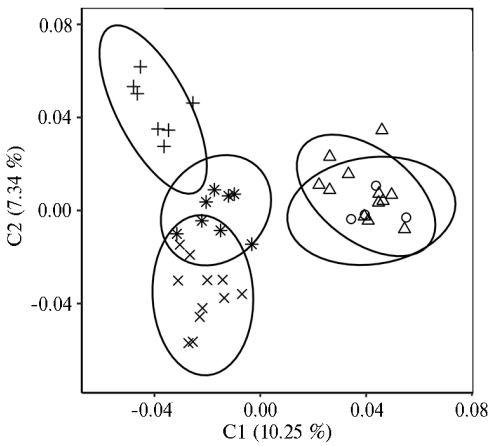

Fig. 2. Graphic representation of results of multidimensional scaling (MDS) of reindeer *(Rangifer tarandus)* samples, genotyped with Bovine SNP50 BeadChip: + — CHU (Chukotka breed), × — EVK (Evenk breed), ✳ — EVN (Even breed); ○ and △ — taiga and tundra populations of wild reindeer, respectively; C1 — component 1, C2 — component 2 (Sakha Republic, 2016-2017).

A diagram of the location of the studied samples based on SNP analysis using the method of multidimensional scaling (MDS) is presented in figure 2. The first component (C1) explained 10.25 % of the total variance and clearly separated domestic and wild populations, while, the second component (C2), explaining about 8 % of the total variability, allowed to differ the domestic reindeer breeds from each other. The individuals of the Even and Evenk breeds were characterized by the greatest genetic closeness with a minor overlapping of breed-specific clusters. The presence of admixed individuals can be explained by the closer geographical location of breeding farms of these breeds. Wild reindeer of the taiga and tundra populations formed a common cluster indicating their genetic similarity.

2. Differentiation of the studied breeds of reindeer (*Rangifer tarandus*) by F_{ST} index at pairwise comparison, based on SNP genotypes (Sakha Republic, 2016-2017)

Groups	CHU	EVK	EVN	WLD
CHU	0.0000			
EVK	0.0953	0.0000		
EVN	0.0981	0.0632	0.0000	
WLD	0.1007	0.0659	0.0597	0.0000

N o t e. Breeds of domesticated reindeer: CHU — Chukotka, EVK — Evenk, EVN — Even; WLD − population of wild reindeer.

Calculation of F_{ST} values at pairwise comparison (Table 2) showed similar trends with the results of the MDS analysis: the lowest value (F_{ST} = 0.006)

was detected between the Even and Evenk breeds confirming their greater genetic closeness. The reindeer of Chukotka breed were equidistant from both breeds: the Even (F_{ST} = 0.098) and the Evenk (F_{ST} = 0.095); and were the most differentiated from the wild population (F_{ST} = 0.101).

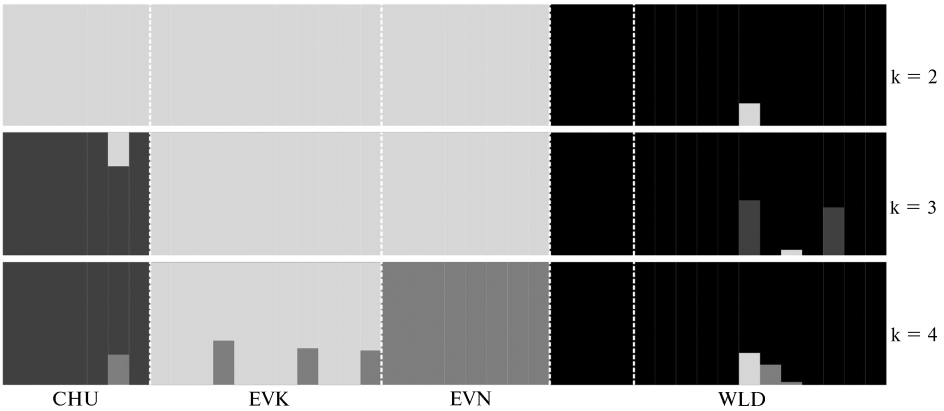

Fig. 2. Genetic structure of studied breeds of reindeer (*Rangifer tarandus*) based on results of admixture analysis of samples genotyped with Bovine SNP50 BeadChip for the cluster number k from 2 to 4: CHU — Chukotka, EVK — Evenk, EVN — Even; WLD – population of wild reindeer (Sakha Republic, 2016-2017).

Admixture analysis of the genetic structure of the studied reindeer breeds (Fig. 2) for the number of clusters k = 2 demonstrated a clear differentiation of domestic breeds from the wild reindeer population. At k = 3, the individuals of Chukotka breed formed their own cluster, while animals of the Even and Evenk breeds clustered together. At k = 4, each of the investigated breeds formed its own cluster with a high degree of genetic isolation. However, it is necessary to indicate that within the Chukotka and Evenk breeds some animals had an admixed genetic origin, which was closer to EVN breed. Individuals of the wild reindeer population, used as a comparison group, retained their membership in their own cluster at k from 2 to 4, although some of them revealed the admixture signals of domesticated reindeer.

Thus, our study showed the successful application of DNA chip designed for cattle to differentiate reindeer breeds from each other and from the wild reindeer, and to assess the genetic structure of breeds.

Numerous publications have demonstrated the successful study of related non-model species using a commercial BovineSNP50K BeadChip, developed for domestic cattle. G.D. Haynes and E.K. Latch [35] gave a description of two representatives of the family *Cervidae*: mule deer (*O. hemionus*) and white-tailed deer (*O. virginianus*). They found that 38.7 % of loci could be genotyped, of which 5 % (n = 1068) were polymorphic. R. Kasarda et al. [37] performed the study of two species from the family *Cervidae*: red deer (*Cervus elaphus*) and fallow deer (*Dama dama*). In total, 94.76 % SNPs were successfully genotyped and 5.24 % (1542) of them were polymorphic. Studying the European bison *(Bison bonasus*), M. Tokarska et al. [46] found 960 polymorphic loci. C. Pertoldi et al. [32] identified 929, 1403 and 1524 polymorphic SNPs in European bison (*Bison bonasus*; EB) and two subspecies of American bison: the plains bison (*B. bison bison*; PB) and the wood bison (*B. bison athabascae*; WB), respectively. Authors showed that SNP genotyping systems developed for domestic species could be effective even in bottlenecked species in which heterozygosity of other markers such as microsatellites may be very low [46, 32]. Along with the study of biodiversity and evolutionary relationships, SNP markers have found application

for the determination of the parentage verification [47]. Thus, above-mentioned studies clearly showed that SNP genotyping systems developed for domestic species represent powerful tools for genetic analysis in related non-model species

Thus, as the result of our study of single nucleotide polymorphisms using DNA chips, designed for cattle, we gave the population-genetic characterization of three breeds of domestic reindeer — Chukotka (Khargin), Even and Evenk, which are bred in the territory of one of the main reindeer herding regions of Russia — Republic of Sakha (Yakutia). Reindeer herding in this region is connected not only to the agricultural sector, but is an integral part of the lifestyle and culture of indigenous people. The obtained data can be used to develop programs for the conservation and management of this species important for agricultural biodiversity.

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GENETIC DIVERSITY IN TYVA HORSES DERIVED FROM POLYMORPHISM OF BLOOD SYSTEMS AND MICROSATELLITE DNA

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Abstract

Tyva horse breed is one of the most promising local breeds of universal use. Tyva horses are well adapted to year-round pasture grazing, resistant to disease, and, therefore, suitable for low-cost meat production. Due to relatively isolated location of the population which has ancient origin uva horses are undoubtedly of interest both genetically and evolutionarily. For allele pool study we sampled blood (n = 32) and hair (n = 35) specimens from Tyva horses reared at two farms of Tyva Research Institute of Agriculture in 2009-2016. Genetic analysis was carried out according to the authority certified by ISAG (International Society of Animal Genetics). The study of polymorphic blood system and microsatellite DNA loci showed the Tyva horses to be fairly high genetically diverse on structural genes and microsatellite DNA. High polymorphism was found in *Tf*, *Al*, *Es* loci and especially in D system of blood groups. D^{cgm}, D^{bcm} and D^{d} alleles were comparatively high-frequent, while Da^{d}, D^{de} and D^{dk} were relatively rare. As to microsatellite DNA polymorphism, there were 113 alleles in 17 loci (6.65 alleles per locus on average), indicating high genetic diversity in the Tyva horse breed. Amon the microsatellite DNA loci found, VHLP, AHT4P, HMS7J, ASB23 L, ASB2B, HMS3 N, ASB17Q, LEX3K, LEX3P, HMS1I, HMS1N and HMS1R were rare, of which HMS1R was unique as not found in horses of European origin (L.H.P. Van de Goor et al., 2010). There were from 4 to 9 alleles in the studied microsatellite loci, and the average number of effective allele per locus (A_e) made 4.20 being rather high even for local breeds. Loci ASB17 (10 alleles), AHT4 (9 alleles), VHL20 (9 alleles), ASB2 (9 alleles) and ASB23 (8 alleles) were the most diverse. The genetic population analysis demonstrated good correspondence between the observed (H_o = 0.748) and the expected (H_e = 0.742) heterozygosis level and the absence of inbreeding (F_{IS} = –0.008) in the Tyva horses. The highest similarity was found out between Tyva horse and Khakass horse (0.823), and also between Tyva horse and Mongolian horse (0.822) which areas border on the South and South-East. The data of whole genome association analysis (J.R. Mickelsn et al., 2012) are also in line with genetic distance that we calculated based on 17 microsatellite loci polymorphism. The visualized dendrogramm indicated common origin of the Tyva and Mongolian horses, which make a common branch in the evolutionary tree of horse breeds. Our findings indicate a high level of genetic plasticity of Tyva horses promising for breeding. In general, we can conclude that the studied population of Tyva horses is characterized by the original allele pool, including a number of rare alleles that must be preserved in the breed at rearing and genetic improvement.

Keywords: genetic diversity, microsatellite DNA, polymorphic blood systems, population analysis, Tyva horse

Domestic stud and local breeds of horses, well adapted to local climatic conditions and resistant to diseases, often have a unique allelic pool which is a significant breeding reserve [1].

Genetic markers allow monitoring of genealogical structure of populations, matching and selection of animals for breeding programs that take into

account the genotype [2-5]. Using the microsatellite analysis, the gene pool, the composition of populations, phylogenetic connections in a number of unique native breeds of horses, Khakass and other aboriginal Siberian breeds [6-8], Budennovskaya [9], Bashkir [10] and Karachai [11], were studied. Microsatellites are used for molecular genetic comparison of lines and families [12, 13], estimation of genetic diversity [14, 15] and breed stability [16], for marking genotypes, control of origin [17-19], and in gene flow studies [20]. Microsatellites can be combined with polymorphism markers of structural or mitochondrial genes [17, 21, 22]. Blood group systems due to the codominant type of inheritance of antigenic factors, broad polymorphism and relatively easy assessment [23, 24] also remain important markers in population studies [25-28].

The Tyva horse (*Equus ferus caballis*), which is related in origin to the Mongolian one but is larger in size, is valued for its adaptability to year-round pasture maintenance in herds out of premises. It is more resistant to abiotic and biotic stressors, including pathogens, and is less susceptible to disease [29]. The horse farms of the republic contain 68.1 thousand heads [30]. They are mainly used for meat, and the body weight of mares by the end of the pasture period averages 316±7.2 kg. In the livestock structure the mares are 35.2-42.5 %, the yield of foals is 58 heads per 100 mares [31]. The production of horse meat is almost 3 times cheaper than beef. This, along with the availability of favorable conditions and demand, determines the desirability of breeding local horses and raising their meat productivity which is relatively small. At the same time, it is important to preserve the valuable adaptive qualities of the breed. Therefore, in selection and breeding, it is necessary to take into account not only the phenotype, but also the genotypic characteristics of animals.

It should be noted that only one work in which the systems of polymorphic blood proteins were studied [24] was devoted to the immunogenetic traits of Tyva horses, and DNA microsatellite loci test was just recently started. Here, this is the first complex study of the Tyva horse population for combination of immunogenetic markers, such as polymorphic proteins (transferrin, albumin, esterase) and blood group systems, together with DNA microsatellites, which allows us to significantly expand the range of markers for genetic selection programs.

The purpose of this work was to analyze the genetic structure of the Tyva horse population based on the prevalence of the loci of polymorphic blood systems and polymorphism of DNA microsatellites.

Techniques. The research was carried out in 2009-2016 at the basic farms of the Tyva Agricultural Research Institute (GUP Bai-Tal and KFKh Beeche-Shivilig; Bai-Taiginskii and Kyzylski regions, Tyva Republic) on Tyva breed horses (*Equus feru scaballis*). The biomaterial was blood samples (n = 32) and hair samples with a hair follicle (n = 35). When selecting horses for the study, the exterior, body color and weight, and the presence of aboriginal features were evaluated. The whole year the animals were kept on pasturage.

Genotypes on the loci of blood proteins and enzymes were determined by horizontal electrophoresis in starch gel according to the recommendations [32], antigens of blood group systems were studied by hem agglutination with monospecific sera produced by the All-Russian Research Institute of Horse Breeding (certified by the International Society of Animal Genetics, ISAG) which were identified with international standards.

DNA was extracted from hair follicles using Diatom™ DNA Prep and ExtraGene™ DNA Prep kits (OOO Isogen, Moscow). Amplification was performed using StockMarks for Horses Genotyping Kit Equine (Thermo Fisher Scientific, Inc., USA) in a 2720 Termal Cycler (Applied Biosystems, Inc., USA)

with a set of primers for 17 microsatellite loci recommended by ISAG. Electrophoresis of the amplification products was carried out in an automatic 4-capillary 3130 DNA Analyzer (Applied Biosystems, Inc., USA). Decoding and documentation of the results was carried out using the GeneMapper™ v.4.0 software (Applied Biosystems, Inc., USA).

To characterize the allelic pool, the average number of alleles (Na) and the number of effective alleles (Ne) per locus, observed (H_o) and expected (H_e) heterozygosity, and polymorphism indexes (A_e) were calculated. Gene balance in the population, genetic similarity and genetic distance were assessed by conventional methods [33, 34].

Cluster analysis was performed on the basis of calculation of genetic distances between breeds based on the frequency of microsatellite loci alleles by M. Nei [34]. In comparing the genetic structure of the Tyva horse with other breeds, previously published data were used [3, 8].

Data statistical processing was carried out in MS Excel 2003 and Statistica 6 softwares (StatSoft Inc., USA). The table shows the mean (X) and standard errors of the mean ($\pm x$). Differences among the values were considered statistically significant at $P > 0.95$.

Results. Analysis of blood group systems ($n = 17$) and polymorphic blood protein systems ($n = 15$) revealed a distinctive pedigree uniqueness of the genetic structure of the Tyva horses, despite a small number of studied animals. In the transferrin system, 5 alleles were detected, Tf^D, Tf^F, Tf^H, Tf^R и Tf^O. In the population allele pool, Tf^F allele prevailed with the frequency of 0.324; the frequency of Tf^H and Tf^R alleles was almost the same (0.235). The Tf^O allele (0.029) was comparatively rare. In the albumin locus, two alleles were found, ALB^A and ALB^B, of which ALB^B prevailed (0.559). In the esterase locus, there were 3 alleles, Es^F, Es^G и Es^I. In the studied horses, the Es^I allele was the most common (0.559) while Es^F (0.206) and Es^G (0.235) were relatively less frequent.

We determined the frequency of antigens of the three genetic blood systems (A, K, and D). For EAA system, the Tyva horses were carriers of the blood marker A^a (Table 1). For EAK system, antigens were found indicating the presence of K^a and K^- alleles with relatively more frequent K^a allele (0.2941±0.1110).

1. Frequency of blood group system loci in Tyva horses ($X \pm x$, $n = 17$, Bai-Taiginsky and Kyzylsky regions, Republic of Tyva, 2009-2016)

EAA system		TAD system		EAK system	
genotype	frequency	genotype	frequency	genotype	frequency
A^a	1,00±0,00	$D^{ad/d}$	0.0588±0.0570	K^a	0.2941±0.1110
		$D^{bcm/dghm}$	0.0588±0.0570	K^-	0.7059±0.1110
		$D^{bcm/cgm}$	0.1176±0.0780		
		$D^{bcm/d}$	0.1176±0.0780		
		$D^{cgm/d}$	0.0588±0.0570		
		$D^{cgm/de}$	0.1765±0.0920		
		$D^{cgm/dk}$	0.0588±0.0570		
		$D^{cgm/cgm}$	0.0588±0.0570		
		$D^{cgm/dghm}$	0.2941±0.1110		

A great variety was found in the frequency of EAD antigenic factors. In horses, this blood group system is the most complex, includes 17 antigens that form more than 30 phenogroups, and is considered as the most informative for understanding breed genesis. The data obtained by us (see Table 1) indicated the dominance of the genotype $D^{cgm/dghm}$ in the local breed, with the frequency of 29.41 %. Alleles D^{cgm}, D^{bcm} and D^d showed a relatively high frequency, and D^{ad}, D^{de} and D^{dk} were relatively rare.

When studying the polymorphism of microsatellite DNA in Tyva horses, 113 alleles were found in 17 loci, or on average 6.65 alleles per locus,

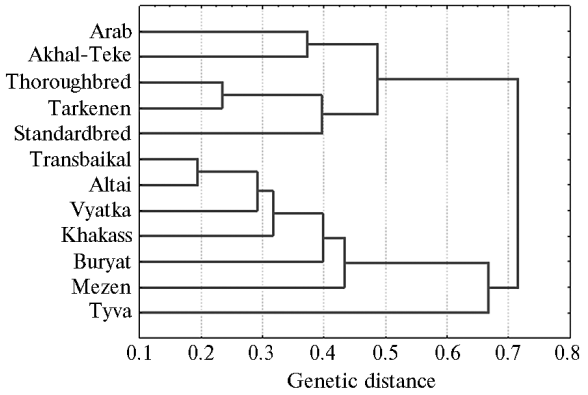
which indicates a high genetic diversity of the breed (Table 2). In a number of loci, rare alleles VHLP, AHT4P, HMS7J, ASB23L, ASB2B, HMS3N, ASB17Q, LEX3K, LEX3P, HMS1I, HMS1N and HMS1R were revealed. It should be specially noted that the Tyva horses have a very rare HMS1R (0.014) allele that is not found in the populations of horses of European origin [18].

2. The frequency of heterozygosity (H_o) for 17 microsatellite loci in Tyva horses ($X \pm x$, n = 35, Bai-Taiginsky and Kyzylsky regions, Republic of Tyva, 2009-2016)

Locus	Alleles		H_o
	number	set	
VHL20	9	I, J, L, M, N, O, P*, Q, R	0.771
HTG4	6	K, L, M, N, O, P*	0.600
AHT4	9	H, I, J, K, L, M, N, O, P*	0.857
HMS7	5	J*, L, M, N, O	0.771
HTG6	5	G, I*, J, M*,O	0.714
AHT5	6	J, K, L, M, N, O	0.743
HMS6	5	K, L, M, O, P	0.857
ASB23	8	G*, H*, I, J, K, L, S, T, U	0.857
ASB2	9	B*, C*, I*, K, M, N, O, P, Q	0.800
HTG10	7	I*, K, M, O, Q, R, S*	0.714
HTG7	4	K, M, N, O	0.829
HMS3	6	I, M, N*, O, P, Q, R	0.886
HMS2	5	H, I, J, K, L	0.794
ASB17	10	G, I, J, K, L, N, P, Q*, R	0.943
LEX3	7	F, H, I, L, M, N, P	0.886
HMS1	6	I*, J, K, M, N*, R*	0.514
CA425	6	I, K, L, M, N, O	0.800

Note. Rare alleles are marked by asterisk. The R allele found in the HMS1 locus does not occur in European breeds of horses.

Test showed that Tyva horses are characterized by significant polymorphism in a number of microsatellite loci. This indicates a very high genetic diversity in the population. In the studied microsatellite loci, from 4 to 9 alleles were identified, the average number of effective alleles per locus (A_e) was 4.20, which is considered to be a sufficiently high index even for local horse breeds. The loci ASB17 (10 alleles), ANT4, VHL20 and ASB2 (9 alleles) and ASB23 (8 alleles) were the most diverse. Cluster analysis confirmed the uniqueness of the genetic structure of the Tyva breed (Fig.).



Dendrogram of genetic distances according to M. Nei between horse breeds based on 17 DNA microsatellite loci (data by V.V. Kalashnikov et al.) [3].

When surveying horses in KFKh Beeche-Shivilig (n = 11), 12 additional alleles were found in 9 out of the 16 studied microsatellite loci, which occurred at a frequency of < 5 % that indicates high genetic plasticity of the breed. The average value of H_o for microsatellite loci in Tyva horses from this farm was 0.721 with fluctuations from 0.480 to 0.960.

Genetic population analysis demonstrated good compliance of the observed (H_o = 0.748) and expected (H_e = 0.742) heterozygosity for microsatellite loci, and the absence of inbreeding in the Tyva horse (F_{IS} = –0.008). When comparing the breeds, Tyva and Khakass (0.823) and Tyva and Mongolian (0.822) horses, which areas border on the south and southeast, showed the greatest genetic similarity. The common origin of the Tyva and Mongolian horses which form a common branch in the tree of caballine breed evolution is also confirmed by a full-genome analysis [35].

Thus, our study of the loci encoding blood systems and microsatellite DNA showed that the Tyva horse exhibits genetic variability on the alleles of both structural genes and microsatellites. Of the structural genes, the transferrin, albumin, esterase, and especially the D blood group loci, with both frequent and

relatively rare alleles, were the most polymorphic. Polymorphism of microsatellite DNA averaged 6.65 alleles per locus which indicates a high genetic diversity. In a number of loci, rare alleles and the HMS1R allele which is not found in European horses are identified. Loci ASB17, ANT4, VHL20, ASB2 and ASB23 were the most diverse. Tyva horse is genetically the most similar to the Khakass (0.823) and Mongolian (0.822) breeds which areas border on that of Tyva horse. It is important to keep the original allele pool of the Ttva horse, which includes a number of rare alleles, for further breeding and selection. Improvement of the Tyva horse which is characterized by genetic plasticity seems to be very promising.

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REPRODUCTIVE FUNCTION IN HYBRID POULTRY. IV. AN IMPACT OF MATERNAL HORMONES ACCUMULATED IN EGG

(review)

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Abstract

Deposit of maternal hormones in the egg yolk is shown to significantly change the pattern of ontogenesis in descendants. Accumulation of maternal sexual steroids in yolk influences behavior, growth, morphology, immune function and viability of descendants (T. Groothuis et al., 2005). Testosterone and androstenedione cause changes in postnatal growth (H. Schwabl, 1996), immunocompetence (M.Tobler et al., 2010), models of competitive and agonistic behavior in non-reproductive relationships between individuals (Müller W. et al., 2009) and sexual intercourse (C .Eising et al., 2006). Such consequences develop as a result of regulation of corresponding functions in the descendant body, including indirect influence through other systems. Stress simulation in females by administration of corticosterone (K) led to a dose dependent change in growth and development in the chicken. Imbalance in fatty acids' ratio and assimilation in descendant embryo occurred in the yolk (S. Yalçın et al., 2011) reduce fertility and shell quality, and embryo mortality and death of chicks increase (M. Eriksen et al., 2003; Saino N. et al., 2005; Y.-H. Kim et al., 2014). Similar effects were found in the offspring of hens lines divergently selected by growth rate (A. Abdelkareem et al., 2013). In the yolk of white shell eggs of unstressed layers the corticosterone level is almost two times higher than that in brown shell eggs (K. Navara et al., 2010). Under the influence of different stress factors the activity of the hypothalamic-pituitary-gonadal axis alters, resulting in an inadequate kinetics of sex hormones and inhibition of the reproductive function. Increased concentration of blood corticosterone in the mother hens is accompanied by changes in the content of gonadal hormones in the egg yolk (A. Janczak et al., 2009; F. Guibert et al., 2013), productivity (A. Bertin et al., 2008; E. de Haas et al., 2013) and the sex ratio (S. Correa et al., 2005; T. Pike et al., 2005; S. Pryke et al., 2011). Migration of the hormones form a mother hen to the egg and the embryo, and their interference in metabolism regulation in the descendant occur during early ontogenesis, when the functions of organs and systems are the most labile. Changes in ontogenesis caused by accumulated maternal hormones can be regarded as an adaptive response in the descendants to be ready to a shift in environmental conditions (T. Mousseau et al., 1998; Z. Kankova et al., 2012). Due to egg-deposited maternal hormones the offspring can form phenotypic traits which are inherited epigenetically (T. Groothuis et al., 2008; D. Ho et al., 2011). All these finding should be taken into account at poultry commercial reproduction. When using technological methods and veterinary measures, it is necessary to appreciate possibility of transovarial transfer of signal information about outer conditions mediated by the maternal hormones to cause adaptations in the descendants.

Keywords: bird, egg, maternal hormones, accumulation, stress, ontogenesis descendants

During ovogenesis, maternal biologically active substances accumulate in the egg deutoplasm, determining the character of ontogeny in descendants. Accumulated hormones significantly affect the metabolism of a developing organism, forming phenotypic characters inherited epigenetically in individuals of the next generation [1-3]. Due to various stress factors in the layers, stress-realizing systems are activated, thus the hormones concentration in eggs increases.

This review is focused on summarizing and analyzing features of reproductive function of the poultry breeding stock caused by the maternal hormones

deposited in the egg deutoplasm.

Influence of maternal sex steroids. Accumulation of maternal hormones in egg yolk (deutoplasm) affects behavior, growth, morphology, immunofunction, and viability of the offspring [4]. Thus, testosterone and androstenedione cause changes in postnatal growth [5], immunocompetence [6], models of competitive and agonistic behavior in non-reproductive relationships between individuals [7] and sexual interactions [8]. The consequences develop due to direct regulation of the corresponding functions by these hormones, or under indirect influence through other systems [2, 9, 10].

The content of sex steroids in the egg yolk depends on the genotype, nature and the impact of stress factors on the female organism during egg-laying. Thus, in quails, unstable communities lead to an increase in testosterone level, whereas the amount of progesterone and androstenedione remains the same [11]. In quails, testosterone and androstenedione decreased and progesterone increased in response to the presence of a person [12]. To study testosterone accumulation in yolk as influenced by social status of the layer, white Leghorn males and females were grouped together, five hens and a rooster per cage. Dominant, two intermediate and subordinate social ranks were studied. The study showed that from dominance to subordination the living weight of individuals and the number of laid eggs decreased from 1862 to 1503 g, and from 66.8 to 63.5 g, respectively, while testosterone increased [13].

Z. Kankova et. al. [14] studied the effect of divergent selection of Japanese quails for the testosterone content in yolk on the immune system activity under limited feeding and weight loss. In the L^+ individuals (here and below, L^+ and L^- are the lines of poultry that underwent contrast selection on a definite trait), the body weight and IgY content were greater, and the ratio of heterophiles and lymphocytes was less than that in L^-. The immune response to the administration of phytohemagglutinin and the corticosterone concentration did not depend on the genotype. An increase in testosterone content in yolk stimulated cell-mediated immune response in *Carpodacus mexicanus* individuals and humoral-mediated immune response in adult *Taeniopygia guttata* individuals [6]. The androgens suppressed both the humoral and cellular immune responses in black-headed gull (*Larus ridibundus*) [15] and western jackdaw (*Corvus monedula*) [16] at different stages of the early ontogenesis

Thus, under the impact of stress factors on the female organism during egg formation, the amount of sex steroids in yolk changes. Concentrations of testosterone, progesterone and androstenedione depend on nature (hierarchical dominance or subordination, fright, feeding regime, etc.) and intensity of a stressor. The author discovered that maternal body weight and the weight of the eggs which the hen laid depend on the maternal social rank. Sexual dimorphism is observed according to the testosterone content in yolk. The immunomodulating effect of maternal androgens varies according to the type of response, depending on the type of bird, the stage of development, and the environmental conditions.

Influence of maternal glucocorticoids. In commercial poultry farming, there is a high probability of stress resulted from some technological factors [17-23]. The risk of developing a metabolic stress is typical of both the egg crosses showing increased aggression, tending to pecking, plucking feathers (PPF), fatty liver syndromes and the sudden cessation of egg laying, and for broiler chickens predisposed to ascites and a reduced thermotolerance [24, 25]. Metabolic stress in birds is realized through the stimulation of sympatic-adrenomedullary and hypothalamic-pituitary-corticoadrenal (HPCA) systems as a result of activity of noradrenaline, adrenaline and corticosterone secreted into

the blood [26, 27]. These hormones, regulating the metabolism intensity, prepare the body for a common "fight or run" reaction [28, 29]. Circulating corticosterone changes the endocrine function of gonads [30], protein metabolism and lipid metabolism [31, 32], vitellogenesis in liver [33], and eventually inhibits reproductive function [34, 35]. These events occur when increasing activity of the hypothalamic-pituitary-gonadal axis which regulates the realization of the genetic potential of egg production with the participation of testosterone, progesterone, androstenedione, and estradiol [36].

Maternal corticosterone deposition in yolk is associated with changes in the characteristics of hatching eggs. In particular, decreased indicators of fertility and shell quality, higher mortality (prior and post hatching), immunodeficiency are developed [37, 38]. These processes are accompanied by a depression of the reproductive function caused by genetic breeding for productive and/or unproductive traits, or by parents' aging [24, 25, 39]. Some authors report that the bird's stress response decreases with age, depends on the genotype, and is higher in hybrids than in specialized lines [40].

Reproductive function during breeding for identical but different productive and unproductive traits or during divergent selection for the same trait differs [24, 25]. The content of maternal glucocorticoids in the deutoplasm of eggs from highly productive poultry of different crosses is different [41-46]. In case of divergent breeding, the differences are revealed in the corticosterone content in egg yolk and white, as well as in the expression of enzymes that regulate the intracellular metabolism of glucocorticoids (11-β and 20-βhydroxysteroid dehydrogenase) in liver of embryos-descendants of meat hens [41). In eggs, more corticosterone was accumulated in L^+ individuals compared to L^- ones. The same regularity (i.e. an increase or decrease in blood corticosterone in response to short-term immobilization) was established in Japanese quails under divergent selection [42]. The concentration of corticosterone was significantly higher in eggs of L^+ than in L^- hens, regardless of they were intact or stressed.

The stock of genotypes with white eggshell is shyer than the one with brown eggshell [43]. The study of manifestation of two associated traits (anxiety and PPF syndrome in chicks of egg crosses Dekalb White and ISA Brown) showed [44] that the first one, being aged 7 weeks, made 16.9 peckings, the second one made only 11.4 peckings during 20 min. The permissible distance to the human observer at 10 weeks of age was at least 153 and 58 cm respectively. In the egg yolk of the Hy-Line W-36 cross layers kept individually, the corticosterone concentration was 2 times as much as that in Hy-Line Brown layers, i.e. 1.6 and 0.8 ng/g, respectively [45]. The author recorded identical corticosterone concentrations in blood and manure of the intact white Leggorne and Hy-Line Brown layers [46]. However, when captured, the Leggorne layers showed higher concentration of this hormone and higher duration of tonic immobility as compared to the Hy-Line Brown layers. Therefore, chickens laying eggs with white and brown eggshells differ in stress responsiveness. The first deposits more maternal corticosterone in yolk, which causes increased aggression.

The correlations were justified between the indices of the immune and endocrine systems in the ISA Brown cross layers due to the divergent selection for the primary antibody production [47]. Under the impact of stress factors of different nature (blood sampling and the number of individuals per cage) in L^+ birds (freely mated control group, initial population) and L^-, the blood corticosterone concentration differed. For example, in blood samples this figure was 46, 28 and 81 ng/ml, respectively. Differences in the HPCA reaction for the studied genotypes were also expressed in coping behavior strategy (reactive style in the livestock of L^-, and proactive in L^+). Thus, divergent selection for primary

immune response caused unequal changes in HPCA reactiveness. All this let it possible to differentiate the population by sensitivity to stress factors, which manifests itself in different coping styles [48].

Dose-dependent changes in pre- and postnatal growth and development of chicken were found in the stress modeling by using corticosterone as an additive to feed. Observations of the stress markers kinetics, including the concentration of uric acid and the blood ratio of heterophyll to lymphocytes, made it possible to determine that the severity of this state is in direct proportion to the dose of the hormone (1.0, 1.5 or 2 mg/head per day) [49]. Only 3 days after the feeding with corticosterone started, the changes of these markers were registered, as well as the accumulation of corticosterone in yolk. Feeding broiler chicken with feeds enriched with corticosterone (2 mg/head per day) for 14 days [50] resulted not only in the accumulation of corticosterone in yolk, but also in a change in the ratio of many fatty acids and their assimilation by embryos. Before the incubation, the content of docosahexaenoic acid in yolk (C 22:6 n3) decreased, and the content of the stearic (C 18:0) and cis-8,11,14-eicosatrienoic (C 20:3 n6) acids increased as compared to the control, whereas in the yolk sac of embryos, the amount of stearic (C 18:0), trans-octadecenoic (C 18:1tr n9), and arachidonic (C 20:4 n6) acids was higher. The content of myristinic (C 14:0), palmitooleic (C 16:1 n7) and linoleic (C 18:2 n6) acids was below the control. Consequently, the development of stress in mothers caused changes in fat metabolism in embryos.

Feeding the egg chicken with a diet containing 30 mg/kg of corticosterone for 14 days led to an increase in the amount of feed intake by 39 %. The body weight changed insignificantly, but the productivity sharply decreased compared to the control [51, 52]. The weight of eggs and shells, as well as the strength of the latter, practically remained the same. The thickness of eggshell increased from 0.41 to 0.47 mm, the height of the protein decreased from 7.8 to 5.5 mm, and the Haugh units diminished from 87 to 69. Moreover, the action of corticosterone decreased the content of calcium and triglycerides in blood, and the concentration of albumin, glucose, uric acid and the activity of a number of enzymes increased.

In the experiment with introduction of 0.2 or 1 μg of corticosterone into eggs, chicks from these eggs formed respectively group 1 and group 2 [53, 54]. The rate of growth from the 8 week age in chickens in group 2 was suppressed more than in group 1 and group 3 (control). The same dependence was noted for the duration of tonic immobility and the frequency of aggressive behavior due to changes in the HPCA response and the serotonergic system, which may be associated with the DNA methylation. In blood and yolk produced by chickens in group 2 the corticosterone concentration was greater than that in groups 1 and 3. The expression of luteinizing hormone receptors and the content of follicle-stimulating hormone in the cells of follicle membranes have decreased in the phases F_1, F_2 and $F_3$of their development. In addition, oviposition in the second group began 1 week later, and the egg productivity and quality were worse than that in groups 1 and 3. In Japanese quails with implanted corticosterone-containing capsules and without hormone (control) blood corticosterone contents were 11.7 ng/ml and 1.3 ng/ml, respectively [55]. During a week after the operation, they laid an equal number of eggs with corticosterone level in the yolk of 2.1 ng/ml and 0.9 ng/ml, respectively. The offspring of the experimental group grew slower only during the first week of life. The HPCA response after capture and restriction of movement at puberty at 8 weeks of age was higher than in control. When corticosterone was injected directly into the egg [56] in an amount that causes almost twofold increase in the concentration of this hor-

mone in yolk thus reaching the level obtained after implantation [55], there was sexual dimorphism of the response to corticosterone. Growth slowed in males, but not in females, and stress response decreased in adult female quails, but not in males.

It should be noted that in implantation of capsules with corticosterone to mothers, the adult offspring showed increased response to the identical stress factors. According to the authors' opinion [55, 56], this contradiction was due to the peculiarities of the corticosterone distribution when injected into layers and directly into the egg. Despite the fact that steroids are liposoluble, their distribution over the layers of yolk is not the same [57]. This may cause the corticosterone impact on the metabolism of embryos at different stages of development, which will lead, for example, to unequal impact of the hormone on the integration of HPCA elements. Thus, rodents showed that the nature of the consequences in descendants after prenatal stress in their mothers was determined by the stage of embryogenesis during which the effect of stress factors and, accordingly, the release of glucocorticoids occurred [58]. HPCA activity increased in guinea pigs, if glucocorticoids were administered at early luteinization phase during the ovarian cycle, but having the hormones introduced during the late phase, this system was depressed [59].

Sexual dimorphism of the dynamics of some blood hormone levels during pre-egg-laying period and sexually active period is described in black-legged kittiwake (*Rissa tridactyla*) by using corticosterone through implants [60]. The blood concentration of luteinizing hormone, released in response to induction by the releasing factor, decreased in females, while the amount of this hormone and testosterone, and the intention to mate did not change in males.

After the implantation of corticosterone (30 mg/head) or placebo (control) under the skin of the white Leghorn and ISA Brown chickens [61], the testosterone and progesterone concentration in blood and egg yolk of test birds decreased, but in different ways. In ISA chickens in the control group for progesterone it was 3654 ng/g, whereas in the experimental group it was 9.3 % less. In white Leghorn it was 3127 ng/g and 4.9 % less, respectively. Egg productivity in the control hens of both genotypes averaged 17.5-18.5 pcs/head for 19 days after the corticosterone implantation. However, the experimental group of white Leghorns for this period showed a 31.4 % decrease in this indicator, while in ISA it almost did not change and amounted to 18.0 pcs/head. In layers of both genotypes from experimental groups, the testosterone and progesterone in blood and yolk decreased if compared to the control. This is consistent with data on the antigonadotropic effect of glucocorticoids which ensures the dominance of physiological functions responsible for survival. It is especially important in case of chronic stress of various nature [62], including hyperthermia [63-66], hypodynamia [67], etc.

Thus, the modeling of stress in female precocial birds and altricial birds, as well as in mammals, with different methods of corticosterone administration made it possible to establish dose-dependent disturbances in ontogenesis of offspring, which is manifested in changes in egg productivity, hatching egg quality, embryo metabolism, and development of young animals. Sexual dimorphism was revealed according to hormonal state, stress reactivity and growth of descendants. Different genotypes of egg hens show an unequal response to the administration of corticosterone, both in the concentration of hormones in the egg yolk and in the parameters of egg productivity [61].

Role of maternal hormones in sex inversion of the offspring. In response to the impact of stressors of different nature, females optionally affect the sex ratio in offspring [68]. This phenomenon is discovered not

only in the natural habitat, but also in experimental conditions and in commercial poultry farming. Two races of Gouldian finch (*Erythrura gouldiae*), red-headed and black-headed, co-exist within one habitat. Females prefer to mate with males of the same coloring, because daughters from mixed crosses have lowered viability due to genetic incompatibility of parents [69, 70]. In females, mating with unpreferable phenotype leads to stress, the marker of which is the increased blood corticosterone (an average of 68 ng/ml), whereas in females from pairs of the same race it is 19 ng/ml. Among the descendants of mixed pairs, males predominate (82 %), while in the offspring of homogeneous pairs their number is lower (about 46 %). The phenomenon of sex inversion in the descendants of peacocks (*Pavo cristatus*) was found as a result of removing a part of feathers, which have the spots typical of the species, from the tails of father birds prior to mating [71]. In the yolk of eggs laid by female partners the corticosterone increased, and there was a tendency to a decrease in the testosterone level with an unchanged 17β-estradiol. In the offspring of the experimental group, there were more females, and the ratio of ♂:♀ significantly decreased if compared to the control (0.35 and 0.54, respectively). Interesting, there are similarities and differences in the reproduction in Gouldian finch [69, 70] and peacocks [71] determined by the male phenotype. In both species when mating females with the males belonging to another race or having a modified feathering patterns the corticosterone concentration in the egg yolk increases. However, the sex ratio in the finch offspring shifts toward the males, which reduces the adverse effect of the low viability of daughters of heterogeneous pairs on the population, while in peacocks this proportion shifts towards the females.

In female Japanese quail, implantation of corticosterone-containing capsules allowed the authors [72] to establish that it is namely the increased concentration of this hormone that causes sex inversion in the offspring and leads to an increase in the female number. Some authors describe other examples of participation of sex hormones in offspring sex ratio regulation [73]. A dose-dependent sex inversion was found in chicken that resulted from injections of progesterone (2.0 and 0.25 mg). The number of cockerels was 25 and 61 % respectively, whereas in control (placebo administration) this figure was 63 % [74]. Differences in social rank among mother hens also lead to sex inversion in offspring [13]. Thus, in the offspring of the dominant and subordinate layers, the ratio of ♂: ♀ was 0.6 and 0.4. Egg yolk testosterone level correlated with the social rank of hens, as well as the sex of developing embryo-descendants, this index varied from dominating hens to subordinates ones from 1.1 to 2.3 pg/mg for female chicks, and only from 1.7 to 1.9 pg/mg for cockerels.

A change in the sex ratio in the offspring of stressed females was found in different birds [75], horses [76], and humans [77]. This phenomenon is widely viewed as the manifestation of adaptive response to changes in environmental conditions, the character of the relationships in the community, and a hierarchy in the population. The mechanism of sex inversion in chickens hatched from eggs with the increased concentrations of deposited hormones in the deutoplasm is not clarified yet. The introduction of corticosterone into the females of chestnut-eared finch (*Taeniopygia guttata*) during meiotic division caused a significant increase in the number of males among the chicks [78]. The same results were obtained on quails and chickens when introduction of corticosterone and testosterone, respectively [79, 80]. However, 5-minute long bag handling of females of this species in about the same period (5 hours prior ovulation), which caused a physiological increase in the concentration of corticosterone, did not affect the primary sex ratio [81]. Such a contradiction may be due to different strength and nature of the acting factor. Moreover, the research results may be affected by the

unequal viability of embryos of different sex [82].

Maternal hormones participation in the formation of epigenetic adaptations in descendants. An increase in the content of androstenedione and 17β-estradiol in yolk from hens kept on the floor was registered if compared to that in layers kept in cages [83]. As the authors believe, the concentration of these hormones may be due to the mechanism by which mothers inform descendants on the state of the environment or living conditions.

Under a chaotic change of illumination in the experimental group of egg hens, in contrast to regular illumination in the control (12 h/12 h), subject to free accessible standard feed and mealworms [84], the individuals from the experimental group preferred feed, had a larger weight and pecked much more often compared to the control (595 and 322 movements for 15 minutes, respectively). The test for pair dominance was negative, and there was no difference between the groups. The descendants of both groups were grown at 12/12 hours lighting regime. The daughters, but not sons, in the experimental group were more likely to peck the feed than in the control. In the pair dominance test, the offspring of the experimental group also pecked the feed more often, and the intervals between the movements were shorter than those of the control groups (199 and 470 seconds, respectively). The former preferred worms and had a larger body weight, and their survival by the 40-week-old age was 65 %, while that of the latter was only 39 %. The egg yolk 17β-estradiol in experimental gropu was 1.04 mmol/l vs. 0.87 mmol/l in the control. Corticosterone, testosterone and androstenedione concentrations were identical. The individuals of both groups had differences in hypothalamus cells; there also were differences in the expression of 9 genes which was preserved in the descendants. The differences were particularly pronounced for several immunoglobulin genes. The data obtained demonstrates the participation of maternal 17β-estradiol in providing the epigenetic adaptation of offspring to environmental conditions (unpredictable regime of illumination) which was unusual for parents.

When adult Japanese quails were exposed to different stressys (blowing, shaking cages, noise) (group 1), and the control population was left intact (group 2) [85], sexual behavior in male F_1-1 changed compared to their fathers, i.e. the numbers of copulation attempts (2.4 and 4.2, respectively) and of completed sexual acts (1.2 and 2.2) decreased. The eggs from F_1-1, when compared to those from their mothers, had lower yolk content (27.5 and 29.3 %, respectively) and fertilization (46.3 and 66.2 %), but not hatchability. However, the yolk of eggs from F_1-1 contained more progesterone and testosterone compared to yolk from eggs from mothers.

Therefore, the embryos' assimilation of maternal steroids from the deutoplasm is the cause of the formation of both short-term phenotypic characters and their long-term programming in offspring [86, 87]. The possibility of such regularities is also justified by the data that maternal 17β-estradiol affects brain development in embryos, causing an increase in anabolism and masculinized behavior in offspring [88].

The phenomenon of maternal hormones impact on the phenotype of descendants is revealed in different classes of animals, like reptiles [89], altricial birds [60], precocial birds [67], and horses [76]. According to L.K. Trofimova [90], the influence of various stress factors changes locomotor and orienting-research activities, anxiety, heart rhythm variability, functions of stress realizing and stress limiting systems in pregnant rats and their offspring. Moreover, physical development is disturbed. Changes in ontogenesis in the offspring under the influence of maternal hormones may be considered as an epigenetic adaptive response to parent's signals about the need to be ready for a change in environ-

mental conditions [1, 91]. The study of the regularities of these processes offers prospects of a better understanding variability in animal populations during phenotypic evolution. It should be noted that the influence of parents on the ontogenenesis of their descendants is realized not only through the parent hormone accumulation in the deutoplasm, but also via other signaling systems. For example, the parents of chestnut-eared finch use sounds to inform embryos in incubated eggs about an increase in the ambient temperature above 26 °C (92).

In natural conditions, the signals about changes in environment transmitted to descendants via maternal hormones seem to serve as a mechanism that ensures the preservation of the species. However, in commercial poultry farming, the adaptive response which results in a decrease in the reproductive function should be regarded as undesirable, especially with regard to a shortage of hatching eggs for highly productive broiler chickens [93]. Breeding for increased performance of the latter has negative consequences as well resulting in a decrease in the reproductive function and efficiency of vital systems, including the cardiovascular problems, e.g. ascitic syndrome [24, 25, 94].

Routine technological and preventive measures in commercial poultry farming can also cause metabolic stress in livestock of breeding studs. Thus, in egg hens, when the feed is changed, aggression increases, and signs of PPF become more severe [95]. Feeding quails with a seed mixture instead of the usual mixed feed causes an increase in HPCA activity and corticosterone response [96]. As a result of limited feeding, ovarian tissue of hens excreted in vitro more progesterone and less testosterone while secretion of 17β-estradiol and arginine vasotocin did not change [97]. In the hypothalamus and ovary of 23-week-old individuals, expression of grehlin and its receptors was activated. In the ovaries of 7-week-old females, the first one decreased, the second one remained the same [98]. Therefore, limited feeding can affect sex hormone production, as well as age-related changes in the function of grehlin which has the properties of gonadotropin releasing hormone. These data proves the need to use limited feeding of the parent herd cautiously because of a possible disruption in the metabolism of hormones that regulate reproductive function. It also seems to influence the storage of hatching eggs. With a 6-fold increase in the ammonia content in air, blood corticosterone in egg hens increased more than twice, 17β-estradiol and progesterone decreased, egg productivity significantly decreased [99]. Obviously, such eggs are not suitable for incubation.

Thus, as a result of stresses, including those developing in female birds due to technological factors, the concentration of maternal hormones in eggs increases. Stress response and the amount of hormones accumulated in the deutoplasm are not the same in genotypes. Selection for productive and/or nonproductive characteristics also changes stress response. The migration of hormones along the mother—egg—embryo chain and their participation in the regulation of metabolism in offspring occur in early ontogenesis, when the functions of organs and systems are most labile. Testosterone accumulation in yolk causes changes in postnatal growth, immunocompetence and behavior patterns. In the offspring, sexual dimorphism is revealed in the manifestation of the effects of increased mother hens' hormone deposition in yolk due to unusual housing conditions and feeding during egg production period. When carrying out technological and veterinary preventive measures, the role of the parent hormones in the transovarial transmission of signals about the acting stressors from a mother hen to the offspring should be taken into account, for it leads to metabolic disorders, suppression of the reproductive function, and reduces the efficiency of commercial poultry farming.

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FUNCTIONAL EGG PRODUCTION. II. THE ROLES OF SELENIUM, ZINC, AND IODINE

(review)

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Abstract

Different aspects of selenium, zinc, and iodine in the nutrition of laying hens are reviewed in relation to the production of functional eggs enriched with these trace elements. Selenium can be easily transferred into the eggs. Selenium is a part of certain antioxidant selenoproteins (primarily enzyme glutathione-peroxidase) improving antioxidant status and the system of antiradical defense in laying hens; these compounds can also be transferred into eggs improving the oxidative stability of yolk and albumen during egg storage (Z.G. Wang et al., 2010). Recent results of the worldwide research proved that diets for layers (and eggs as a result) should by advisably enriched simultaneously with selenium (M. Fasiangova, G. Borilova, 2017) and vitamin E since this combination of the two most active dietary antioxidants provides the best antioxidative defense in layers and the best antioxidative status of the eggs (Z. Zduńczyk et al., 2013). The organic forms of selenium are shown to be the most effective selenium sources (compared to inorganic sources) due to less toxicity for poultry, better selenium transfer to eggs and deposition into the body selenium pool, primarily in muscle tissues, which can be activated during an oxidative stress (P.F. Surai, V.I. Fisinin, 2016). The combination was also shown to be an effective protector for polyunsaturated fatty acids in yolk lipids (A.Sh. Kavtarashvili et al., 2017). Determination of optimal proportion of selenium and vitamin E in diets for layers requires further research and justification. Zinc is an integral part of antioxidative enzyme superoxide-dismutase (SOD) and lowers oxidative stresses due to the antagonism to the ions of transition metals with high redox potentials. Enrichment of eggs with zinc via high dietary zinc levels improves quality and stability of the albumen during egg storage (H. Aliarabi et al., 2007), eggshell quality, bone development, feather condition and immunity in layers (K.M. Martin, 2016). Supplementation of diets for layers with 50-80 ppm of inorganic or 500-100 ppm of organic zinc will generally not affect their productivity (K. Sahin et al., 2009). Simultaneous enrichment of eggs with selenium and zinc using their high dietary levels is complicated by the antagonism between the two elements which will be possibly overcome due to the development and investigation of their new dietary forms and sources. High dietary iodine levels provide the possibility for the production of iodine-enriched functional eggs; according to EU legislation, however, iodine level in diets of laying birds should not surpass 5 ppm (EU Commission, 2005). Several studies reported the absence of detrimental effects of higher dietary iodine doses (5-10 ppm) on overall productivity in layers while certain egg quality parameters (eggshell thickness and strength, relative albumen weight, Haugh units) decreased with the increase in dietary iodine content (M. Lichovnikova, L. Zeman, M. Cermakova, 2003). Simultaneous enrichment of eggs with selenium and iodine is possible (Yu.A. Ponomarenko, 2015) since these two elements are not antagonists (especially in their organic forms) but rather synergists; the efficiency of different sources and doses of selenium and iodine in combined diet supplementation and transfer to eggs is still to be elucidated.

Keywords: functional eggs, laying hens, selenium, zinc, iodine, dietary levels and sources, egg composition and quality

In the previous part of our review we considered the issues related to the

content of polyunsaturated fatty acids (PUFA) of the ω-3 series in chicken eggs, and also showed the need for their additional enrichment with antioxidants on the example of vitamin E [1]. Another important field in the production of functional eggs is their enrichment with trace elements that are deficit in human nutrition. Some trace elements that show antioxidant properties increase antioxidant capacity and add value to eggs as a functional food product. These elements are successfully transferred into eggs via the layers diets. Complex enrichment of eggs with target trace elements is also possible. However, the interaction of these elements may cause certain problems.

This paper is the first to comprehensively summarize various aspects of eggs enrichment with trace elements possessing antioxidant properties (Se, Zn and I) separately and jointly.

S e l e n i u m. Se is one of the most significant antioxidant trace elements for humans and animals [2]. High doses of Se are toxic, but its natural content and rates of additional uptake in feeds are usually 10-20 times lower than dangerous ones [3]. Selenium can be incorporated into proteins, replacing sulfur atoms in sulfur-containing amino acids (in particular, in methionine and cysteine) forming selenoproteins (SeP). In addition, plant SeP contain predominant selenomethionine (SeMet), while animal SeP contain selenocysteine (SeCys). SeMet in vertebrates does not show neither any coding triplets, nor transfer RNA [4], while SeCys has UGA coding triplet [5]. Selenoproteins are an essential part of a proteome. The chickens have about 25 SeP genes the expression of which affects the regulation of cell growth and apoptosis, as well as functions of cellular signaling systems and transcription factors [6].

Some SeP (the family of glutathione peroxidases — GP, thioredoxin reductases, etc.) are antioxidant enzymes, the SeCys fragment [7] being the site of their activity. GP are the most significant in the system of protecting tissue damage from free radicals. One of the GP form is found in the gastrointestinal tract. It suppresses the absorption of hydroperoxides [8]. Phospholipid GP is a part of the lipid fraction of membranes and inhibits lipid peroxydation, protecting the structural integrity of membranes [9]. In eggs, Se, particularly GP, protects against peroroxydation of yolk lipids and albumen [10-12].

Formerly, it was considered that the laying hens have low need in selenium (0.06 mg/kg of feed), but later it was found that requirement in this element rapidly increases due to stress, particularly in case of intensive husbandry technology [13]. It was also reported that Se preparations can mitigate the harmful effects of feeds contamination with mycotoxin by improving anti-radical protection of the organism [14]. Selenium deficiency (hyposelenosis) causes exudative diathesis [15], food-related encephalomalacia [16], food-related pancreas atrophy [17] in poultry. The combination of Se and vitamin E deficiencies are especially dangerous. Se excess is also toxic to poultry: food-related hyperselenosis manifests itself in the inhibition of growth and egg productivity, anemia, decreased mobility of the tibiotarsal joints [18] and the relative weigh of heart and liver [19], degeneration and/or necrosis of the liver, myocardial degeneration, and necrosis of the convoluted kidney tubules [20]. Thus, the amount of Se in the diet should be optimal, so as not to reduce the productivity and viability of poultry. The literature indicates different LD_{50} of Se: 9.7 mg/kg of live weight (selenite, per oral) for hens [21]; 24.6 mg/kg with a low toxic dose of 1.7 mg/kg for chickens [22]; 33.4 mg/kg with a low toxic dose for laying hens being 15 mg/kg [23].

Three main reasons for enriching the laying hens' diets with Se [13] are maintaining their health and productivity, including the condition of skeleton [24], achieving optimal quality of albumen, yolk and eggshell, and selenium

providing to egg consumers. Formerly, selenium was introduced into animal diets only in inorganic forms in the form of selenite (Se^{+4}), selenate (Se^{+6}) and selenide (Se^{-2}). Later, the organic forms appeared. Among the inorganic sources of Se, selenate and sodium selenite are now the most common. In poultry diets selenite has two important advantages: it is much cheaper that all organic sources and is metabolized quickly, which allows an organism to make an accelerated synthesis of selenoproteins at the Se deficiency [3]. The disadvantages of inorganic forms of selenium are relatively high toxicity, Se interaction with other trace elements, low incorporation into muscles, and low efficiency of its transfer into eggs [13]. Moreover, selenite (especially in high doses) shows pro-oxidant activity [25]. SeMet is the most common among the organic preparations. Apart from the two main selenated amino acids, hydroxy-selenomethionine (as a more persistent and stable analogue of SeMet) [13], and selenium homolanthionine [26] were studied as another source of Se. Selenated yeast and algae (chlorella) were also used, where Se was found mainly in the form of SeMet. Organic preparations of Se diacetophenonyl selenide, or bis-(benzoylmethyl)-selenide (DAFS-25), and 9-phenyl-sym octahydro selenoxanthene (selenopyran) have been developed and are being used in Russia. Se nanopreparations ensure more precise delivery of the element to the target organs and tissues and have a low toxicity, which increases the bioavailability and effectiveness of Se application [27-29].

Prior comparative studies on various sources of Se led to the conclusion that inorganic sources are preferred to the organic ones for they are better metabolized by the organism and more efficiently fit into SeCys, synthesized in the liver from serine and H_2Se [30]. Some authors still claim that feed organic preparations of selenium for inclusion in an organism's proteins require preliminary metabolization of Se in H_2Se, therefore they do not have advantages over inorganic forms both in the efficiency of selenium absorption and use, and in toxicity [31]. However, this is not exactly right. Indeed, the exogenous SeCys do not fit into the functional endogenous selenoproteins (such as GP), which is proved by the work with preparations labeled with ^{75}Se [32]. However, SeMet is not recognized by the mammal's intestine as a selenium-containing substance, therefore it is absorbed and used in the body similarly to methionine [33], that is, with very high speed and efficiency (most likely exceeding that in case of passive absorption of Se from inorganic sources). Absorbed SeMet is nonspecifically fit into various structural and non-functional proteins (for example, into muscle proteins that accumulate half of all selenium reserves in a mammal organism), creating a Se repository. It is mobilized in case of stress or other conditions, when the body's need for metabolically active selenium increases.

A similar mechanism of active absorption and incorporation into proteins is shown for feed SeCys, though to a lesser extent. Probably, the organism partially recognizes it as a source of Se and metabolizes it on the “inorganic way”. A study of selenium tissues distribution in chickens during the feeding of selenated yeast showed [34] that in the muscles the SeMet content had truly and significantly increased, while it was SeCys content that increased in liver and kidneys. The amount of SeMet increased in all organs and tissues with the dose of organic Se, whereas when selenium was fed, this effect was much less pronounced, and an increase in the CeCys content was seen in the heart tissues only. Inorganic forms of selenium (unlike the organic ones) cannot participate in the creation of the Se repository, since the non-metabolized residue of inorganic Se is rapidly excreted renally [35]. As for the relative toxicity of inorganic and organic forms of Se, DAFS-25 (rats, orally), for example, shows the toxicity that is 20 times lower than that of sodium selenite [36].

Another important aspect of selenium feed of the poultry is this element's interaction with vitamin E. Se-dependent enzyme thioredoxin-reductase is involved in the reduction of oxidized forms of vitamin E, into which it is converted through the neutralization of free peroxide radicals in biological membranes [37]. Thus, both antioxidants work both separately and together in the anti-radical protection system. It is no wonder that plenty of research dealt with seeking their optimal correlation in poultry diets in recent years. Enriching diets with organic forms of Se allows reducing the amount of expensive vitamin E added to the diet by 25-30 % [38].

It has also been reported that the inclusion of Se in laying hen diets increases the vitamin E content in egg yolk [39]. This index made respectively 297, 311 and 370-375 mg/kg of dry yolk solids without addition of Se and in the groups receiving selenite and Se-enriched yeast or algae. Therefore, in the interaction between vitamin E and Se, organic forms of selenium are more efficient. When selenite was fed to lambs, the content of vitamin E in their liver decreased if compared with the control group (without the addition of Se), and the highest doses of Se (3-4 mg/kg of body weight), the decrease was proved ($p < 0.01$). After feeding SeMet, a significant but not proved decrease in the amount of vitamin E in liver was observed only at the highest dose of Se (8 mg/kg of body weight) [40]. An increase in the dose of Se and vitamin E in hens' diets raised the antioxidant blood status [41] and improved liver function, statistically ($p < 0.05$) decreasing the concentration of bilirubin, aspartate aminotransferase and alanine aminotransferase in blood plasma [42]. The immune status of layers also improved: the amount of immunoglobulin IgA in the blood [41] and the antibody titer against the Newcastle disease virus [42] increased.

Some authors believe [31] that so far there is no firm evidence of the advantages of organic forms of Se over inorganic ones in their effect on the productivity of animals. However, numerous studies show that organic forms more effectively improve the selenium and antioxidant status, health, egg and meat poultry productivities, and the quality of eggs and other poultry products. Thus, when comparing the effects of selenite, Se-yeast and DL-SeMet, it was found that in the same doses (0.3 mg Se/kg of feed) both organic forms more effectively increased the activity of GP and the amount of Se in the albumen ($p < 0.01$), pectorals and leg muscles of laying hens ($p < 0.001$) than the inorganic form [43]. Interestingly, the Se accumulation in the albumen and muscle protein in case of feeding DL-SeMet was significantly higher than when feeding Se-yeast ($p < 0.05$). Se deposition in the yolk was not significantly different between the organic forms. With the increase in the amount of SeMet introduced into the diet (apart from selenite), the Se content in albumen increased [44].

Among other parameters that are possibly affected by the enrichment of the layers diet with selenium, are the pH of the egg contents, the fatty acid profile of the yolk lipids [45], and the oxidation stability of the yolk and albumen [46]. Eggs enrichment with Se increases their resistance to internal oxidation processes during storage. The slower the pH grows inside the egg, the better its quality is maintained. This slowdown in case of layers diets enrichment with Se and with vitamin E at the same time is accompanied by an increase in the strength of the vitelline membrane and/or the putamen [47]. Improvement in the preservation of the yolk oxycarotenoid pigments in eggs was also reported: feeding Se-yeast to laying hens resulted in a significant increase in the yolk color grade on the Hoffmann-La Roche scale from 4.77 in the control to 5.04 [48]. The slowing down of yolk and albumen oxidative products accumulation in eggs after introducing Se into the diets of laying hens is usually associated with an increase in the activity of GP. Both organic and inorganic forms of Se influ-

enced the concentration of malondialdehyde (MDA) in fresh and stored eggs, however inorganic selenium was less effective after long storage periods [49]. Feeding Se (in 0.3 mg/kg) in the form of Se-yeast significantly reduced the concentration of MDA in the yolk compared to the control (without feeding selenium) [12]. Concentration of carbonyl compounds in albumen also decreased, making 4.55 and 4.43 nmol/mg of 2,4-dinitrophenylhydrazine with doses of Se of 0.3 and 0.6 g/t respectively (control: 4.67 nmol/mg).

Zinc. Zn is part of and/or mediates the activity of several hundred enzymes [50], including carbonic anhydrase, which provides carbonate anions to form eggshell [51]. Zn is necessary for antioxidant protection as one of the cofactors of superoxide dismutase (SOD) that converts superoxide anions to hydrogen peroxide [52]. It also reduces oxidative stress by being an antagonist of ions of transition metals with a high redox (inorganic copper and iron), preventing the formation of hydroxyl radicals from hydrogen peroxide. In the Fenton reaction, Zn competes with other transition metals (Cu and Fe) beyond the binding locations and serves as an electron donor for these reactions [53-55]. The acute antioxidant effect of zinc is related to its antagonism to other transition metals and the protection of sulfhydryl groups of proteins. The chronic effect manifests itself in protection from prooxidants through participating in the synthesis or activation of other antioxidants, for example metallothioneins. Metallothioneins are cysteine-rich proteins. They include the Zn-dependent ones, which are capable of neutralizing oxygen radicals [56] and binding divalent metal cations, regulating their homeostasis [57].

Zinc deficiency in the diets of young animals causes abnormalities and defects in bone development, poor bone mineralization, growth retardation, coat deterioration and dysfunction of immune system [57]. High content of this element worsens the mineralization of the skeleton due to impaired absorption and use of Ca and P [58]. Most likely, this effect is related to the competition between trace elements for inhausting sites in the digestive tract of birds. Superhigh doses of Zn (~ 10-20 g/t of feed) cause pancreatic and muscular stomach cankering in birds [59]. Another negative consequence of high doses of Zn is an increase in its excretion and quantity in litter. Since plants poorly accumulate Zn, it can reduce soil fertility [60]. At the same time, an increase in nitrogen retention was reported in response to the Zn introduction into the diets. Zn inhibits the microbial enzyme uric acid-degrading uricase [61].

Formerly, zinc was added to poultry diets in the form of inorganic sulphate or oxide. The National Research Council (NRC, USA) [62)] establishes the following standard rate of Zn in the diets of egg-producing poultry: 38-40 g/t for the young, 33-35 mg/t for adults, whereby the requirement of brown crosses is slightly lower than that of white ones. Later, they used organic forms of Zn, mostly chelate compounds with different amino acids (most often with methionine), small peptides and proteins, and picolinic acid (pyridine-2-carboxylic). Availability of feed zinc depends on the composition of the diet, for example, the content of phytates (inositol-polyphosphates). The latter include some that are able to firmly chelate Zn. Adding phytase to a diet rich in phytate increases the bioavailability of Zn, feed intake and the increase in body weight of broilers [63]. Other divalent metals competing with Zn for inhausting sites and carrier molecules can also affect the availability of feed Zn [64]. For broilers, the bioavailability of the Zn with amino acids complex was 64 % higher than that of zinc sulfate, while the poultry that received the organic form showed improved feed conversion [65]. Similar results were obtained by N.M. Salim with contributing authors [66]. However, some studies almost did not make any difference between the bioavailabilities of organic and inorganic Zn [67]. A number of studies

stated that the bioavailability of Zn from different preparations can be evaluated by its solubility in a buffer with pH 5.0 [68], where Zn proteinate sample showed better bioavailability of Zn. The positive effect of combined use of organic and inorganic Zn is shown. Since zinc is absorbed in the small intestine both passively (diffusion) and with the involvement of proteins that perform the transmembrane transport of divalent cations, the simultaneous presence of two forms of Zn in the chyme allows using different absorption mechanisms in case of competition or inhibition of one of them [69, 70]. Comparing the two doses of Zn (25 and 50 g/t) in the form of sulfate and organic preparation in layers it was established that neither the dose nor the form of Zn influenced egg production capacity, egg weight and feed conversion. However, both forms increased the height of albumen and the Haugh index [71].

It is known that in adult egg laying poultry the amount of zinc in the diet is related to the quality of the eggshell and the bone structure (osteoporosis), the latter being especially important for the cage housing of layersdue to the syndrome of cellular fatigue. The recent study that used different forms of zinc and other trace elements [72] pointed out that the strength and specific gravity of eggshells showed proved linear increase ($p < 0.05$) with an increase in the amount of Zn in the diet (from 30 to 120 g/t). The egg breakage rate decreased. The authors attribute the eggshell quality to an increase in the density of the palisade layer and a decrease in the density of the mamillary layer. Laying hens that received Zn in doses from 0 to 80 g/t in the form of sulfate or chelate with hydroxy methionine showed that the organic form caused better confirmed improvement ($p < 0.05$) of the thickness and strength of the eggshell, the strength of the tibia, as well as the titer of antibodies against sheep erythrocytes [73]. Egyptian researchers [74] studied the possibility of producing zinc-enriched eggs in the chickens of the local breed Golden Montazah, feeding it in the form of sulfate or chelate with methionine (0, 50, 100, and 150 g/t). At the maximum studied dose of ZnMet, the highest concentration of Zn in eggs was recorded (2.23 mg/100 g of content), while the excess over the control was proved in all test groups ($p < 0.01$), except for the one that got Zn sulfate in the dose of 50 g/t. In terms of productivity, the most effective dose came out to be 100 g/t: this group showed better egg production capacity, egg weight and significantly improved feed conversion. In all experimental groups, regardless of the source of Zn, the concentration of Zn, total protein, albumin and globulin fractions were increased in the plasma of the layers. The quality of albumen (relative mass, thickness, Haugh index) also improved.

A series of experiments [75] on 19-60 weeks old layers of the Bovans (White Leghorn) cross, having compared the effect of Zn sulfate and the organic preparation Availa-Zn (Zinpro, USA) in doses of 40, 80 and 120 g/t, did not justify significant influence on feed intake, egg production capacity, feed conversion, bogy weight gain, egg weight, relative shell weight, bone strength, and keel bone fractures rate. A dose of 80 g/t in both forms increased the eggshell strength ($p < 0.05$). The organic form increased the relative weight of the yolk ($p < 0.05$) more effectively compared with the same dose of sulphate. The best coat condition was in birds that received 80 g/t of sulphate, the worst one — in case of feeding 120 g/t of the organic form. Fecal excretion of Zn increased in proportion to its dose in the diet and regardless of its form. A regression equation linking Zn concentrations in the diet and litter ($p < 0.0001$, $R^2 = 0.78$) was derived as follows: Zn_d, g/t = –70.057 + 3.706 × Zn_l, g/t. The positive effect of zinc for laying hens in case of thermal [51] and cold stresses [76] and in protection against coccidiosis and eimeriosis [77] was reported. After feeding organic or inorganic Zn to layers, its maximum accumulation was 18 μg/g of egg content.

In vitro bioavailability of Zn for humans was 75 % in raw eggs, 69 % in boiled eggs, and 65 % in fried eggs [78]. It has been concluded that Zn-enriched eggs can provide up to 150 % of the daily requirement of a year old children in this trace element.

A positive effect of combined organic forms of Se (0.3 g/t) and Zn (60 g/t) in quails' diet on egg quality during storage (4 °C or 20 °C) was reported [79]. Zn or Zn + Se contributed to a better preservation of albumen compared to the control without additives or fed Se only. This combination looks promising, for Zn improves the quality and oxidation stability of albumen, Se — the same of yolk. However, selecting dosages and forms should be very careful, since there is antagonism between these trace elements due to competition for intestinal inhausting sites [80].

I o d i n e. This is one of the irreplaceable trace elements in human and animal nutrition. According to the World Health Organization, iodine deficiency is found in about 30 % of the world's population (over 2 billion people). At the same time, 655 million have thyroid gland hypertrophy, and over 50 million suffer from mental disorders caused by iodine deficiency in their mother's diets [81]. In Russia, 75 % of the population has its deficiency, manifested to a varying degree [82]. Many countries approved iodine deficiency prevention programs that use iodized salt and iodized food products — bread, milk and eggs [83]. Iodine is a part of thyroid hormones - thyroxine and its derivatives. They regulate metabolism (particularly the cellular oxidation processes) and significantly affect the growth and productivity of poultry. Iodine deficiency in laying hens can interrupt metabolism, reduce egg production capacity, and cause hypertrophy of the thyroid gland [84]. Thyroid hormones participate in the function of the pituitary gland, responsible for birds' photosensitivity and puberty [85]. Thyroid hormones (T3 and T4), as well as iodide, accumulate in the ovary, resulting in iodine easily transferred to the oocysts [86]. Thus, when feeding layers with iodized feed, the concentration of iodine in the egg yolk is usually ten times higher than that in the albumen.

Iodine is absorbed in the stomach and small intestine, and hormone-like iodine compounds can enter the bloodstream without cleavage [87]. Inorganic iodine is absorbed mainly in the form of iodide. The availability of feed iodine depends not only on its form, but also on the composition of the diet. For example, goitrogenic anti-nutritional factors (thio- and cyanoglycosides, etc.) of some cruciferae (canola) and leguminous crops (soybean, lupine, pea) impair absorption and use of any form of iodine [88]. It is also known that the assimilation of feed I is affected by the content of K, Ca, Sr, F, and Co in the diet [89]. As far as iodine absorbed in the intestine is metabolized mainly by the thyroid gland and its hormones, the process of stabilizing iodine concentration in eggs when feeding layers with iodine-rich food takes some time.

Having fed layers with I in the form of iodinated yeast in doses of 1 and 2 mg/kg (control — without I supplement) [90], the first 3 weeks deposition of I in yolk and shell either remained the same or slightly increased in all groups. Between the weeks 3 and 6 it was almost twice lower than before the beginning of the experiment. From week 6 to 7 I deposition slightly increased, and after the 9th week it increased rapidly, reaching significantly higher proved values by the week 12 (for a dose of 2 mg/kg twice as high) compared with the original ones. Since the same dynamics were observed in the control group, it could be concluded that in laying hens it was associated with the adaptation of I metabolism to the egg-laying stage, but not to the doses of feed iodine. Another experiment [91] with different doses of iodine (from 0.45 to 13.0 mg/kg), its concentration in eggs after 5 and 10 weeks of feeding was almost the same. However, this experiment did not involve young layers that started the egg laying, but the

old ones (55 weeks old or older), which were at the peak of egg production. It is possible that their metabolism has quickly adapted to the intake of I with food, because at the beginning of the productive cycle they have already passed the maintenance phase of I concentration in eggs. In laying Hisex Brown after feeding I (3.5 mg/kg of feed) for 10 weeks, its deposition in the yolk was about 5 mg/kg in the first 3 weeks and 17-20 mg in the next 7 weeks [92]. Another experiment (93) indicated that after 1 month of feeding the diet with 4.0 mg/kg of iodine, its content in eggs increased from 75.96 μg/100 g of egg content in the control to 184.5 μg/100 g.

Iodine is fed to birds with food or water, most often in inorganic forms (potassium iodide or sodium iodide, potassium iodate or calcium iodate: anhydrous, monohydrate or hexahydrate one). However, inorganic sources are unstable for they are prone to oxidation and/or reduction. Moreover, I evaporates (note that iodates are more stable than iodides) when processing and storing mixed fodders and premixes; light and moisture accelerate the decomposition of salts and sublimation of free I. Iodine losses from iodized salt can start from 50 % after 1 week of storage, from finished feed — up to 70 % after 2 months of storage. The incompatibility of inorganic forms of I with the salts of certain trace elements (especially, copper) in premixes has been reported. Moreover, the released I can destroy vitamins and other biologically active substances [94]. It should be noted that the amount of iodine in the feed will be lower than expected: for example, for a calculated value of 5 mg/kg, the analysis of the chemical composition of the feed revealed the actual amount of I of only 4.20 mg/kg [95]. Due to the instability and high reactivity of I compounds, a scatter of evaluation results is possible depending on the method of analysis. In most of the research on layers, the I content was determined either with the aid of ion-selective electrodes, or spectrophotometrically using the classic Sandel-Kolthoff method [96].

A number of stabilized sources of feed iodine like iodinated yeast, seaweed (kelp, etc.), iodine casein, and iodized extruded soybean have been developed. Russia uses Monclavit-1 preparation (Orgpolmersintez LLC, St. Petersburg), an aqueous-polymer system with I as a complex of poly-N-vinyl amide cyclosulphonic iodide, which performs an additional function of the drinking system disinfectant [82]. Another one is Yoddar (Innbiotekh LLC, Moscow), a dry fodder preparation based on iodized casein of cow's milk (iodine casein) [94]. Ukraine developed a liquid thermostable concentrate of iodine (Jodis R&D and manufacturing company, Kiev) that can be given with water or food [97]. Organic sources of iodine (for example, iodotyrosine, where I is bound covalently and can be cleaved almost exclusively enzymatically) are more stable. Despite the greater preservation of iodates in feeds and premixes if compared to iodides, at the same dose of I, the element was better absorbed and transferred to eggs from potassium iodide than from potassium iodate [88] or calcium iodate [95] when used in doses (for I) 1-5 and 0.25-5.0 mg/kg, respectively. Comparison of calcium iodate with iodized yeast [98] showed that organic iodine preparation is more effective. After feeding in a dose of 1 mg/kg of feed (for iodine) for 12 weeks in the form of iodate and I-yeast, the concentration of iodine was 58.0 and 104.5 μg/100 g of yolk ($p < 0.05$), at initial indexes in the groups of 50.57 and 51.75 μg/100 g of yolk, respectively. Note that feeding I-yeast (at a dose of I 2 mg/kg) resulted in the accumulation of the element in an amount of 110.5 μg/100 g of yolk. That is, only 6 % more than at a dose of 1 mg/kg. Perhaps, in case of higher content of organic and inorganic forms of iodine in feeds this comparison will produce different results. NRC recommendations [62] establish the requirement of growing and adult egg hens in iodine of 0.33-

0.48 mg/kg of a diet. At the same time, the maximum allowable amount of iodine in the rations of laying hens in the European Union in 2005 was reduced from 10 to 5 mg/kg of feed at a humidity of 12 % [99].

High doses of iodine are toxic to poultry and worsen its health and productivity. It was reported that a dose of iodine of 6.07 mg/kg in the form of calcium iodate in the diet of ISA Brown layers fed for 52 weeks of the production period slightly reduced the egg production capacity, egg mass and feed conversion if compared to a dose of 3.57 mg/kg. The decrease of the relative mass of the yolk (to the egg mass), the albumen Hau index and the absolute weight of the eggshell was proved ($p < 0.05$) [100]. Similar results were obtained in the experiment that compared doses of iodine of 0; 3; 6; 12 and 24 mg/kg in the form of calcium iodate. These doses were fed to brown layers for 30 weeks. Doses of 12 and 24 mg/kg resulted in a decrease in the Hau index and in the relative mass of albumen, as well as in the deterioration of feed conversion. The doses of 3 and 6 mg/kg [101] were considered safe for laying hens' productivity and egg quality. In both experiments the content of iodine in yolk, albumen and egg increased in proportion to its dose in the diet.

The Egyptian researchers' experiment [102] with different contents of I in the feed in the form of KI in the doses of 0.3; 0.6; 1.2; 2.4; 4.8 and 9.6 mg/kg resulted in recognizing the dose of 2.4 mg/kg the most effective in affecting the productivity of chickens. The authors note that it allows obtaining iodine-functional eggs that can satisfy 44% of the daily requirement for iodine in children aged 1-10. With the increase in the amount of iodine in the chickens' diets, it was proved that the concentration of hormones T3 and T4, phosphorus and alkaline phosphatase in the blood plasma increased, and the amount of calcium decreased. The authors associate this to the effect of increasing the concentrations of thyroid hormones on gonadotropin secretion. Previous studies reported that in rats hyperthyroidism induces hyperphosphatemia and hypocalcemia [103]. The experiment conducted in India [91] compared doses of iodine (calcium iodate) of 0.45 (control); 3.25; 6.50; 9.75 and 13.0 mg/kg according to the economic efficiency of enriched eggs production. Eggs were checked for iodine content after 5 and 10 weeks of layers' consumption of enriched diets. The iodine concentration in the egg increased with the increase in its dose. The lowest cost for feeding was recorded in the group receiving a dose of 6.50 mg/kg. In the groups receiving I in the amount of 3.25 and 9.75 mg/kg, this indicator was not different from the control. According to the same authors [104], high doses of iodine (9.75 and 13.0 mg/kg) significantly and reliably ($p < 0.05$) reduced digestibility and the use of macronutrients in the diet. The doses of 3.25 and 6.50 mg/kg were considered to be the most effective ones. The experiment [105] that compared doses of iodine of 0; 5; 10; 15 and 20 mg/kg of feed (calcium iodate) showed that a dose of 10 mg/kg had no adverse effect on the productivity indices of the layers. On the one hand, in this case, the minimum content of cholesterol in the yolk was reliably recorded ($p < 0.05$). On the other hand, the minimum relative mass of eggshell and the maximum egg breakage rate and eggs with soft shell were also recorded.

Another important aspect of enriching eggs with iodine is the stability of this element in eggs after cooking. Previous research reported that after boiling the eggs, iodine of the albumen is usually destroyed, while the I concentration in the yolk decreases averagely by 10 % [106]. However, other authors [91] did not note significant differences in the content of iodine in raw and boiled eggs. Iodine accumulates not only in the egg contents, but also in its shell, where being enriched with iodine, its deposition into the eggshell may exceed its deposition in yolk by an order of magnitude greater [90]. The growing interest in eggshell

preparations as a source of trace elements in human nutrition (including Russia) [107] may promote using iodized eggshell for their production.

Since the absorption mechanisms of selenium and iodine are different, their high content in the diet does not interfere with the absorption and assimilation of both trace elements, which allows them to simultaneously enrich eggs. In rats, it was found that high intake of feed iodine against a background of selenium deficiency leads to an increase in oxidative damage to thyroid tissues due to a decrease in the activity of the thyroid gland. At the same time, relatively moderate doses of Se on the background of iodine deficiency compensate for a decrease in T4 concentration in the blood plasma [108]. The series of experiments conducted by Russian and Belorussian scientists, it was found that the inclusion of iodine organic compounds (kelp, I — 0.90 mg/kg) and selenium (mixed SeMet and SeCys, Se — 0.29 mg/kg) allows receiving enriched eggs without negative consequences for the productivity of laying hens and the quality of eggs [109, 110]. A decrease in MDA content in the layers' blood during week 15 of life and an improvement in other parameters of the body antioxidant status were recorded. Ukraine produces a version of the Yodis preparation (a dose of active iodine is 80 mg/l), additionally enriched with Se as a citrate (0.05 mg/l).

Thus, it makes most sense to enrich diets of layers and, consequently, eggs with selenium and vitamin E, since this combination of the two most active feed antioxidants provides the best protection of the chickens and the high antioxidant status of eggs. The organic forms of Se are the most effective. They are better transferred to the egg and allow the creation of selenium repository in the body, which is mobilized in case of oxidative stress. This combination also ensures the maximum preservation of polyunsaturated fatty acids in egg yolk lipids. The optimal ratio of these two antioxidants in the layers diets is still to be determined. It would possibly be determined by the type and composition of the diet and housing conditions, as well as by the cost of the preparations used. Enrichment of eggs with zinc, when introduced into laying hens diets, enhances the quality and preservation of albumen, improves the condition of the eggshell, skeleton and coat, and have positive effect on the immunity of the layers. Inorganic sources of Zn can be introduced into diets at a dose of 50-80 g/t, organic ones at a dose of 50-100 g/t without reducing productivity. The combined egg enrichment with Se and Zn through feed is still unpromising due to the antagonism between these trace elements, which, perhaps, will be overcome with time by developing and investigating the interaction of new feed forms of elements. Increasing the iodine content in the layers' diets up to 5 mg/kg, it is possible to obtain an egg, functional for this element. As some authors state, doses of 5-10 mg/kg also do not adversely affect the productivity, but lead to a certain decrease in the quality of eggs (eggshell thickness and strength, relative mass of albumen, Haugh index). The combined enrichment of eggs with iodine and Se is possible because there is no antagonism between these trace elements for absorption, but there is a certain metabolic synergy. However, the effectiveness of using different sources of these elements in diets requires further research.

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VITAGENE REGULATION AS A NEW STRATEGY TO FIGHT STRESSES IN POULTRY PRODUCTION

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Abstract

Commercial poultry production is associated with various stresses leading to decrease of productive and reproductive performance of growing chickens, parent birds and layers. In commercial poultry/animal production, there are four major types of stress: technological, environmental, nutritional and internal stresses. A growing body of evidence indicates that most of the stresses in poultry production at the cellular level are associated with oxidative stress due to excess of free radical production or inadequate antioxidant protection. The vitagene concept of fighting stresses emerged as a new direction in a nutritional research and it was successfully transferred from medical sciences to agricultural sciences, including poultry and pig production. In fact, vitagene regulation by nutritional means appeared as a new approach to realise a full potential of the body for adaptation to stress conditions in poultry/animal production. Therefore, the aim of the study was to test if supplementation of the vitagene-regulating antioxidant mixture (Magic Antistress Mix/PerforMax) with drinking water can improve broiler performance in stressful conditions of commercial chicken production. The experiment was conducted at the poultry farm AO «Prodo Tumenskiy Broiler» (Tumen region, Russia) in a special poultry house designed and equipped for conducting experimental trials and measuring growth parameters weekly. Twelve thousand and four hundred newly hatched Arbor Acres chicks were divided into two equal groups with four replicates in each group and placed in poultry house. The results confirmed the idea that using vitagene-regulating mixture with drinking water can improve chicken performance, including improvement in FCR (1.50 vs 1.56 in control) and vaccination efficacy, as shown by increased vaccination index by 40 %).

Keywords: chicken, vitagenes, stress, nutrition

Commercial poultry production is associated with various stresses leading to decrease of productive and reproductive performance of growing chickens, parent birds and layers. Indeed, domestication and genetic selection based on rapid growth rates, improved feed conversion, and heavier body weight (BW) of broilers has made domestic birds, including broilers and turkeys, particularly susceptible to oxidative stress [1]. From a physiological point of view, stress is related to a deviation from optimal internal and external conditions. Under stressful conditions, the hypothalamic-pituitary-adrenal axis, the autonomic nervous system and the immune system are responsible for re-establishing homeostasis. Therefore, a cascade of regulatory mechanisms is involved, resulting in a mobilisation of energy and a shift in metabolism, with detrimental effects on growth

performance and feed efficiency [2]. In modern commercial poultry production, oxidative stress-related nutritional metabolic diseases (e.g. encephalomalacia, exudative diathesis, muscular dystrophy, etc.) have practically disappeared [3, 4], however, various disorders in the antioxidant defence system still cause substantial problems. For example, the amount of a particular nutrient in the diet may be insufficient to meet requirements, the diet may contain substances that inactivate the nutrient or inhibit its absorption/utilisation, or metabolism may be upset by the interaction of dietary and environmental factors causing oxidative stress [5].

A growing body of evidence indicates that most of the stresses in poultry production at the cellular level are associated with oxidative stress due to excess of free radical production or inadequate antioxidant protection [3, 4]. Therefore, dietary antioxidants are considered to be the main protective means to deal with various stresses in poultry production [3, 4, 6-8]. Recently, a concept of the cellular antioxidant defence has been revised with a special attention paid to cell signalling. Indeed, in animals, redox signalling pathways use reactive oxygen species (ROS) to transfer signals from different sources to the nucleus to regulate a number of various functions including growth, differentiation, proliferation and apoptosis.

The vitagene concept of fighting stresses emerged as a new direction in a nutritional research and medical sciences. In accordance with Calabrese et al. [9] the term vitagenes refers to a group of genes that are strictly involved in preserving cellular homeostasis during stress conditions and playing an essential regulatory role in cell and whole organism adaptation to various stresses. The vitagene family includes heat shock proteins (HSPs; Heme oxygenase-1 and HSP70), the thioredoxins (Trx)/thioredoxin reductase (TrxR) system, sirtuins [9] and superoxide dismutases (SOD) [10]. The vitagene concept has been applied to various human diseases, involving neurodegenerative disorders [11], neuroprotection [12], schizophrenia [13], vascular dementia [14], autism spectrum disorders [15], aging and longevity [9, 16-18], dermatology [19], free radical-related diseases [20], osteoporosis and Alzheimer pathology [21]. The vitagene concept was successfully transferred from medical sciences to agricultural sciences, including poultry and pig production [22-28] and was reviewed in previous issue of this journal 5 years ago [23]. Upregulating vitagenes, for-example, via the Nrf2/ARE system, and improving adaptive ability of animals to stress it is possible to decrease negative consequences of various stresses in poultry and farm animal production. Indeed, vitagene regulation by nutritional means [10, 23, 27, 28] appeared as a new approach to realise a full potential of the body for adaptation to stress conditions in poultry/animal production. Indeed, modulation of endogenous cellular defence mechanisms may be an innovative approach to deal with commercially relevant stresses in poultry/animal production.

Therefore, when testing whether supplementation of the vitagene-regulating antioxidant mixture (Magic Antistress Mix/PerforMax) can improve broiler performance in stressful conditions of commercial chicken production, in this well organised and controlled experiment, conducted in accordance with requirements set in many countries with developed poultry production, it was clearly shown that Magic Antistress Mix/PerforMax supplied with drinking water to Arbore Acres broilers, decreased negative impact of commercial stresses. This led to improvement of feed conversion ratio (FCR) and vaccination index (VI).

The aim of the study was to test if our previously developed vitagene-regulating antioxidant mixture (Magic Antistress Mix/PerforMax) can be used to improve liveability and productivity of broilers in commercial conditions.

Techniques. The experiment was conducted at the poultry farm AO Pro-

do Tyumen Broiler (Tumen region, Russia) in a special poultry house designed and equipped for conducting experimental trials and measuring growth parameters weekly. Twelve thousand and four hundred newly hatched Arbor Acres chicks were divided into two equal groups with four replicates in each group and placed in a poultry house. Temperature, light and other parameters of chicken growth were maintained in accordance with recommendations of Arbor Acres accepted at the poultry farm. The diet was balances in all major nutrients in accordance with the same Arbor Acres recommendations accepted at the poultry farm. In additional to the balances died the experimental birds were supplemented with Magic Antistress Mix/PerforMax with drinking water at a dose of 1 mg/l in stressful period of chicken growth in accordance with recommendations of the preparation developer (Feed-Food Ltd., UK). The preparation composition was shown earlier to contain vitagene-regulating mixture of vitamins, minerals, amino acids, carnitine, betaine, organic acids, etc. [35].

Chickens were grown up to 35 days and information was collected weekly. Body weight and daily gain (g), mortality (%), daily feed consumption (g) and FCR were assessed in three growth periods (15-21 days, 22-28 days and 29-35 days) and for whole growth period (1-35 days). To compare broiler results from different flocks and different regions, the European Broiler Index (EBI) or European Production Efficiency Factor (EPEF) is used (this factor standardises technical results, taking into account feed conversion, mortality and daily gain):

$$EPEF = (\text{Average daily gain, g} \times \text{survival rate, \%})/(FCR \times 10).$$

At the end of the experiment intestinal weight was assessed and internal organ examination was conducted to check nephritis, enteritis and intestinal atony.

1. Effect of usage with drinking water of vitagene-stimulating additive Magic Antistress Mix/PerforMax on growth and development of Arbor Acres broilers (*M*±SEM, the experiment, AO Prodo, Tyumen broiler, Tyumen region)

Parameter	Control (n = 6200)	Experimental (n = 6200)
15-21 days of age		
Body weight, g	935.50±8.75	926.00±19.50
Daily gain, g	68.60±2.40	68.30±1.60
Mortality, %	0.40±0.04	0.30±0.05
Feed consumption, g/chicken	101.40±1.80	99.50±1.90
FCR	1.48±0.03	1.46±0.03*
22-28 days of age		
Body weight, g	1457.50±6.50	1457.80±29.80
Daily gain, g	74.60±0.60	76.10±3.10
Mortality, %	0.50±0.05	0.30±0.08*
Feed consumption, g/chicken	119.60±1.40	116.50±3.70
FCR	1.61±0.02	1.54±0.04*
29-35 сут days of age		
Body weight, g	2019.00±7.50	2007.50±27.50
Daily gain, g	80.20±0.80	78.60±2.80
Mortality, %	1.90±1.30	0,90±0.20
Feed consumption, g/chicken	132.40±3.30	126.70±2.70
FCR	1.65±0.04	1.62±0.04
1-35 days of age		
Body weight, g	2019.00±7.50	2007.50±27.50
Daily gain, g	56.50±0.20	56.20±0.80
Mortality, %	96.20±1.20	97.70±0.50*
Feed consumption, g/chicken	3.15±0.07	3.02±0.02*
FCR	1..56±0.04	1.50±0.02*
European Production Efficiency Factor	356.80±10.80	372.8±9.6**

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

Efficacy of vaccination against infectious bursitis was assessed by ELISA in accordance with recommendations of test system producer (Bio-Tek Instruments, Inc., USA). Vaccination Index (VI) was calculated as a ratio of average antibody titre by ELISA to variation coefficient (*Cv*, %).

During statistical analysis, average parameters (*M*) and the standard errors (±SEM) were determined. Significance of differences with Control was assessed by *t*-test by using ANOVA, differences were considered to be significant at $p < 0.05$.

Results. Chicken viability (Table 1) in both groups was within standards for the poultry farm. However, the mortality for 35 days in the control group (3.8 %) was numerically higher in comparison to the control group. In commercial conditions such a difference could be significant for a meat producer. Usage of Magic Antistress Mix/PerforMax with drinking water was associated with

decrease number of enteritis, nephritis and intestinal atony as well as pododermatitis (data not shown). For the first two weeks of the experimental period data in the control and experimental groups were not different (data not shown). Later (see Table 1) there was an improvement in FCR in the experimental group in comparison to the control group resulting in decreased feed consumption per week. There was no difference in body weight and daily gain in the control and experimental groups. Therefore, the main advantage of usage of vitagene-activating composition for growing broilers was associated with improvement of FCR, which in the experimental group (1.50) was significantly better than in the control group (1.56).

EPEF is used to compare results of broiler growth in different areas and flocks. Taking into account all data of the chicken growth and development for 5 weeks EPEF in the control group was shown to be 356.8, while in the experimental group it was 372.8, being comparable with an efficacy of broiler production at the best farms in Russia and abroad.

It is important to mention that in experimental chickens at day 35 intestinal weight was higher (by 5 %) in comparison to the control chickens (Fig. 1, A).

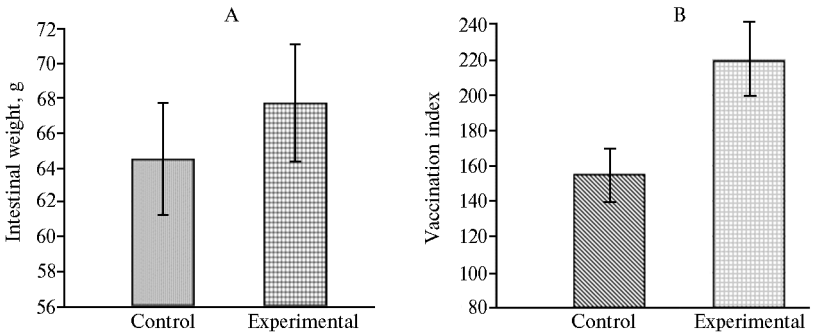

Fig. 1. Intestinal weight (A) **and vaccination index** (B) **in 35-day old Arbor Acres broilers as a result of providing with drinking water the vitagene-stimulating additive Magic Antistress Mix/PerforMax** (the experiment, AO Prodo Tyumen broiler, Tyumen region, Russia).

Vaccination efficacy against infectious bursal disease assessed by ELISA methodology (BioTeck) at day 35 includes antibody titres, *Cv* expressed in % and a vaccination index calculated as a ratio of average antibody titre to *Cv*. Our data indicates that average antibody titre in 35 days old chickens was 4500 in the control group and 5258 in the experimental group. It is interesting that *Cv* in the experimental group (24 %) was lower than that in the control group (29 %). As a result, the vaccination index in the experimental group was significantly higher than that in the control group (see Fig. 1, B).

2. Economics (in roubles) **of Arbor Acres broiler meat production with usage with drinking water of vitagene-stimulating additive Magic Antistress Mix/PerforMax** (the experiment, AO Prodo Tyumen broiler, Tyumen region, Russia)

Economical parameters	Control	Experimental	Difference
Cost of 1 kg meat production :			
salary with taxes	2.09	2.09	0.00
Feed	33.74	32.45	–1.30
Veterinary	1.99	2.19	0.20
Other	8.21	8.13	–0.08
Cost of day old chick per 1 kg meat production	6.67	6.61	–0.07
Cost of 1 kg live weight	52.71	51.47	–1.24
Cost 1 kg of meat production	70.24	68.59	–1.66
Cost of slaughtering and sale per 1 kg meat	19.61	19.61	0.00
Average price of meat sales per 1 kg	120.69	120.69	0.00
Income per 1 kg meat produced	30.84	32.49	1.66

As a result of FCR improvement due to water application of the antistress composition economics of meat production was improved (Table 2). Indeed, due to the PerforMax application an additional income per kg of meat production comprises 1.66 roubles.

In discussion of the data it is necessary to mention that in poultry production, there are four major types of stress [25-27], including technological, environmental, nutritional and internal stresses [28-33] (Fig. 2).

Technological stresses
Chick placement
Increased stocking density
Weighing, grading, group formation, catching, transferring to breeder houses
Prolonged egg storage, egg transportation, inadequate egg storage conditions, incorrect incubation regimes
Environmental stresses
Inadequate temperature
Inadequate ventilation and increased dust
Inadequate lightning
Nutritional stresses
Mycotoxins
Oxidised fat
Toxic metals (lead, cadmium, mercury, etc.)
Imbalance of minerals (Se, Zn, Cu, etc.) and other nutrients
Low water quality
Usage of coccidiostats and other drugs via feed or water
Internal stresses
Vaccinations
Microbial or virus challenges
Gut dis-bacteriosis
Pipping and hatching

Fig. 2. Stresses in poultry production, adapted from [30].

It has been suggested that in broiler production stress of chicken placement is one of the most important stresses. Indeed, chick viability is an important factor in determining profitability. In fact, from fertilisation to placement at the broiler farm, factors such as egg quality, egg storage conditions, incubation conditions and post-hatch environment will all affect chick quality [34]. It has been proven that the first 24 hours of the chick's life are the most important [34-37]. It is believed that a chick should have an access to the feed and water as soon as possible after hatching to stimulate the development of the digestive and immune systems. It is well appreciated that time between chick hatch and placement is stressful due to dehydration and yolk sac reserve depletion. Indeed, when putting together hatching time inside the hatcher, time of chick processing and transportation, and finally, placement at the farm, it could take up to 36-48 h before a newly hatched chick has access to feed and water and during this time body weight decreases quickly [38]. It has been shown that in the hatching chick the most dramatic changes in the small intestine occur within the first 24 h post-hatch [39]. In fact, there is an inverse relationship between duration of post-hatching holding time and subsequent chick performance [34-37, 40, 41]. Therefore, immediate access to feed and water help achieving an increased body weight of the growing chick at 3 weeks of age [42] or at market age of broilers [43]. It should be also mentioned that there is the hatch window (24-36 hours) or the spread between late and early hatchers which depends on the homogeneity/heterogeneity of the incubating eggs which is dependent on breeder age [36, 37]. A spread in the hatching period will increase the numbers of chicks sitting extra hours in stressful conditions of the hatcher without food or water. Furthermore, any delay in accessing food [44, 45] and/or water intake after hatching as well as hatchery treatments such as vaccination, sexing and transport to the farm can result in additional stress [46]. Indeed, extended time in the hatcher (36 h) was associated with decreasing antioxidant defences indicative by decreased vitamin E and coenzyme Q concentrations in chicken tissues [47].

Given the relatively high temperature and humidity in the hatcher, it is possible to make the argument that the chick may be under chronic oxidative stress during this holding time [23, 47]. Therefore, antioxidant protection at hatching time is considered to be an important determinant of chick viability during first post-hatch days [3, 48-50]. During chick embryo development there is an antioxidant/prooxidant balance in the tissues which supports normal embryonic development and post-hatch chick viability [48-51]. It has been suggested that an accumulation of the natural antioxidants like vitamins A, E and carotenoids as well as an increase in GSH-Px activity in the embryonic liver may have an adaptive significance, evolving to protect unsaturated lipids against peroxidation during the stress imposed by hatching [3, 49, 50].

Postnatal nutritional exposures are considered to be critical for the developmental maturation of many organ systems and optimal physiological functions. There is a growing body of evidence indicating that environmental exposures including nutritional exposures during these critical and sensitive periods of life can cause permanent changes in many physiological processes, which is known as "programing" [52]. Our previous investigations indicate that low quality neonatal nutrition resulted in long-term impairment in the capacity to assimilate dietary antioxidants [53]. It seems likely that early programming associated with epigenetic mechanisms plays a key role in chicken growth and development and at time of chicken placement. Furthermore, scientific evidence is accumulating that the programming effects of conditions during early development can be transmitted to the offspring [54]. Therefore, transgenerational effects of stress are potentially mediated via modulation of the hypothalamic—pituitary—adrenal axis as well as epigenetic mechanisms causing heritable changes in gene expression and it was suggested that early experiences may shape phenotypes of chickens in a long-term way [55]. In addition to the aforementioned stresses chicks are exposed to such stresses as hatching without maternal contact, transportation and social isolation. Indeed, the early life social isolation stress resulted in a dampened corticosterone response to restraint stress in affected birds and in their male offspring. Furthermore, stress-specific genes, such as early growth response 1 and corticotropin-releasing hormone receptor were upregulated immediately after restraint stress [55].

Research data is accumulating to support the hypothesis that the vitamin E status of chickens and turkey poults and chickens may be inadequate during the first weeks after hatching [56]. A variety of approaches aimed at improving the vitamin E status of turkey poults have, in fact, been investigated including dietary supplementation of the poults with high levels of α-tocopherol [3, 57], bile salts [58] and fat [59], as well as vitamin E injection [60] and alterations in provision of n-6 and n-3 polyunsaturated fatty acids [60]. When d-α-tocopherol was added in the drinking water, there was an increase of α-tocopherol in tissues and a decreased susceptibility of red blood cells to hemolysis [61]. Moreover, day-old chickens were treated per os with 3.25 mg vitamin E/bird per day, via the drinking water, for two weeks. The vitamin E content of both the liver and the blood plasma was significantly higher in the treated chickens than in the untreated controls [62]. It seems likely, that provision of vitamin E and other fat-soluble vitamins (A, and D_3) with water at time of chicken placement can solve the problem of their low availability for newly hatched chicks [23]. Such a supplementation helps chickens overcome stress of placement and has positive effects on chicken growth and development.

When chicks are placed in winter while outside temperature is quite low there is always a temptation to decrease ventilation to keep energy usage to the minimum. However, it is very important to provide good quality, warm, fresh air

that is rich in oxygen for the recently hatched chicks. Indeed, the chick's trachea is very often irritated from being boxed and shipped in the chick trays, often for many hours. Furthermore, chicks can be exposed to formaldehyde gas and contaminated air during hatch [23, 50]. Excessive amounts of irritants such as carbon dioxide and ammonia can cause depression, dehydration, emaciation as well as various problems with the respiratory system of the chick [23, 50]. The increased lipid peroxidation and reduced activities of antioxidant enzymes in healthy chickens reared under unfavourable microclimatic conditions such as higher air temperature and humidity, higher ammonia concentrations, and lower light intensity were indicative about an induced oxidative stress [63]. It should also be mentioned that poor ventilation is often associated with toxic carbon monoxide accumulation. Toxicity causes irreversible physiological and biochemical changes that cannot be corrected with successive additional ventilation [25, 26]. Therefore, to deal with oxidative stress at chicken placement, there are several important options. They include: a) electrolyte supplementation via drinking system to increase water consumption by chicks and keep optimal electrolyte balance in the body [23]; b) fat-soluble vitamin supplementation via drinking water to overcome low efficacy of vitamin assimilation from the diet [3, 23]; c) other protective nutrient supplementation (ascorbic acid, Se, carnitine, betaine, lysine, methionine, etc.) with water to decrease oxidative stress related to chick placement and gut adaptation to a new type of feed [23, 28]. Improved antioxidant defences during first days of postnatal life are suggested to help immune system development in this critical period of time [65, 66].

As it was shown in our study, supplementation of the vitagene-regulating composition with drinking water helps chicken to maintain optimal FCR which was significantly improved in comparison to the control chickens. Therefore, it could well be that by decreasing placement stress it is possible to give chickens a better start which later will be translated into better gut development and health. In fact a trend indicating increased intestinal weight in the experimental chicks found in our study could be one of those factors reflecting the aforementioned feature. Our previous idea about antioxidant-prooxidant balance in the gut [67, 68] recently has been updated in relation to chicken placement [51]. Indeed, antioxidant protection in chicken gut is based on natural antioxidants, including vitamin E and carotenoids, and enzymatic systems [69], including SOD, an important part of the vitagene network [10]. Therefore, by using vitagene-promoting composition with drinking water it is possible to improve antioxidant defences of the gut and this could contribute to a better gut integrity translated into a better FCR.

The second possibility of better FCR is an improvement of immunocompetence. Indeed, immune system is a crucial factor determining not only chicken health but also chicken production efficacy. In fact, immune system is quite expensive to run and only optimal immune response gives a reliable protection against pathogens with minimal expenses for the body. Our data indicates that index of vaccination in the experimental chickens was increased and this could be due to immunomodulating properties of the vitagene-regulating mixture used in this experiment. Our previous studies clearly showed that upregulation of vitagenes is an important approach for high immunocompetence in commercial poultry production and PerforMax was shown to decrease vaccination stress [23, 66]. It has been proposed that communication between various immune cells is a key for high immunocompetence [3, 4]. Indeed, immune system includes trillions of lymphocytes and billions of phagocytes and all those cells have to communicate with each other and other types of immune cells to make a right decision on the strategy of immune defence. It seems likely that receptors on the

surfaces of immune cells are responsible for such a communication and the receptors can be damaged by free radicals in stress conditions. Furthermore, production of communicating molecules such as cytokines and eicosanoids can also be affected by oxidative stress. Therefore, by upregulating vitagenes responsible for synthesis of such protective molecules as heat shock proteins, thioredoxin and thioredoxin reductase, sirtuins and superoxide dismutase it is possible to provide a protection for receptors and maintain high immunocompetence in stress conditions.

Our previous studies indicated positive effects of the vitagene-regulating mixture (Magic Antistress Mix/PerforMax) on growing broilers, including improvement FCR [71, 72] and improved immunocompetence [70]. In this study previous results were confirmed in conditions providing growth, development and FCR comparable with the best examples of chicken production in Russia and abroad generating additional income.

Much attention has recently been paid to mycotoxins as the main nutritional stresses in poultry and animal production [73-76]. Oxidative stress caused by mycotoxins is the main molecular mechanism of their action [77-79]. This is true first of all for ochratoxin [80-83], T-2 toxin [84-86], DON [87, 89], fumonisins [90-92] and aflatoxins [93, 94]. It is very difficult to prevent feed contamination by mycotoxins. That is why various adsorbents are used to decrease negative effects of mycotoxins on animal/poultry with varied success [95-98]. However, absorptive capacity for mycotoxins in different adsorbents is greatly varied and, therefore, feed adsorbents are not able to solve mycotoxin problem in animal/poultry industry. It seems likely that key strategy in fighting the mycotoxin problem includes usage of various antioxidant compositions in the diet to support functions of the liver, a place where detoxification of most mycotoxins takes place as well as to support intestine where microbiota is involved in DON detoxification [78, 99]. Indeed, successfully tasted in our experiment the vitagene-stimulating composition of wide spectrum of action may become a very important instrument in the hands of poultry technologists/nutritionists [22, 23].

The importance and efficacy of the vitagene-regulating composition for rearing birds and adult egg type breeders (Hy-Line) have been studied at one of the biggest egg producing farms in Russia (Borovskaya poultry farm, Tumen region) and have been recently reviewed [28, 33, 100]. In particular, it was shown that usage of the antistress composition containing vitagene-activating nutrients (PerforMax/Magic Antistress Mix) with drinking water at specific periods of the increased stress can improve breeders' performance. In particular, there was an increase by 2 % of the egg peak production and peak plateau lasted about 50 days longer than that in the control birds. It is interesting to note that egg production in the control hens (260.8 eggs) was higher than the target one for the line in Russia (253.4 eggs), and in the experimental group this parameter increased by 6 eggs. Furthermore, improved egg production was associated with increased weight of the oviduct in the experimental layers. It is also important to mention that FCR (feed per 10 eggs) was also improved by usage of the antistress composition and was better than the target one for the line. Notably, shell strength at age 26, 36 and 56 weeks was improved in the experimental group by 2.8, 5.6 and 5.6 %, respectively. The most interesting finding was related to a significant increase of the carotenoid level in the egg yolk of experimental birds. Since carotenoids were not supplied with the antistress composition, this increase could be due to improved absorption of nutrients resulting from antistress composition usage. This can also explain improved FCR in the experimental birds. Vitamin A level in the egg yolk from the experimental layers was also increased probably reflecting its transfer from the antistress composition. In particular, antistress

composition usage was associated with improved fertility at weeks 16, 40, 48 and 56 by 2.5; 2.7; 2.8 and 3.7 %, respectively. In the same experimental group the hatch of condition chicks improved at weeks 26, 32, 40, 48 and 56 by 3.6; 2.1; 3.4; 4.9 and 4.3 %, respectively [28, 33, 100, 101]. Similarly, effects of the antistress composition on the rearing birds were studied [28]. In fact, the usage of the antistress composition positively affected testes development of 15-, 26- and 56-week old cockerels. The liver of experimental birds was characterised by a significant increase in vitamin A content at various ages [28, 102]. It was clearly shown that in laying type breeders PerforMax increased egg production, improved percentage of eggs suitable for incubation, as well as improved hatchability. This gave a profitability of hatching egg production at 29.3 % in comparison to 19.7 % in the control group [28]. There is also a possibility of increasing hatching egg production in broiler breeders due to PerforMax usage [103]. Furthermore, possible effects of supplying the antistress composition at 1 g/l drinking water to Hy-Line breeders on their progeny chicks with specific emphasis to chick uniformity as an important determinant of rearing birds' quality were studied [104]. The obtained data indicates that supplying the antistress composition to breeders was associated with a significant improvement of the uniformity (at day 28) in progeny chicks obtained from breeders of various ages: 26 weeks (81.3 vs 67.3 %), 32 weeks (85.5 vs 76.8 %), 40 weeks (83.2 vs 68.8 %), 48 weeks (75.5 vs 68 %) and 56 weeks (73.7 vs 62 %). Therefore, it seems likely that changes in egg composition due to supplying to breeders with important nutrients, including methyl donors (betaine, methionine, vitamin B_{12}, etc.), could have epigenetic effects of the progeny chicks [23, 104, 105]. Furthermore, protective effect sof the product in pig production were also shown [24, 51, 106]. Our preliminary studies also indicate that upregulation of vitagenes by nutritional means is a universal approach for fighting stresses, since usage of vitagene-regulating composition in other avian (turkey, duck, goose, quail) and animal species (young calves, lambs, foals, rabbits, fur animals, companion animals, fish, etc.) is quite effective (P. Surai, unpublished).

A study of relationship between vitagenes and gut microbiota can open new chapter in understanding of the role of gut immunity and structural integrity of the intestine in poultry/animal health [107-110]. It seems likely that targeted action on the vitagene expression in the intestine could help find new approaches for increasing poultry/animal resistance to microbial and viral pathogens. However, this question remains to be studied in the future.

Thus, our new concept of fighting stresses is based on an idea that supplying birds with various antioxidants via drinking water could help them deal with stress conditions more effectively. In the aforementioned study it was confirmed that usage of the vitagene-regulation composition supplied with drinking water is an effective means in fighting stresses and improving growing birds' performance, including improvement in FCR and improved vaccination efficacy. The cooperative mechanisms of the vitagene network are considered in detail in recently published comprehensive reviews with a major conclusion indicating an essential regulatory role of the vitagene network in cell and whole organism adaptation to various stresses. Indeed, the products of the vitagenes actively operate in detecting and controlling diverse forms of stress and cell injuries. Furthermore, our analysis of recent data indicates that vitagenes can be regulated by nutritional means. In particular, vitamins A, D, E, C, as well as selenium, carnitine and betaine are shown to affect vitagenes and improve adaptive ability of animals/birds to various stresses. In fact, protective effects of the aforementioned nutrients on vitagenes are most pronounced in stress conditions. Indeed, these results are the first step to go from the development of

the vitagene concept to designing a commercial product which can help fighting stresses in the commercial conditions of poultry and pig production. However, further work is required to understand molecular mechanisms of the interactions of vitagenes with various signaling pathways and transcription factors in the cell to build an adequate adaptive response to minimize detrimental consequences of commercially-relevant stresses in poultry and pig production. This would give an opportunity to improve our universal antistress composition and develop more specific compositions to deal with liver disorders in poultry associated with a commercial production of eggs and meat. It is likely that the vitagene concept would help solving other problems of modern poultry production, including losses associated with egg shell quality and eggs cracks in layers at the second part of their production.

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DEVELOPMENT OF AVIAN INFLUENZA VACCINE ON THE BASIS OF STRUCTURALLY MODIFIED PLANT VIRUS

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Abstract

Avian influenza is an infectious viral disease that affects various species of birds, including poultry (chicken, turkeys, ducks and geese). Vaccination is a key strategy in the prevention of epizootics and epidemics of influenza. Now, the actual problem is the development of rapid, safe and effective methods for the production of avian flu vaccines. The using of recombinant flu antigen is a promising approach for creating universal, safe and effective poultry flu vaccines. To increasing of low immunogenicity of recombinant antigens plant viruses and virus-like particles can be applied. We have previously shown that spherical particles (SPs) with unique adsorption property and immunostimulating activity. Here we design the candidate vaccine against the avian flu virus (H5N1). The complexes containing the antigenic determinants of the avian flu virus — hemagglutinin (HA) and the extracellular domain of the M2 matrix protein (M2e) presented on the surface of SPs were developed. By indirect immunofluorescence microscopy the specific antigenic activity of recombinant proteins adsorbed on the SPs surface were demonstrated. The SPs—HA-M2e (SPs—HA62/284-M2e) complexes were highly immunogenic. The candidate vaccine induced a strong humoral immune response to both antigenic determinants of avian flu A virus. HA and M2e adsorption on the SPs allowed a 10-fold increase in the production of blood antibodies to antigens in immunized animals. The advantage of this approach for the vaccine development is high efficiency based on the stability and adjuvant activity of SPs, safety and low cost of using plant viruses. Obtaining veterinary vaccines based on structurally modified plant viruses allows the creation of marker veterinary vaccines. Development and creation of marker vaccines is extremely important for the recovery of the poultry population in agro-industrial complexes. The proposed vaccine can be considered as a candidate recombinant vaccine against avian influenza virus.

Keywords: plant viruses, vaccines, spherical particles, influenza virus, recombinant proteins

Flu is one of the most contagious and rapidly spreading infectious diseases [1]. Its causatives are RNA-containing viruses of the family *Orthomyxoviridae*. Despite the fact that wild birds are a natural reservoir of the flu A virus, these viruses can also infect poultry and several species of mammals, including humans. Epizootic outbreaks, etiologically associated with the flu A virus, cause significant damage to agriculture. At the moment, combatting avian flu involves the introduction of quarantines, as well as the slaughter of infected and contact individuals, which leads to significant economic losses. The greatest danger to humans carried the H5N1 strain of all the flu viruses circulating among birds. Its hazard is due to the high incidence of human infection after the contact with infected birds and the highest rate of deaths [2].

Vaccination is considered to be the most effective way of preventing epidemics and panzootics of flu. In order to obtain modern licensed vaccines

against flu viruses, researchers use chicken embryos. Production of such vaccines for each strain takes up to 6-9 months, which in the case of the emergence of a new pandemic strain can have unpredictable consequences. Moreover, the use of traditional methods is limited by the high virulence of the H5N1 strain for chicks and chicken embryos, and a potential hazard to humans.

Constructing recombinant proteins is an alternative approach to the creation of universal, safe and efficient vaccines against the avian flu virus [3]. However, a number of studies reported that the viral proteins that are the most promising for inclusion in the vaccine (the surface protein haemagglutinin HA, which serves as the main target for neutralizing antibodies, and the conservative matrix protein M2) have low immunogenicity and cannot stimulate an effective immune response [4-7]. One of the solutions to the problem is using plant viruses [8-10] and virus-like particles to present the determinants of protective antigens and enhance the immune response [11-15].

Earlier, we showed that structural rearrangement of tobacco mosaic virus (TMV) protein after heating spiral virion to 94 °C led to formation of spherical particles (SPs) of a controlled size. Such SP are biodegradable, they do not contain RNA and have high stability. In addition, SP are safe for humans, since plants and animals do not share common pathogens [16-19]. At the same time, SP are effective immunostimulants [11].

In this work, unique SPs having no analogues were used to create the veterinary candidate vaccine. For the first time, we obtained SP-HA62/284-M2e complexes, containing spherical particles, which were formed during thermal rearrangement of TMV, with recombinant antigens HA62/284 and M2e of the flu A viruses adsorbed, and demonstrated the antigenic specificity of the recombinant proteins. Such complexes appeared to be highly immunodominant: the immunization of laboratory animals shows the production of specific antibodies to both antigenic determinants of avian flu A virus.

The purpose of this study was to develop a new generation of recombinant candidate vaccines against the avian flu virus by assembling in vitro complexes that include immunogenic determinants of HA and M2 proteins of flu A virus on the surface of spherical particles based on the virion of the tobacco mosaic virus.

Techniques. The virions of TMV (strain U1) were isolated from the infected *Nicotiana tabacum* L. plants of Samsun variety, as it was formerly described [20]. The SPs was obtained from purified TVM preparation (5 mg/ml) at 94 °C according to develoeped protocol [18].

Recombinant protein that contains a fragment of the HA molecule of flu A virus (16 kDa) was constructed based on strain A/Kurgan/5/05. The corresponding complementary DNA fragment of the haemagglutinin gene was synthetized in reverse transcription PCR with primers H562-284-P (5′-GCGGATCCGGAGTGAAGCCTCTAATTTTA-AGAGATT-3′) and H562-284-M (5′-CGTCTAGATTATTCACTTTTCATAATTA-TTGTTAGAGTCCCCT-3′). The amplified fragment was cloned in pQE30 vector (Qiagen N. V., Germany) with BamHI and XbaI restriction sites. The recombinant HA62/284 protein was expressed in *Escherichia* coli M15 and purified according to the standard procedure [21]. Recombinant 26 kDa protein containing the M2e determinant (23 amino acid residues long) of the flu A virus protein M2, fused to the protein with dehydrofolate reductase, was expressed and purified as described [11]. SP-HA62/284-M2e complexes were prepared in vitro by incubation of HA62/284, M2e and SP proteins in a weight ratio of 5:5:100 at 25 °C for 20 min.

Sample preparation for electron microscopy was as previously described [22]. The preparations were examined in an electron microscope JEM-1011

(JEOL, Japan) equipped with a digital camera ES500W Erlangshen (Gatan, Japan). The photomicrographs were analyzed in the ImageJ program (National Institutes of Health, USA).

The antigen specificity of the SP-HA62/284-M2e complex was studied with the use of immunofluorescent microscopy [23]. Rabbit antiserum to HA62/284 protein and mouse antiserum to flu virus M2e protein diluted 1:100 were the primary antibodies. The control samples lack the addition of primary antibodies. The binding of primary antibodies to the complexes was detected with secondary donkey anti-rabbit antibodies conjugated with Alexa Fluor® 546 fluorophore (Invitrogen, USA) or secondary chicken anti-mouse antibodies conjugated with Alexa Fluor® 488 fluorophore (Invitrogen, USA). The analysis was carried out with the use of Axiovert 200M fluorescence microscope (Carl Zeiss, Germany) equipped with an integrated ORCAII-ERG2 camera (Hamamatsu, Japan).

Immunogenicity of SP-HA62/284-M2e complexes was studied in female white lab mice aged 6-8 weeks with a body weight of 15-18 g divided into 4 groups of 5 animals each. Mice were intraperitoneally (i.p.) immunized with PBS (phosphate-buffered saline, negative control, group 1), free recombinant proteins HA62/284 and M2e (group 2), proteins mixed with Freund's adjuvant (group 3), and SP-HA62 complexes/284-M2e (group 4). The dose per injection was 5 μg HA62/284, 5 μg of M2e, and 100 μg of SP; the volume of the mixture injected was 0.2 ml per animal. A total of 3 immunizations were performed at a 2-week interval. Blood for analysis was taken 1 week after the last immunization.

The titer of the antisera pool was determined by indirect immunosorbent assay using a Multiscan FC (Thermo Scientific, USA), as described previously [12]. HA62/284 and M2e proteins were used as antigens at a concentration of 10 μg/ml. The titer of the antiserum was a dilution in which the optical density of the product of the enzymatic reaction was twice the corresponding value in the negative control (nonimmune mouse serum).

Results. In order to create a candidate vaccine from a purified TMV preparation by means of thermal denaturation at 94 °C, spherical particles were obtained. Transmission electron microscopy was used to control their characteristics (Fig. 1). The size of the obtained SPs was 612±41 nm.

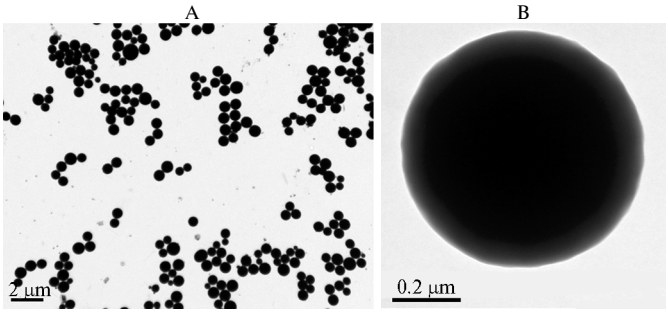

Fig. 1. Electron microphotographs of spherical particles obtained by thermal denaturation of the tobacco mosaic virus. Transmitting electron microscopy (JEM-1011, JEOL, Japan); contrast agent 2 % uranyl acetate; ×10,000 (A) and ×200,000 (B).

The site of the hemagglutinin molecule (amino acid residues 62-284) of strain A/Chicken/Kurgan/05/2005 (H5N1) of influenza A virus was chosen as the antigens that contains the main virus neutralizing epitopes, and the conserved N-terminal extracellular domain of the M2 matrix protein (23 amino acid residues long peptide M2e). Hemagglutinin is one of the two major surface proteins of the flu virus. This protein is responsible for the binding the virion to the cell receptors and the fusion of the viral envelope with the cell membrane. HA is the main surface antigen of the flu virus and serves as the main target for neutralizing antibodies. The research showed that the region of the molecule which

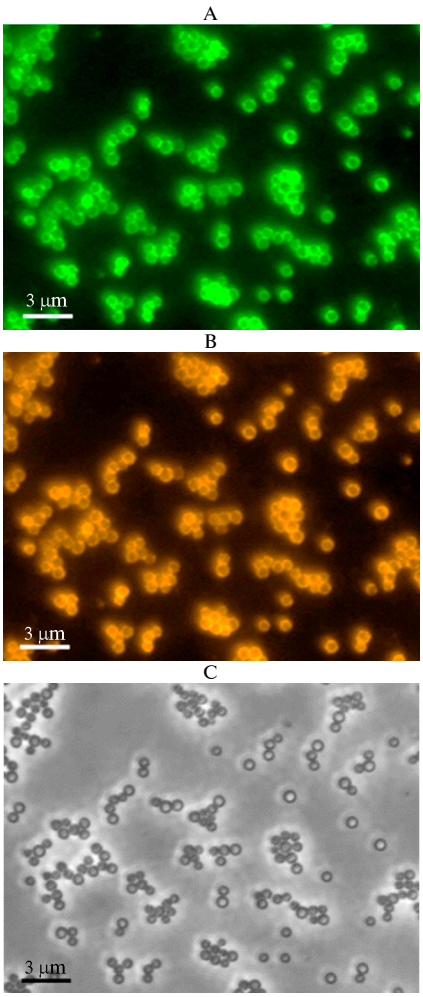

Fig. 2. Immunofluorescent microscopy of the complexes of spherical particles (SPs) **obtained by thermal denaturation of tobacco mosaic virus, with the flu virus proteins HA62/284 and M2e simultaneously adsorbed on the SP surface:** A — protein M2e detected with primary mouse antibodies and secondary antibodies conjugated with Alexa Fluor® 488 fluorophore; B — protein HA62/284 detected in the same sample with primary rabbit antibodies and secondary antibodies conjugated with Alexa Fluor® 546 fluorophore; C — the corresponding image of the sample obtained in the phase contrast mode. Fluorescence microscope Axiovert 200M (Carl Zeiss, Germany).

includs the amino acid residues form 62 to 284 contains most of the neutralizing determinants, as well as the structural elements necessary for efficient folding the recombinant protein [24]. The membrane protein M2 forms ion channels in the virion lipoprotein membrane. An extracellular domain of M2 (M2e), the 22 amino acid residues long fragment of this protein, is exposed on the outer surface of the viral particle. The M2e peptide is evolutionarily conservative and almost identical for all flu viruses circulating in animal populations, including the pandemic viruses. Hence, M2e peptide can be considered as a promising determinant for the development of a universal vaccine against flu [25].

In in vitro obtained SP-HA62/284-M2e complexes containing SPs along with the recombinant flu virus antigens HA62/284 and M2e, proteins on the SP surface retained the ability to bind with the specific antibodies to recombinant proteins, as confirmed by indirect immunofluorescence microscopy with two different fluorophores. Fluorescence on the SP surface indicated that both antigens (HA62/284 and M2e) were adsorbed on the same spherical particles and retained antigenic activity in the SP-HA62/284-M2e complexes formed (Fig. 2, A, B). Comparison of the obtained images in the fluorescence (see Fig. 2, A, B) and phase contrast (see Fig. 2, B) modes showed that all the SPs are associated with the molecules of the target protein, while aggregates of antigenic complexes not associated with the SPs, are absent. Therefore, the SPs free of antigen, was not found in the studied preparation. In a negative control, when recombinant proteins or primary antibodies were not used, fluorescence was not observed (data not shown). It indicates the absence of a nonspecific interaction of anti-species antibodies conjugated with the fluorophore with the SP surface. Thus, it has been shown that the adsorption of HA and M2e proteins on the SP surface does not prevent their binding with specific antibodies to recombinant influenza A viruses.

In assessing the ability of SP complexes with HA and M2e proteins of avian flu A to stimulate a specific immune response, laboratory mice were immunized with free HA62/284 and M2e proteins or with the SP-HA62/284-M2e complex. In the sera of mice immunized with the SP-HA62/284-M2e complex, a significant increase in antibody titer to viral antigens was observed compared to that of free antigens (in case of the SP absence). Thus, when using SPs on the

surface of which M2e and HA62/284 proteins were simultaneously adsorbed, the titers were 2.0×10^5 for antibodies to M2e protein (Fig. 3, A) and 2.4×10^5 for antibodies to HA62/284 protein (see Fig. 3, B). In the absence of the SPs, in mice immunized with HA62/284 and M2e, the antiserum titer was less than 4.0×10^4. Antiserum titers obtained by immunizing mice with a mixture of HA62/284 and M2e proteins using Freund's adjuvant, one of the strongest immune response stimulants used only in laboratory practice, were 3.0×10^5 and 3.6×10^5, respectively (see Fig. 3, A, B). Thus, the adsorption of HA62/284 and M2e on the SP surface significantly enhanced the immunogenicity of recombinant proteins under intraperitoneal injections resulting in an almost 10-fold increase in the concentration of antibodies to HA62/284 and M2e proteins in the serum of immunized animals compared to that in immunization with free proteins, which is comparable in effectiveness to using Freund's adjuvant.

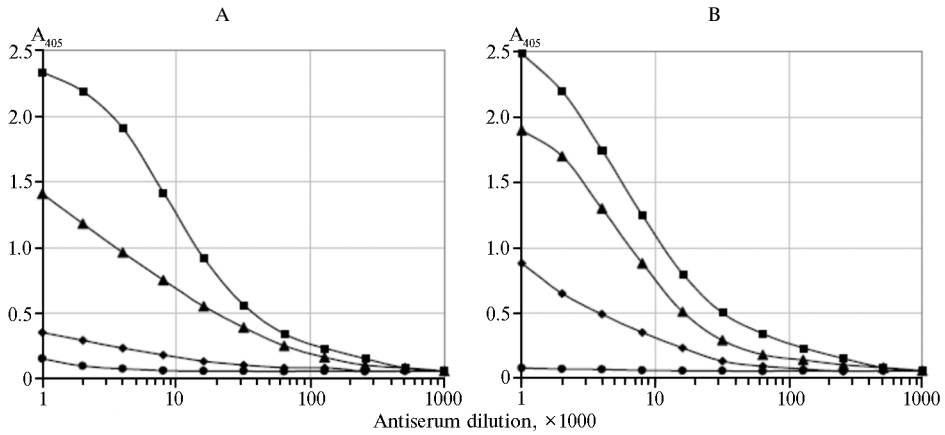

Fig. 3. Adjuvant properties of spherical particles (SPs) **obtained by thermal denaturation of tobacco mosaic virus in the candidate vaccine complex with antigenic determinants of avian flu A virus HA62/284 and M2e (SP-HA62/284-M2e)** (enzyme immunoassay). The antigens M2e (A) and HA62/284 (B) were immobilized at a concentration of 10 μg/ml on the microplate. The titration curves of antisera during immunization of lab mice with a mixture of free antigens (♦), their mixture with addition of Freund's adjuvant (■) and a SP complex (▲) are given. The control was non-immune serum (immunization with phosphate-buffered saline (PBS) solution (•).

It is important to note that the production of veterinary vaccines based on structurally modified plant viruses (SPs) allows the creation of domestic marker veterinary vaccines, i.e. spherical particles, on which a certain part of antibodies is produced during vaccination with the SP pathogen complex [26], can act as a marker which will further distinguish vaccinated birds from carriers of the field virus.

So, the obtained complexes SP-HA62/284-M2e may serve as a basic unit for creating a modern recombinant vaccine against avian flu virus. The advantage of this approach to development of vaccines is their high efficiency, based on the stability and adjuvant activity of spherical particles, as well as the safety and low cost of using plant viruses. The inclusion of the conservative peptide M2e in the developed vaccine preparation should provide protection from both seasonal and probable pandemic viruses. Moreover, it is a marker vaccine that makes it possible to distinguish between vaccinated and unvaccinated individuals that is important for the improvement of commercial poultry populations.

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STUDY OF MICROSATELLITES IN THE RUSSIAN BREEDS OF TURKEY

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Abstract

Currently, in the total volume of poultry meat production turkey (*Meleagris gallopavo*) meat takes the second place in the world. According to the analytical agency (Global Reach Consulting, Russia), this segment has grown more than 8 times over the last 10 years. Positive dynamics is also observed in Russia, i.e. a 34.9 % increase has been achieved in 2015, mainly due to the use of modern genetic approaches and highly efficient technologies. The high competition in the market of the world's poultry genetic material defines the importance of monitoring its origin and genetic consolidation. It is known that one of the most informative methods for studying genetic biodiversity of different species in animals and birds is the analysis of microsatellite loci. The estimation in microsatellite loci in turkey breeds and commercial lines, bred in USA, Turkey, Hungary and other countries has allowed us to establish genetic profiles of breeds, their differentiation, similarities and differences. However, till now the study of microsatellite polymorphism in the Russian breeds of turkeys was not conducted. The aim of this work was to study polymorphism and genetic differentiation on microsatellite loci of turkeys' breeds of the Russian selection. The work was carried out at the North Caucasian Zonal Experimental Station for Poultry Breeding using the turkey breeds of the Russian selection maintained in the Breeding and Genetic Center of North Caucasian Zonal Experimental Station for Poultry Breeding. MLVA (multiple locus variable, number tandem repeats analysis) genotyping was performed on 12 VNTR loci (MNT9-MNT20). Blood samples were taken from turkeys of seven breeds: broad-breasted White (n = 12), North Caucasian Bronze (n = 12), North Caucasian White (n = 9), North Caucasian Silvery (n =15), Moscow White (n = 12), Tikhoretskaya Black (n = 10), Uzbek Buff (n = 8). DNA was isolated using guanidine thiocyanate. The quality of DNA samples was determined spectrophotometrically. The detection of PCR amplification products was carried out by the presence of specific bands on the electrophoregram in the agarose gel. To quantify the discriminating ability of the typing method, we used the Hunter-Gaston index. For grouping, the pairwise unweighted clustering with arithmetic averaging (UPGMA) was performed, the dendrogram was constructed using a computer program START-2. It is established that 3 loci (MNT11, MNT15, MNT17) of 12 investigated VNTR loci were monomorphic, and 6 loci (MNT9, MNT10, MNT12, MNT14, MNT19 and MNT20) had two alleles each. For MNT16, 3 alleles were revealed, and only MNT13 and MNT18 produced 4 alleles each. Twenty one of 26 identified alleles have not been previously described or deposited in specialized international databases that specifies in originality of the investigated Russian turkey breeds. Phylogenetic analysis of genetic distances allowed us to allocate two unequal clusters — I and II. The cluster I is formed by a part of broad-breasted White genotypes and from all genotypes of Uzbek Buff breed. The cluster II is formed by two large C and D groups: C group is formed by genotypes of North Caucasian Bronze, North Caucasian White and broad-breasted White breeds, and D group is formed by genotypes of Tikhoretskaya Black, North Caucasian Silvery and Moscow White breeds. Features of genetic differentiation in turkey breeds of the Russian selection are due to their history and the participation of the gene pool of sertain breeds in creation of others. Allelic variants identified by us in the investigated loci for the first time allow further researches on genetic differentiation of turkey breeds of the Russian and foreign selection.

Keywords: turkey breeds, microsatellites, VNTR loci, phylogenetic analysis, MLVA, genetic diversity

Currently, in the total volume of poultry meat production turkey (*Meleagris gallopavo*) meat takes the second place in the world. According to the analytical agency Global Reach Consulting (GRC, Russia), this segment has grown more than 8 times over the last 10 years. Positive dynamics is also observed in Russia: a 34.9 % increase has been achieved in 2015, mainly due to the use of modern genetic approaches and highly efficient technologies. The high competition in the market of the world's poultry genetic material defines the importance of monitoring its origin and genetic consolidation.

Microsatellite loci analysis is one of the most informative methods for studying genetic biodiversity of different species in animals and birds [1-3]. It is known that microsatellites, SSR (simple sequence repeat) or STR (short tandem repeat), are the tandem repeats with a length of 2 to 4, sometimes up to 6 pairs of nucleotides in non-coding regions of the genome. The priority of their use in DNA diagnostics is due to a relatively uniform distribution on chromosomes and a wide variety. Polymorphism of microsatellite repeats is so high that it makes it possible to distinguish chromosomes during family analysis, tracing their transmission in generations, and also attributing a biological sample to a species, breed, and population [4].

Since the data on the populations genetic structure are extremely important for the rational use of the genetic resources of farm animals and poultry, the Food and Agriculture Organization of the United Nations (FAO) carried out the Global Project for the Measurement of Domestic Animal Genetic Diversity (MoDAD) [5, 6]. Genotyping from 6 to 50 breeds of the same species was performed with the help of 30 microsatellite loci. An example of the successful project approbation was the results of studying the genetic diversity of microsatellite markers in more than 50 populations of chickens [7, 8]. Despite the fact that a significant number of microsatellite loci in turkeys are identified and mapped [9-11], a unified microsatellite panel for *M. gallopavo* has not been proposed to date [12].

Scholarly papers on genetic diversity in turkeys demonstrated the possibility of using the microsatellite panel recommended by FAO for hens to evaluate it. So, when describing the genetic profile of the Nero d'Italia, Brianzolo and Colli Euganei breeds, raised in Turkey, 22 out of 31 chicken markers were informative. 63 common alleles were identified for the three breeds, according to them. At the same time, 10 markers were examined using multiplex panels (Multiplex Master Mix 1 — ADL0268, ADL0278, LEI0094, MCW0216, MCW0248; Master Mix 2 — MCW0034, MCW0069, MCW0081, MCW0222, MCW0295), and 12 loci (ADL0112, LEI0192, LEI0234, MCW0014, MCW0016, MCW0037, MCW0067, MCW0098, MCW0103, MCW0111, MCW0165, MCW0183) were studied separately in single PCR. No PCR products were detected for 9 markers (LEI0166, MCW0020, MCW0078, MCW0080, MCW0104, MCW0123, MCW0248, MCW0284 and MCW0330) [13].

K. Reed et al. [14] performed the most ambitious study of the chicken microsatellite loci informativeness for estimation of the genetic characteristics of the turkey. They used 520 microsatellite chicken markers and obtained amplification products with turkey DNA only for 280 markers (54 %). For the further analysis, 57 loci were selected, in 20 of which a low allelic polymorphism was established, an average of 1.4 alleles per locus. The authors concluded that about 20 % of microsatellite chicken markers are informative when studying genetic biodiversity in turkey populations [14]. Genetic profiles of two turkey breeds (BIG6 and BIG10) were constructed using eight microsatellite

chicken (*Gallus gallus*) markers, MCW0111, MCW0067, LEI0104, MCW0123, MCW0081, MCW0069, MCW0104 and MCW0183. The polymorphism of 7 loci out of 8 loci studied was established, which allowed the authors to conclude that the microsatellite loci of *G. gallus* are informative for characterizing the gene pool of *M. gallopavo* [3].

The study of multiple locus variable (number tandem repeats) analysis, MLVA, was performed on turkey breeds in several countries. Thus, E.K. Latch et al. [15] surveyed populations of the Eastern wild turkey (*M. gallopavo silvestris*) and domestic turkey (*M. gallopavo*), bred in Turkey, for 7 microsatellite loci. The number of alleles per locus varied from 5 to 15, while the average heterozygosity was high for almost all loci. Domestic turkey was characterized by a much smaller number of alleles per locus with a low overall heterozygosity compared to the Eastern wild turkeys [15]. In their study, K. Reed et al. [16] discovered eight new microsatellite loci in turkeys.

D. Kamara et al. [17] examined the genetic relationships between commercial and non-commercial turkey breeds (Narra-gansett, Bourbon Red, Blue Slate, Spanish Black and Royal Palm) from the gene pool of the Virginia College farm, using 10 microsatellite loci (RHT0009, RHT0011, RHT0024, RHT0095, RHT0131, RHT0216, RHT0294, TUM16, TUM20, ADL0023). Phylogenetic analysis revealed that the Narra-gansett, Bourbon Red and Blue Slate breeds had a greater genetic similarity with commercial breeds than the Spanish Black and Royal Palm [17)]. Similar results for these breeds were obtained using three marker systems: RAPD (randomly amplified polymorphic DNA), microsatellite and SNP (single nucleotide polymorphism) [18].

In a joint study, US and Turkey scientists, while identifying microsatellite loci that were convenient and informative for the study of the turkey genome, designed primers for 164 genomic DNA regions containing microsatellites, based on a data library. The authors conclude that 154 detected genetic markers are quite acceptable for analysis, but further research is required to develop an informative panel [19].

S. Kusza et al. (20), studying populations of turkeys of Hungarian Bronze and broad-breasted White breeds using 15 microsatellite loci, found that the first population was highly polymorphic (the average number of alleles per locus was 3.20). The obtained data and the genetic analysis by M. Nei made it possible to clearly differentiate the studied populations.

For the most complete description of genetic diversity of turkey breeds and lines bred in Turkey, their genome was sequenced [21]. It is established that all modern Turkish commercial lines have a common origin and are derived from wild populations. At the same time, wild turkeys are characterized by higher heterozygosity compared to commercial lines. The authors concluded that the turkey genome is much less diverse than that of other farm animals and poultry.

By now, the study of the genetic profile of turkey Russian breeds, including microsatellite loci, has not been carried out, which determines the relevance of our study.

For the first time, we performed MLVA to genotype Russian turkey breeds for 12 microsatellite (MNT9-MNT20) VNTR (variable number of tandem repeats) loci. The genetic uniqueness of the studied breeds is established: out of the 24 identified alleles, 18 have not been previously described and annotated in specialized international databases. Phylogenetic analysis based on genetic distances revealed grouping of the part of broad-breasted White genotypes and all genotypes of the Uzbek Buff breed in one cluster, while genotypes of the North Caucasus Bronze, North Caucasian White, broad-breasted White, Tikho-

retsky Black, North Caucasian Silvery and Moscow White breeds formed another cluster. The obtained data showed that the patterns of genetic diversification of Russian turkey breeds are due to the history of their origin and the participation of the gene pool of some breeds in the creation of others.

The purpose of this work was to study polymorphism and genetic diversification of microsatellite loci in Russian turkey breeds.

Techniques. The study was carried out on breeds raised in the Selection-genetic center of the North Caucasian Zone Experimental Station for Poultry Farming (Stavropol Krai). To determine the genetic diversity against 12 VNTR-loci, blood samples were taken from seven turkey breeds: broad-breasted White (n = 12), North Caucasian Bronze (n =1 2), North Caucasian White (n = 9), North Caucasian Sylver (n = 15), Moscow White (n = 12), Tikhoretsky Black (n = 10), Uzbek Buff (n = 8)

The DNA was extracted by guanidine thiocyanate method according to the protocol to the AmpliPrime DNA-sorb-AM commercial set (InterLabService, Russia). The purity of the DNA samples was evaluated spectrophotometrically ($A_{260/280}$).

For the MLVA genotyping, 12 VNTR loci (MNT9-MNT20) [22] were selected. PCR was carried out in a final volume of a 20 μl mixture containing following amounts of reagents per reaction: 1 μl forward primer (F) and 1 μl reverse primer (R) (FKUZ StavNIPChI Rospotrebnadzor, Russia), 2 μl dNTPs, 4 μl RNA eluent, 10 μl PCR-mixture-2red solution (InterLabService, Russia) and 2 μl DNA from the blood sample. All reactions were carried out in the following mode: 15 min at 95 °C (initial denaturation); 30 s at 95 °C, 30 s at 58 °C (for the loci MNT10, MNT11, MNT20 the temperature was 56 °C), 30 s at 72 °C (35 cycles); 5 min at 72 °C (final elongation).

PCR amplification products were detected by the presence of specific lines during electrophoretic separation in a 1.5 % agarose gel. The exact size of the alleles of the identified markers for all VNTR loci in each test sample was determined using an automated microcapillary electrophoresis station Experion System (Bio-Rad Laboratories, USA).

To evaluate the discriminatory ability of the genotyping protocol, the Hunter Gaston Discriminatory Index (HGDI) was used [23]. Cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), and START 2 [24] software was used to construct dendrograms.

Results. The Selection-genetic center of the North Caucasian Zone Experimental Station for Poultry (Stavropol Territory) is the only enterprise in Russia that creates new breeds and crosses, and ensures the preservation of the turkey gene pool.

1. Primers used to amplify 12 VNTR loci in Russian turkey breeds (North Caucasian Zone Experimental Station for Poultry, Stavropol Krai**)**

VNTR locus	Nucleotide sequence of primer (5′→3′)	
	forward (F)	reverse (R)
MNT9	TGGGAGTGGAAAGGTGAAAG	TTCTCCTCAGCTCAGCAACC
MNT10	TTCCCAGTGCACTACCTGAAC	TGAACAGTGATTCCACTGAAGC
MNT11	TTTCTGACACAGGTACAAGGAAAC	GCCCTCGAGTATTAGCCACTC
MNT12	AGGTGTTTTTGGGCAGTCTC	TGCAAGCACCATCTGCTAAG
MNT13	TTAGGGGATGCTGAACTGTG	GCGTAATTGGTGCTTTCTCC
MNT14	AAACAGAACAACCTCAAGGACAG	GAATTGGGTTTGCATTTGAG
MNT15	TTGTTGCTGTTGTTTTTGTGG	TTTCTGTGCCTAAGCTTAATGTG
MNT16	TGTTTGCCTGCAATAAGCTG	GCACCCTCCCACTGACTG
MNT17	GGAGCACCCAGCTCAAAG	GAGTAATACCAAGGAAAAGTGTGC
MNT18	GCAGGCACAGAGAGCTACG	CCAATGTTGAAGCAGGTGAG
MNT19	GCAGGAGGCTCTGAGCTATG	TTATACGGAAGGCGGTTGAG
MNT20	TAACTGTCTGCCAGGTGGTG	GATCTCGGGTGGTGATTGC

Sequences of the primers used to amplify the 12 MLVA loci are shown in Table 1.

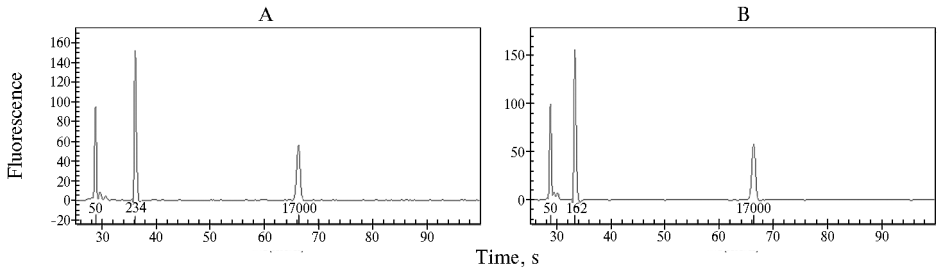

Fig. 1. Examples of determining the allele size of VNTR loci found in Russian turkey breeds, based on microcapillary electrophoresis of PCR products: A — previously not described allele 234 bp (locus MNT16, sample No. 140, Tikhoretsky Black breed), B — previously not described allele 162 bp (locus MNT18, sample No. 107, Moscow White breed); 50 and 1700 — peaks corresponding to the standards of 50 bp and 17,000 bp (Experion System, Bio-Rad Laboratories, USA).

The carried out research has allowed us to reveal some features of variable VNTR loci in the studied Russian turkey breeds (Table 2).

2. Variability of VNTR loci in the studied Russian turkey breeds (North Caucasian Zone Experimental Station for Poultry, Stavropol Krai)

Locus	Number of alleles	Size of an allele, bp
MNT9	2	164*, 168
MNT10	2	67*, 78*
MNT11	Invariable	90*
MNT12	2	121*, 145
MNT13	4	183*, 185*, 187*, 235*
MNT14	2	177, 181*
MNT15	Invariable	188
MNT16	3	219*, 226*, 234*
MNT17	Invariable	181*
MNT18	4	158, 159*, 161*, 162*
MNT19	2	224*, 250*
MNT20	2	192*, 195*

N o t e. The asterisk denotes allelic variants of VNTR loci not described earlier.

Out of the 12 examined loci, MNT11, MNT15 and MNT17 were monomorphic. In six loci (MNT9, MNT10, MNT12, MNT14, MNT19, MNT 20), two alleles were detected, three alleles were detected in the MNT16 locus, and two loci, MNT13 and MNT18, showed four alleles each. Consequently, the average number of alleles per locus was 2. The obtained data to some extent confirms the conclusions of other scientists that turkeys' genome is less polymorphic and more conservative at microsatellite loci compared to other farm animals [21, 25].

At the same time, the data obtained by us make it possible to draw a conclusion about the genetic originality of the studied Russian turkey breeds. Thus, out of the 26 detected alleles of VNTR loci, 21 have not been previously described in other breeds and were not annotated in specialized international databases. Identified variants of alleles made it possible to divide the individuals of the studied breeds into 14 types against 12 variable VNTR loci, on the basis of which a dendrogram of genetic distances was constructed, reflecting phylogenetic relationships (Fig. 2).

Two unequal clusters, I and II, emerged on the dendrogram. The first cluster grouped a part of the genotypes of the broad-breasted White breed (branch A) and all genotypes of the Uzbek Buff breed (branch B). The combination of these breeds in one cluster seems quite reasonable, since the Uzbek Buff breed was developed by crossing of local Uzbek Bronze turkeys with broad-breasted White ones.

The second cluster, in turn, was formed by two large groups C and D. Group C included the genotypes of the North Caucasian Bronze, North Caucasian White and broad-breasted White. The similar location of the North Caucasian Bronze and North Caucasian White breeds in subcluster C1 (branches C1.1 and C1.2, respectively) is explained by the use of the gene pool of one breed in

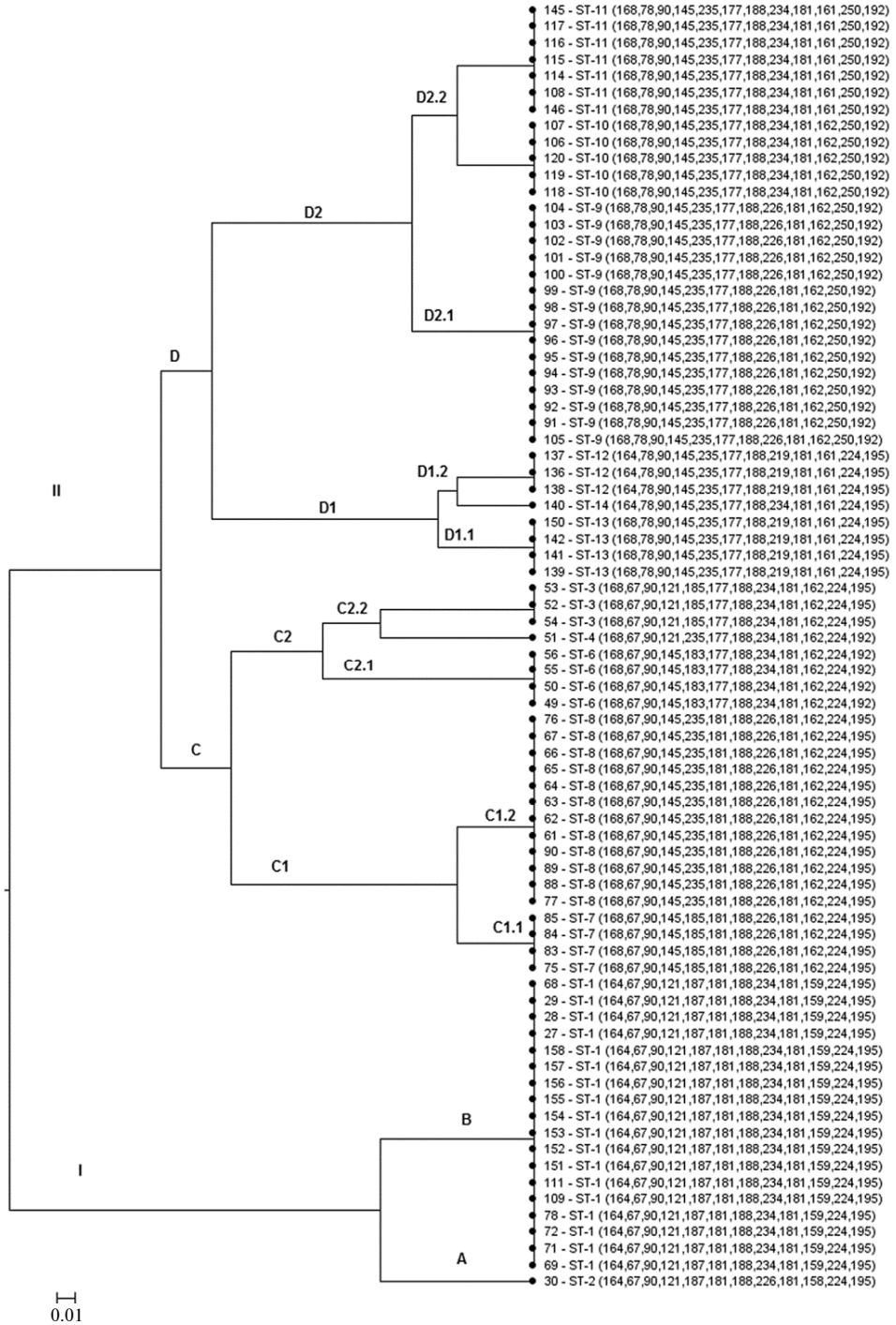

Fig. 2. Dendrogram of genetic distances between the Russian turkey breeds based on 12 variable VNTR loci (MLVA): I and II — clusters; A, B — groups; C1, C2, D1, D2 — subclusters, C1.1, C1.2, C2.1, C2.2, D1.1, D1.2, D2.1, D2.2 — branches of subclusters (see the text for rescription).

Sample numbers by breed: broad-breasted White — 27-30, 49-56 (n = 12); North Caucasian Bronze — 61-69, 75-77 (n = 12); North Caucasian White — 71, 72, 78, 83-85, 88-90 (n = 9); North Caucasian Sylver — 91-105 (n = 15); Moscow White — 106-111, 114-120 (n = 12); Tikhoretsky Black — 137-142, 145, 146, 150 (n = 10); Uzbek Buff — 151-158 (n = 8); St 1-14 — 14 genotypes found in the studied turkey breeds against 12 microsatellite loci (the numbers of the alleles corresponding to the genotypes St 1-14 are indicated in parentheses) (North Caucasian Zone Experimental Station for Poultry, Stavropol Krai).

the creation of another one. Thus, the first domestic turkey breed, the North Caucasian Bronze, was bred in the 1950s and 1960s by a prolonged use of the Bronze and broad-breasted Bronze gene pool breeds during the breeding of local turkeys. The second domestic breed, the North Caucasian White, was created in the 1970s and 1980s by crossing the North Caucasian Bronze turkeys with males of broad-breasted White breed of English origin. Genotypes of broad-breasted White breed formed their own subcluster C2, branches C2.1 and C2.2, were located very close to each other, which confirms belonging to the same breed and indicates its high genetic consolidation.

It is known that the broad-breasted White breed of turkeys is one of the oldest in the world. It was imported from Britain to Russia in the early 1960s. In 1980, the North Caucasian Zone Experimental Station for Poultry received four Hidon crossing lines, A, B, C, D, from the Netherlands. Using two lines, B and D, parent forms have been obtained, pure breeding of which is being maintained to date. Group D (see Fig. 2) also consisted of two subgroups: subgroup D1 was formed solely by genotypes of the Tikhoretsky Black breed which divided into branches D1.1 and D1.2. In the D2 subgroup, the D2.1 branch was formed only by the North Caucasian Silvery breed, and the D.2.2 branch included genotypes of the Moscow White breed.

A certain genetic isolation of these breeds is explained by the history of their creation. Thus, the modern North Caucasian Silvery breed, approved in 2008, was created based on the gene pool of the Uzbek Buff and broad-breasted White breeds. To increase the reproduction and improvement of meat types, a repeated introductory crossing of hybrids with maternal males of broad-breasted White breed (line O4) was carried out. During the next step, the population was improved by breeding in itself. The Tikhoretsky Black breed, formerly known as the Krasnodar Black, was created in the years 1950-1960 in the Krasnodar Krai during prolonged mass breeding of local turkeys with black plumage. The Moscow White breed was derived during the same period based on a complex high-performance crossing of local white turkeys with Dutch White and Beltsville Small White turkeys. In subsequent generations, the crossbred females were improved by crossing with the Beltsville Small White males and further the desired genotypes were bred in themselves.

The obtained results allow us to conclude that turkeys of the same breed, as a rule, had the same MLVA12 type and clustered as a single subgroup on the dendrogram.

Calculations showed that the discriminating ability of our analysis of 12 variable VNTR loci (MLVA12 method) was satisfactory. According to common practice, the typing method is considered acceptable if the Hunter-Gaston index is ≥ 0.9. In our study, the value of this index (HGDI = 0.9) corresponded with the acceptable values

Thus, as a result of the analysis of a phylogenetic tree constructed on the basis of genotyping native turkey breeds at the North Caucasian Zone Experimental Station for Poultry for 12 variable VNTR loci (MLVA12 types), the groups formed were characterized by different degree of discrimination. Turkeys of the same breed usually have the same MLVA12 type and clustered together on a dendrogram. In the studied breeds, the average number of alleles per locus was 2, which agrees with the notion that the turkey genome is more conservative for microsatellite loci. The discriminating ability of the used MLVA12 method was satisfactory (HGDI = 0.9). The alleles of the studied loci which we identified for the first time (a total of 21 allelic variants) allow us to continue the study of genetic differentiation in turkeys of Russian and foreign breeding. For an in-depth analysis of genetic characteristics of turkey

breeds, it is advisable to use a comprehensive approach using additional methods of genetic analysis, such as SNP, MLST (multilocus sequence typing) and Sanger sequencing.

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INVOLVEMENT OF CHOLESTEROL, PROGESTERONE, CORTISOL AND LIPOPROTEINS IN METABOLIC CHANGES DURING EARLY ONTOGENESIS OF BROILER CHICKS OF AN INDUSTRIAL CROSS

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Abstract

Morphophysiological changes in body are influenced by environmental factors, what is more, the specific nature of the body reactions depends on the reaction rate and the stage of ontogenesis on which the physiological stimuli act (I. Schmalhausen, 1982). Also it is known that hormones participate in the regulation of metabolism, growth and development, in adaptation processes. We determined the concentration of high-density (HDL) and low density (LDL) lipoproteins directly involved in protein and lipid metabolism, total cholesterol (TCS), progesterone (P_4), 17-hydroxyprogesterone (17-OHP), and cortisol in blood plasma of Hubbard F15 broiler chickens at early postnatal ontogenesis using four groups of poultry of the industrial herd of Chebarkulskaya Ptitsa LLC (Chelyabinsk Province, Russian Federation), 10 animals in each, aged 1, 7, 23 and 42 days, respectively. The role and interrelations of these substances in metabolism were assessed using Pearson's correlation analysis and factor analysis by the principal components method with Varimax factor rotation. Thus, in 1-day aged chicks, the integration of factors HDL, LDL, TCS, P_4 and cortisol involved in metabolic processes and metabolism regulations was noted with *r*-Pearson for P_4 and Cortisol at $r = 0.69$, $p = 0.027$; for P_4 and TCS at $r = 0.82$, $p = 0.004$; for HDL and LDL at $r = 0.83$, $p = 0.003$; and for HDL and TCS at $r = -0.67$, $p = 0.033$. On day 7 day, the principal components were progesterone and cortisol (*r*-Pearson for P_4 and cortisol of $r = 0.73$, $p = 0.016$), and a cholesterol donation factor with LDL and TCS as the leading elements ($r = 0.73$, $p < 0.05$). In 23-day-old chicks the components which have become principal were HDL ($r = 0.91$, $p < 0.05$) and 17-OHP ($r = 0.74$, $p < 0.05$), which we attribute to growth, and also P_4 ($r = -0.88$, $p < 0.05$) and cortisol ($r = -0.77$, $p < 0.05$) viewed as regulatory ones. On day 42 we revealed cholesterol donation factor (*r*-Pearson of $r = 0.86$, $p = 0.002$ for LDL and TCS) and an integral factor with the principal components HDL ($r = 0.74$, $p < 0.05$), P_4 ($r = 0.76$, $p < 0.05$) and cortisol ($r = 0.84$, $p < 0.05$). Thus, here we described the age-specific features of interaction between lipoproteins and hormones of cholesterol—progesterone—cortisol system involved in broiler metabolism, and found out the change of principal components and functional relationships among the hormones of progesterone group and lipoproteins during early growth, which, according to our thought, makes a physiological basis for chicken performance under commercial poultry production. In further studies, it can be reasonable to assess the role of these hormones and metabolites in the control of reaction norm and adaptive capability of broilers, and the physiological cost of adaptation (i.e. adequate or pathological response) to reproduction at commercial farms.

Keywords: progesterone, 17-hydroxyprogesterone, cortisol, high density lipoproteins, low density lipoproteins, cholesterol, broiler chicks, early ontogenesis, homeostasis

It was I.I. Schmalhausen who noted that morphophysiological changes are directly related to the effect of environmental factors on the totality of development processes [1]. Body response to external actions becomes a kind of stimulus, which causes subsequent responses. Though, the specificity of the form-building reactions primarily depends on the organism itself, its norms of reaction and the stage of ontogenesis which is affected by the stimuli [1].

The homeostasis that consolidates the cycles of synthesis and decomposition of chemicals into the viable morphofunctional structure of the open system, the organism [2], can be defined as maintaining the relative dynamic constancy of the internal environment on the basis of interrelated systemic regulatory and metabolic processes involving endogenous and exogenous factors that determine ontogenetic development [3, 4]. Broiler chickens in their early neonatal period of development have critical stages, when physiological and biochemical mechanisms are being formed. These mechanisms ensure the sustainable performance and adaptive capabilities of the organism under intensive commercial poultry farming technologies [3, 5-7]. The endocrine regulation serves as the basis of homeostasis, formed during the species evolution, and as the primary link between endo- and exogenous flows of energy, substrates, and signaling pathways. It includes a hierarchical chain of hormones; the blood content of these hormones according to the type of feedback determines, among other things, the amount of blood cholesterol and lipoproteins, which reflect both the processes of synthesis of adrenal hormones and their regulatory effects [2, 8-10]. One of these hormones, the progesterone synthesized from low-density lipoprotein cholesterol, is best known for its participation in the endocrine control of the formation, growth and development of the fetus. 17-Hydroxyprogesterone is the main circulating metabolites of progesterone and one of the leading precursors of steroids [11, 12]. Recent studies have established that progesterone and its synthetic derivatives have a direct, due to the neurosteroids of the group of pregnenolone synthesized de novo from cholesterol in the brain of birds [12], and mediated [13, 14] effects on the vegetative and central nervous system. They also participate in the neurotransmitter control over the chronological rhythms of postnatal growth and development due to receptor interaction with γ-aminobutyric acid [12, 13] and dopamine, affect the organogenesis of blood vessels, locomotor apparatus [15], skeletal muscles (which has hypertrophic development in broiler chickens) [5, 9], and myelination of nerve fibers [13]. Progesterone, 17-hydroxyprogesterone and its metabolite cortisol are among the primary activators and effectors of the hypothalamic—pituitary—adrenal axis [10, 16]. However, the information on the role of progesterone and its derivatives in lipid and protein metabolisms (despite the direct link between the biosynthesis of these metabolites, particularly the low and high density lipoproteins and hormones) is extremely limited. The available information is mainly obtained in medicine, whereas in livestock husbandry, where such studies are of great practical importance, the data are single, scattered or almost unavailable.

This paper is the first to report a systemic study of high and low density lipoproteins (directly involved in protein and lipid metabolism), total cholesterol, progesterone, 17-hydroxyprogesterone, cortisol in blood of Hubbard F15 cross chicks aged 1, 7, 23 and 42 days (with regard of growth and development), and to generalize these biochemical parameters by correlation analysis and the principal components (factor analysis). This resulted in finding age-related profiles of concentrations of these metabolic and regulatory factors that may reflect their changing roles during growth and adaptation.

Here, we studied dynamics of the blood content of some metabolites and hormones (lipoproteins, cholesterol, progesterone and its derivatives) in broilers in relation to changes in body weight, in order to assess the age-related changes of these factors and their role in ontogenesis.

Techniques. The experiments were carried out on broilers of the Hubbard F 15 cross (Chebarkul'skaya Ptitsa LLC, Chelyabinsk Province). The groups (P1, P7, P23, P42, $n = 10$ each) were formed in the growing room (cage housing), the age of the bird by groups was 1, 7, 23 and 42 days, respectively. Feeding and

housing were according to the zoo hygienic norms with unlimited feed access [17].

Blood samples were collected after decapitation at the age of 1 and 7 days, according to the principles of humanity set out in the directives of the European Community (86/609/EEC) and the Helsinki Declaration, and intravitally, in 23- and 42-day old chicks, by vacuum puncturing the axillary vein. In the plasma stabilized with EDTA, total cholesterol (TC), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) were assessed by enzyme assays using commercial kits (Vektor-Best, Russia; Olveks Diagnostikum, Russia) [18, 19]. The hormones were determined by solid-phase enzyme-linked immunoassay (ELISA) using sandwich-type test systems (XEMA Co., Ltd, Russia), namely Progesterone-IFA K207 for progesterone (Pregn-4-ene-3,20-dione, P_4) [20], 17-OH-Progesterone-IFA K217 for 17-hydroxyprogesterone (17-OHP) and Cortisol-IFA K210 for cortisol [11]. In the analysis of hormones, the samples were incubated in an ELMI Sky Line Shaker ST-3 thermostate shaker (ELMI, Ltd, Latvia). The absorbency was measured on a photometer for MINDRAY MR-96A Elisa Microplate Reader (MINDRAY Ltd, PRC).

The average daily body weight gain for the age periods was calculated as follows: $A_{adbwg} = (W_1 - W_0)/(T_1 - T_0)$, where W_0 and W_1 are the body weight (g) at the beginning and the end of the period studied at the age T_0 and the subsequent age T_1 (days).

To identify the structure of the relationships of the analyzed elements, the Pearson correlation (r-Pearson) analysis and factor analysis [21] for normal distribution of the biochemical parameters (STATISTICA 8.0, StatSoft, Inc., USA) were performed. The factors were isolated by the principal components method, Varimax method was used for the factor rotations [21]. The numerical data is represented by the arithmetic mean (X) and standard mean error (±SEM). The degree and reliability of the differences for the results obtained were evaluated by the Student's t-test using STATISTICA 8.0 software. Differences were considered statistically significant at $p \leq 0.05$.

Results. By day 7, the curves of dynamics of the TCS and lypoprotein

1. Dynamics of blood biochemical parameters and body weight in Hubbard F15 cross broiler chickens in early postnatal ontogenesis (X±SEM, n = 10, Chebarkul'skaya ptitsa, LLC, Chelyabinsk Province, 2014)

Показатель	Возраст, сут			
	1-е	7-е	23-и	42-е
High density lipoproteins, mmol/l	1.79±0.04	1.17±0.03***	1.36±0.05***	1.69±0.04
Low density lipoproteins, mmol/l	5.68±0.27	1.39±0.28***	1.81±0.16***	2.32±0.13***
Total cholesterol, mmol/L	8.67±0.57	3.14±0.25***	4.65±0.32***	4.84±0.14***
Progesterone, nmol/l	63.00±3.59	65.28±2.20	57.13±2.40	51.07±4.28
17-hydroxyprogesterone, nmol/l	10.76±1.61	19.43±3.40*	17.87±3.36*	8.30±1.42
Cortisol, nmol/l	2274.31±59.47	2341.42±44.29	2351.38±35.37	2256.00±45.18
Average daily body weight gain, g/day		16.37±0.21	49.98±0.11***	79.28±1.05***

*, **, *** Differences with 1 day age are statistically significant by t-test at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

concentrations were parallel to the graphics of reduction in HDL level up to 34.64 % ($p < 0.001$), in LDL level up to 75.53 % ($p < 0.001$), and in cholesterol up to 63.78 % ($p < 0.001$) (Table 1). From day 23, a gradual increase and stabilization of the amount of total sterol and lipoproteins occurred. However, the greatest approximation of HDL content to the value in the group of a day-old chicks was at the age of 42 days (see Table 1). The changes in the progesterone and cortisol levels were unreliable. The graphics of cortisol and its precursor progesterone concentrations (Cortisol and P_4) were inversely symmetric to the curves for HDL, LDL and TCS, with relatively high values from 7 to 23 days of life (see Table 1). The dynamics of 17-OHP concentration and body weight gain in broiler chickens had a significant similarity to the peak of max-

imum values at the age of 7-23 days. The average daily body weight gain was the largest in 23 day-old chicks, 305.32 % ($p < 0.001$) (see Table 1). Except the HDL, the 17-OHP content at day 42 of life was restored to that at the age of 1 day, and the absolute value of the daily body weight gain from days 23 to 42 was the highest (158.62 %, $p < 0.001$), although remaining below the reference one which was registered from day 7 to day 23 (305.32 %, $p < 0.001$) (see Table 1).

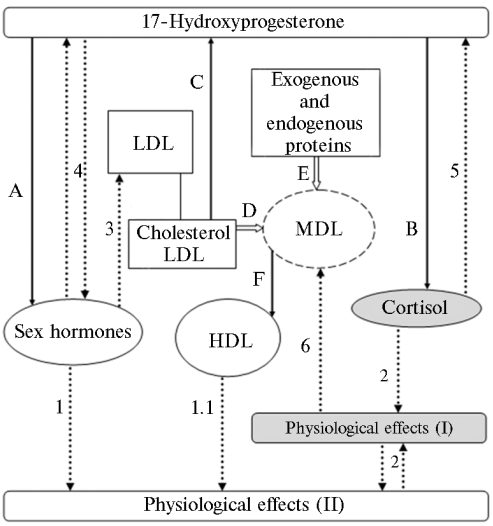
We calculated the mutual correlations (including the Pearson correlations) and determined factors and corresponding principal components among the studied biochemical indicators at different periods of broiler growth and development (Table 2).

2. Factor analysis with principal component method of blood biochemical parameters in Hubbard F15 cross broiler chickens in early postnatal ontogenesis

Parameter	Age, days						
	1	7		23		42	
	factor						
	1	1	2	1	2	1	2
Lipoproteins:							
high density	0.80*	0.07	0.66	0.91*	0.07	–0.40	0.74*
low density	0.83*	0.40	0.73*	0.68	0.29	0.97*	–0.03
Total cholesterol	–0.88*	–0.17	0.73*	–0.53	0.06	0.89*	–0.24
Progesterone	–0.88*	0.91*	–0.17	–0.26	–0.88*	0.20	0.76*
17-Hydroxyprogesterone	–0.60	0.62	0.25	0.74*	–0.55	0.66	0.39
Cortisol	–0.74*	0.88*	0.31	0.34	–0.77*	–0.11	0.84*

N o t e. Factors rotation by Varimex method. Correlations and the principal components were calculated and determined for each identified factor.

* Correlations are statistically significant at $p < 0.05$.



The scheme showing the involvement of cholesterol, progesterone, cortisol, and lipoproteins in growth and development of Hubbard F15 broiler chickens: LDL — low density lipoproteins, MDL — medium density lipoproteins, HDL - high density lipoproteins; A, B and C — the synthesis of sex hormones, cortisol and 17-hydroxyprogesterone, respectively; D, E and F — conversion of low density lipoproteins and proteins to high density lipoproteins; 1 and 1.1 — regulation to provide growth and development with structural proteins, 2 — mutual regulation of adaptation, growth and development, 3 — regulation of LDL metabolism, 4 — regulation of 17-hydroxyprogesterone and sex hormone metabolism, 5 — regulation of 17-hydroxyprogesterone metabolism, 6 — regulation of HDL synthesis. Solid lines mark biosynthetic processes, and dotted lines mark regulation of biosynthetic processes.

The method of principal components revealed one common factor in 1-day old broiler, and there were two factors for each of periods from day 7 to day 23 and from day 23 to day 42 of life (see Table 2). The results we obtained and published data allow us to propose a generalized pattern of the studied hormone and metabolite interactions (Fig.). Here, the processes are divided into synthetic and regulatory ones, and their effects appear sequentially. At any current stage of development, a physiological and biochemical platform is formed (these processes we designate as adaptive; physiological effects I), which provides growth and development in the next stage (physiological effects II). Based on the data from the Table 2 and the scheme, the factors that were allocated for each of the studied

periods included the principal components which we mainly considered as adaptive and growth factors, or as an aggregate factor combining components with adaptive and growth functions (see Table 2, Fig.). Thus, in a day-old chicks, there was an integrative factor of metabolic and adaptive processes that included hormonal and metabolic components (see Table 2). This characterizes their significant relationship (r-Pearson: P_4 and Cortisol — $r = 0.69$, $p = 0.027$; P_4 and TCS — $r = 0.82$, $p = 0.004$; HDL and LDL — $r = 0.83$, $p = 0.003$; HDL and TCS — $r = -0.67$, $p = 0.033$) in the earliest postnatal period, when the metabolism is still not fully formed [3, 5, 6, 9], and reflects the strength in all functional systems during transition from prenatal to postnatal stage and also the impact of environmental factors [22]. This determines the homeostatic parameters that ensure de novo formation of structural and energy resources for subsequent growth and development (see Table 2, Fig.) [3, 9, 23]. On day 7, the broilers showed a factor (see Table 2, Fig.) which included progesterone and cortisol as principal components (r-Pearson: P_4 and Cortisol — $r = 0.73$, $p = 0.016$), and also a factor of cholesterol donation through active conversion of LDL cholesterol to progesterone and, therefore, the activation of endocrine metabolic pathway of 17-OHP and its products, the sex hormones, cortisol and other steroids [15, 16] (see Table 1, 2, Fig.). Progesterone (along with somatotropic and thyroid hormones) provides an increase in body weight, primarily due to the regulation of the synthesis of proteins (mainly the proteins of skeletal muscles and tissues of internal organs) [24]. Progesterone affects metabolism and increases its intensity in chicks. Cortisol directly participates in complex adaptive responses at molecular, membrane, cellular, tissue, and organ levels [10, 16]. Cortisol (see Fig.) also systemically strengthens or weakens adaptive responses, depending on the "expenses" for adaptation and the stage of ontogenesis, and regulates 17-OHP metabolism [25]. It is known that estrogens and other hormonal products of 17-hydroxyprogesterone normally promote utilization of LDL, enhance HDL synthesis, and are involved in synthesis of structural substances necessary for body growth and development [24, 26-28] (see Fig.).

At the age of 23 days, chickens showed two factors. One included HDL and 17-OHP, the other one comprises progesterone and cortisol (see Table 2), which agrees with the data of S. Rettenbacher et al. [29]. These authors showed that an increase in the glucocorticoid content due to stress or exogenous experimental impact (for example, glucocorticoid administration to intact chickens) can lead to a decrease in the conversion of progesterone to 17-OHP and its derivatives due to inhibition of specific enzymes (e.g. 17α-hydroxylase, 17,20-lyase, 17p-hydroxysteroid dehydrogenase) or competitive interaction of glucocorticoids with nuclear and membrane progesterone receptors involved in regulation of expression of the glucocorticoid-synthesizing enzymes [29]. At the same time, in incubated in vitro ovarian tissues of female chickens and testicles of males (i.e. without exogenous stress impact), glucocorticoids do not interfere with the progesterone transformation into 17-OHP and then into androstenedione, testosterone and other steroid products [29]. It was shown [30] that stress inhibits the enzyme responsible for the conversion of cholesterol to pregnenolone. Moreover, as it was noted by us (see Table 1), progesterone, like cortisol, had stable dynamics with a statistically unreliable difference in content from day 7 to day 42. The peaks of 17-OHP and body weight gain, vice versa, reliably occurred at the period from day 7 to day 23 (see Table 1). Achieving a balance of intensive adaptive and growth responses is based on high resource consumption, and may be the most apparent at the beginning of the formation of functional systems [3, 22, 31]. Thus, in the egg-type chickens, the greatest body weight gain was ob-

served in the decades II and III [8] that agrees with our data (see Table 1). The cholesterol donation detected at the age of 42 days (see Table 2) (r-Pearson: LDL and TCS — $r = 0.86$, $p = 0.002$) apparently provides an endocrine pool for the synthesis of progesterone and its derivatives [15, 16, 25] (see Table 1, 2, Fig.). It was reported [8] that in white Leghorn cross chicks the growth stabilized after 30 days of life. In this study, the principal components, revealed when the chicks aged 42 days (see Table 2), reflect stabilization and consolidation of adaptive and growth processes during the decade IV—the beginning of the decade V of the birds' life.

These complex interactions are aided by the hormones of the progesterone axis [14, 28], including glucocorticoids. There is a modulation of the lipoproteins metabolism and the implementation of their physiological effects [28] (see Fig.), particularly, the vascular ones. The events involve intracellular nuclear receptors [14] and the so-called extragenome integral membrane progesterone receptors [14, 15], involved in express responses not accompanied by gene transcription [14, 15], and, apparently, being necessary in growth and development.

Thus, in the whole, the data we obtained indicate that during the growth and development of chicks, the rate of cholesterol used for biosynthesis of steroid hormones changes. It affects the blood concentrations of lipoproteins and hormones, as well as their correlation. The age of chicks and, respectively, the nature of physiological processes in their body, including those mediated by progesterone, 17-hydroxyprogesterone and cortisol, determine formation of homeostasis which provides the development of resistance to the physiological states caused by technological stresses. The research may be continued to assess the role of these hormones and metabolites in determining the response patterns and the adaptive capabilities of broilers, and the body resources consumed for adaptation to the factors of intensive reproduction that, in turn, is related to the boundaries of the norm and pathology.

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AGE DYNAMICS OF PANCREAS SECRETORY FUNCTION AND INTESTINAL MICROBIOTA IN MEAT BROILER CHICKS AND THEIR PARENTAL LINES

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Abstract

The distinctive feature of avian digestion is high activities of the digestive enzymes. The digestion of poorly hydrolysable feed ingredients is known to be partially performed by microbial communities of cecum and large intestine (B. Svihus et al., 2013). The aim of our study was the investigation of embryonic and postembryonic enzymatic and microbial digestive processes in the intestine of meat-type chicken of parental lines B5 (Cornish), B9 (Plymouth Rock) and final hybrids (B59 Smena 8 cross) of Smena Selective-Genetic Centre (Russia) on ontogenesis (7- and 14-day old embryos, and 1-, 7-, 14-, 21-, 28-, and 35-day old chicks; 20 incubated eggs and 20 chicks per age in total; the experiment was carried out in the vivarium of All-Russian Research and Technological Poultry Institute). Activity of pancreatic enzymes (amylase and lipase) was detected in homogenates of the whole embryos (day 7 of incubation) and the embryonic intestinal and pancreatic tissues (day 14 of incubation). In day-old chicks high levels of pancreatic enzymes in the pancreas were found with no significant differences between parental lines and final hybrids in early postnatal period. According to the exocrine function of the pancreas, postnatal ontogenesis can be divided into two periods. In 1-14 day-old chicks the pancreas and its digestive function intensively develop, and the next period (from day 15 to day 35) is necessary to reach physiological maturity of the organ which becomes capable of enzyme production and secretion adequate to the diet. The activity of blood pancreatic amylase and proteases tended to decrease with age, and lowered significantly on day 35. The lipase activity followed the inverse trend and sinuously increased to day 35 ($P \leq 0.05$). The percentage of cellulolytic bacteria in intestinal microbiota reached its peak on day 14 without significant differences between the hybrids (50.79±1.84 %) and the parental lines (50.84±2.32 and 53.23±2.47 %). This percentage subsequently decreased by 60.0 % in the hybrids from day 14 (50.80±1.84 %) to day 35 (20.30±0.85 %), while in the parental lines there were sinuous variations throughout this period with 41.00±1.87 % and 44.80±2.27 % on day 35 in Plymouth Rock and Cornish, respectively. These data suggests a negative correlation between activity of pancreatic proteases and intestine cellulolytic bacteria. The highest r values were noted for *Clostridium* (–0.64, –0.83 and –0.64 for Cornish, Plymouth Rock and the hybrid chicks, respectively). The proportion of *Lactobacillales* that participate in feed fermentation positively correlated with the activity of amylase, lipase and protease in the hybrids ($r = 0.71$, $r = 0.56$, and $r = 0.83$) and in the Plymouth Rock line ($r = 0.60$, $r = 0.46$, and $r = 0.45$). A positive correlation was mostly found between the activity of pancreatic enzymes and the development of opportunistic and pathogenic microflora, i.e. *Enterobacteriaceae* (for amylase, lipase and proteases $r = 0.65$, $r = 0.59$, $r = 0.68$ in the hybrids, and $r = 0.34$, $r = 0.68$, $r = 0.64$ in the Cornishes, respectively), *Staphylococcus* (for protease $r = 0.46$ in the Cornishes, for amylase and proteases $r = 0.70$ and $r = 0.91$ in the Plymouth Rock line), *Campylobacterium* (for proteases $r = 0.86$ in the hybrids) and *Fusobacterium* (for amylase and proteases $r = 0.41$ and $r = 0.90$ in hybrids, for lipase and protease $r = 0.63$ and $r = 0.83$ in the Cornishes, for amylase and proteas-

es $r = 0.99$ and $r = 0.92$ in the Plymouth Rock line). Thus, the intensive development of individuals is due to the activity of digestive enzymes which is interrelated with the quantitative and qualitative composition of the intestinal microbiota. Regarding the analyzed indicators, embryogenesis and early post-embryonic periods should be considered crucial for chicken development.

Keywords: meat-type chicken, broiler chicks, Plymouth Rock, Cornish, exocrine pancreatic function, gut microbiota, pancreatic enzymes in blood serum

Effective feed cleavage for further assimilation of nutrients is one of the most important factors in livestock growing. Fundamental research of the digestive system is based on revealing the molecular and cellular mechanisms of its functioning, while being interrelated with other body systems. Deterioration of digestion and assimilation of nutrients may be a result of violation of physiological and biochemical processes caused by the problems with intestinal health that are related to feed quality and the state of the microbiota [1-3]. This has serious implications for commercial poultry farming, in particular, leads to deterioration in feed conversion, increases risk of diseases and reduces profitability [4, 5]. Moreover, recent changes in legislation in the EU countries on using antibiotics and feeding, as well as the genetic improvement of the poultry itself, require a deeper study of intestine function and health.

The results of studying preparations alternative to antibiotics show that the body weight gain in broilers can significantly increase via the effect on the composition of the intestinal microflora [6-10]. It is known that digestion in poultry occurs due to its own digestive enzyme, secreted by the digestive glands, and a certain role in the digestion of hardly hydrolysable feed components is played by the microflora in cecum and large intestine [11-13]. It is unlikely that digestive enzymes do not affect the composition of microflora in the poultry intestine. However, the available scholarly publications lack such data, especially the data considering the age dynamics and comparing the parent lines and hybrids on the parameters in question.

In this study, having examined the activity of pancreatic enzymes in ontogenesis in meat chicks and their hybrids, and having compared it with the quantitative composition of the intestinal microbiota, we established a correlation between these indices.

The aim of our work was to study embryonic and postembryonic enzymatic and microbial processes in the intestines of parental lines and hybrids of meat chicken.

Techniques. The experiments were carried out on the Cornish (line B5) and Plymouth Rock (line B9) chicken breeds and their hybrids (B59, the cross Smena 8) of the Smena Selective-Genetic Centre (Russia).

During eggs incubation (units Stimul-1000, Stimul-Ink, Russia), embryos aged 7 days and digestive system of the embryos aged 14 days were examined in the groups (hybrids, maternal and paternal lines). For each variant, 10 eggs from each group were used. Experiments were conducted in two replicates. The embryo samples were homogenized with a cooled Ringer's solution at a ratio of 1:10 and centrifuged at 5000 rpm for 5 minutes. Enzymatic activity was determined by Smith-Roy-Ugolev method using an automatic biochemical analyzer Hemwell T2900 (Awareness Technology, Inc., USA) with Pancrease-amylase liquicolor and Lipase liquicolor kits (HUMAN GmbH, Germany) according to the manufacturers' instructions.

The development of pancreatic exocrine function during ontogenesis was studied in the experimental farm conditions (All-Russian Research and Technological Poultry Institute, the feeding and housing schedules met the zootechnic requirements). In each age period (1, 7, 14, 21, 28 and 35 days of life), 10 chickens were used. Experiments were conducted in two replicates. Blood for analysis was taken after slaughter by decapitation. Sodium citrate was added to the sam-

ples, and they were centrifuged at 5000 rpm for 5 minutes. The abdomen wall was dissected and the pancreas was removed, weighed and ground with cooled Ringer's solution. The homogenate was centrifuged at 1200 rpm for 3 minutes. The appropriate supernatant dilution was used for biochemical analysis. The intestine was tied, taken out and exposed to deep freezing for subsequent microbiological assay. The intestinal content samples for molecular genetic analysis were collected simultaneously with those collected for biochemical studies, as established by corresponding requirements [14]. In pancreatic tissue, the amylase activity was determined by starch cleavage (Smith-Roy-Ugolev method); protease activity was determined colorimetrically by a decrease in casein concentration [15]; lipase was measured with the use of a semi-automatic biochemical analyzer BS3000P (SINNOWA, PRC) with an appropriate kit of diagnostic reagents (DIAKON-VET, Russia). The blood amylase and lipase activities were examined with a Hemwell 2900 (T) biochemical analyzer (Awareness Technology, Inc., USA) using required kits (HUMAN GmbH, Germany). Protease activity was studied on a biochemical analyzer BS3000P (SINNOWA, PRC) with N-benzoyl-DL-arginine-p-nitranilide (BAPNA) as a substrate [16].

Total DNA was extracted using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to manufacturer's recommendations. DNA amplification was performed on a Verity device (Life Technologies, Inc., USA) using eubacterial primers 63F (3′-CAGGC-CTAACACATGCAAGTC-5′) labeled at the 5′-end (D4 WellRed fluorophore) and 1492R (3′-TACGGHTACC-TTGTTACGACTT-5′), which allow amplifying fragment of the 16S rRNA gene at positions from 63 to 1492 (numeration is indicated for the *Escherichia coli* 16S rRNA gene). The amplification protocol was as follows: 3 min at 95 °C (1 cycle); 30 s at 95 °C, 40 s at 55 °C, 60 s at 72 °C (35 cycles); 5 min at 72 °C. The fluorescently labeled amplicons of the 16S rRNA gene were purified with 3 M guanidine isothiocyanate. The final concentration of total DNA was determined with a Qubit fluorimeter (Invitrogen, Inc., USA) using the Qubit dsDNA BR Assay Kit (Invitrogen, Inc., USA) according to the manufacturer's recommendations. Restriction of 30-50 ng DNA with endonuclease HaeIII, HhaI and MspI (Fermentas, Lithuania) was carried out for 2 hours at 37 °C. Restricts were precipitated with ethanol, then 0.2 μl of Size Standart-600 molecular weight marker (Beckman Coulter, USA) and 10 μl of Sample Loading Solution formamide (Beckman Coulter, USA) were added. The samples were analyzed using a CEQ 8000 device (error 5 % max, Beckman Coulter, USA).

The height of peaks and their areas were calculated in the Fragment Analysis software (Beckman Coulter, USA). Based on the calculation, the subtypes (phylotypes) with a one nucleotide error accepted for this study were distinguished and their relative amount in the microbial community was evaluated. Phylogenetic status of bacteria was determined using Fragment Sorter software (http://www.oardc.ohiostate.edu/trflpfragsort/in-dex.php).

Real-time PCR to estimate the total bacterial abundance was performed using a DT Lite-4 amplifier (OOO NPO DNA Technology, Russia) with the Reagents for RT-PCR in the presence of intercalating EVA Green stain Kit (ZAO Syntol, Russia) and Eub338/Eub518 (5′-ACTCCTACGGGAGGCAGCAG-3′ and 5′-ATTACCGCGG-CTGCTGG-3′) primers, in the following regime: 3 min at 95 °C; 13 s at 95 °C, 13 s at 57 °C, 30 s at 72 °C (40 cycles).

The average (X) and standard errors of the mean (±SEM) were calculated for the enzyme activity indices. The reliability of the differences was determined by the Student's t-test, considering them significant when $p \leq 0.05$. To explain the cause and effect between the factor and performance traits, the Pearson correla-

tion coefficients were calculated, assuming them high at $r \geq 0.5$. Mathematical and statistical processing, as well as calculation of Pearson correlation coefficients, was performed in Past software (http://folk.uio.no/ohammer/past/).

Results. The hybrids showed the amylolytic activity in the week-old embryo homogenate 8-19 times higher than that of the maternal line and 16-22 times higher than that of the paternal line. Lipolytic activity was 28.5 % and 25.1 % higher than that in the maternal and paternal lines, respectively. Proteolytic activity was not observed in 7-week old embryos. Given the development of the intestine and pancreas in embryos begins since day 3 to day 4, the presence of amylolytic and lipolytic activity in embryonic tissues was quite understandable. In eggs, there is a small amount of monosaccharides, and most proteins are present in the form of glycoproteids. Probably, as a result of the degradation of these proteins during embryogenesis, the released carbohydrates monomers are used for the glycosilation of newly synthesized polypeptides. On day 14 of incubation, the amylase activity in hybrids was 1.4-2.0 times higher compared to the parental lines. The lipase showed the opposite trend: the hybrids had almost twice lower activity than that of parental lines. At this stage, the embryos already have functioning digestion, and the results indicate a more intensive fat hydrolysis in the Plymouth Rocks and Cornishes intestines, if compared to their hybrids. Hence, this data indicates that the amylase activity in hybrid embryos, which afterwards have a high growth rate, is greater than that of their parents. This indicator may serve as a criterion for assessing the development of embryos and poultry performance in the future.

1. Age-related changes in enzyme activity of pancreatic tissue in hybrids and parental lines of poultry of different breeds (X±SEM, experimental farm)

Group	Age, days					
	1	7	14	21	28	35
	Amylase, mg/(g·min)					
Hybrids	14040±828.3	12287±1017.3	18440±443.8	13800±2057.2	15160±1254.7	17343±617.5
Plymouth Rock	13080±1149.2	8712±628.5*	14075±1205.0	11500±538.1	15480±1234.0	16320±1120.0
Cornish	14200±1225.2	10780±1248.2	13300±1116.1	9440±401.3	10680±2670.7	14840±1594.3
	Proteinases, mg/(g·min)					
Hybrids	323±30.7	200±12.1	317±48.9	278±30.1*	325±46.9	487±43.2
Plymouth Rock	314±21.1	125±15.7	246±32.2	192±50.0	206±22.6	402±32.2
Cornish	261±36.1	126±14.1	175±26.6	217±68.6	226±24.7	403±87.4
	Lipase, EU/l					
Hybrids	31288±4401.7	90489±6648.5*	99882±4621.7*	107645±6196.0*	83430±7873.0*	105753±4095.5*
Plymouth Rock	38582±4391.7	67074±6735.0*	81209±7965.0*	95390±8985.0*	88906±10569.3*	85940±7808.0*
Cornish	39670±5551.0	64255±7044.3*	92307±4371.3*	94250±8300.0*	70135±3634.0*	88039±11537.5*

Note. In each group, 20 samples were tested.
* Differences with a day old chicks are statistically significant at $P \leq 0.05$.

Analysis of pancreatic tissue homogenates in a day-old chicks revealed significant amylolytic, lipolytic and proteolytic activity, but there were no significant differences in these parameters in different groups (Table 1). In course of ontogenesis, the exocrine action of the pancreas develops unevenly [17]. During the first weeks of postembryonic development, chicks adapt to new living conditions, and formation of the digestive system occurs. Therefore, there are differences in the growth rates of chickens of different groups (hybrids, the Cornishes and the Plymouth Rocks) [18, 19].

When the chicks aged 14 days, the amylase activity in the pancreatic tissue of the hybrids was 31.3 % higher ($P \leq 0.05$) compared to a day old chicks, but did not change significantly in the parental lines (see Table 1). Proteinase activity after a decrease at the age of 7 days (by 38.1 % in hybrids, and by 60.2 and 57.1 %, respectively, in the chicks of the maternal and paternal lines, $P \leq 0.05$) increased by day 14, reaching the values recorded in the first day of life. Lypolytic activity tended to increase, and in 14-day old chicks it significantly ($P \leq 0.05$) ex-

ceeded the indices in day-old chicks, being 3.2 times higher in the hybrids, 2.1 times higher in the maternal line, and 2.3 times higher in the paternal line. Comparative analysis shows that at the age of 14 days the amylolytic activity in the hybrids was significantly ahead of that in the parental lines (by 23.7 % for the maternal line, and by 27.9 % for the paternal line). Taking into account that amylase is involved in the hydrolysis of carbohydrates and determines the energy balance in the body, the intensity of metabolic processes in hybrids in this period is significantly higher than that of peers from the parental lines. The 2-week old chicks did not show significant differences between groups in lipase activity, whereas pancreatic proteases were much more active in hybrids than in parental lines (paternal score was lower by 44.8 %, $P \leq 0.05$, and maternal score was lower by 22.4 %). The difference in the hydrolysis of feed protein was due to the unequal nutritional value of diets for hybrids and parental lines (in broiler chickens, the content of crude protein in feed was 22.0 %, and in parental lines it did not exceed 19.0 %). The difference in proteolytic activity in pancreatic tissue in chicks from different groups indicated the features determined by the genetic potential and the adaptation of pancreatic secretion to feed quality. Thus, by age of two weeks, the exocrine function of the pancreas in meat-type chickens clearly adapts to the quality of the feed taken. This fact indicates the onset of the gland physiological maturity, since according to functional criteria (including neurohumoral regulation), the organ corresponds to that of adult chickens.

The second period (days 15-35) was characterized by a decrease in proteinase activity in 21-day old age compared to the figures recorded at a day old age, by 14 % in hybrids ($P \leq 0.05$) and by 38.9 % in chicks of the maternal line with a subsequent growth up to 35-day old age. The 35-day old hybrid chicks exceeded a day old ones by 23.5 % ($P \leq 0.05$) for amylase activity, by 50.8 %f or proteinase activity, and by 238.0 % for lipase activity. Between the groups at the age of 35 days, no significant differences in the activity of the enzymes were found, except the lipase. Its activity in the maternal line chicks was 18.7 % lower ($P \leq 0.05$) than that in the hybrids.

The role of pancreas is not limited to digestion, but includes endocrine function, i.e. production of hormones (insulin, glucagon, and somatostatin). Pancreas is also involved in metabolic control due to the presence of pancreatic enzymes in blood [20, 21]. Therefore, we studied their activity in blood in growing hybrids and chicks of the original lines (Table 2).

2. Age-related changes in blood pancreatic enzyme activity in hybrids and parental lines of poultry of different breeds (X±SEM, experimental farm)

Group	Age, days					
	1	7	14	21	28	35
			Amylase, EU/l			
Hybrids	671±50.5	1001±21.4*	1296±358.3*	525±95.0	516±86.7	298±28.2*
Plymouth Rock	929±92.9	926±107.9	832±136.3	445±103.7*	642±82.3*	605±86.3*
Cornish	827±132.9	704±66.3	583±56.6*	382±39.0*	564±124.0	510±71.3*
			Proteinases, EU/l			
Hybrids	14±1.3	15±1.5	11±0.5	10±3.6	13±1.6	8±1.6*
Plymouth Rock	14±1.4	13±0.9	13±1.1	6±0.7*	10±1.1	9±0.7*
Cornish	15±0.8	12±0.7	11±0.6	7±0.1	8±0.8	13±1.4
			Lipase, EU/l			
Hybrids	14±0.3	23±1.5*	16±1.7	18±2.2	10±1.4	20±2.8*
Plymouth Rock	15±0.9	23±1.5*	21±2.3*	14±1.3	11±0.6	18±1.1
Cornish	15±0.8	25±1.7*	20±3.7	15±0.5	10±1.0*	22±2.2*

Note. In each group, 20 samples were tested.
* Differences with a day old chicks are statistically significant at $P \leq 0.05$.

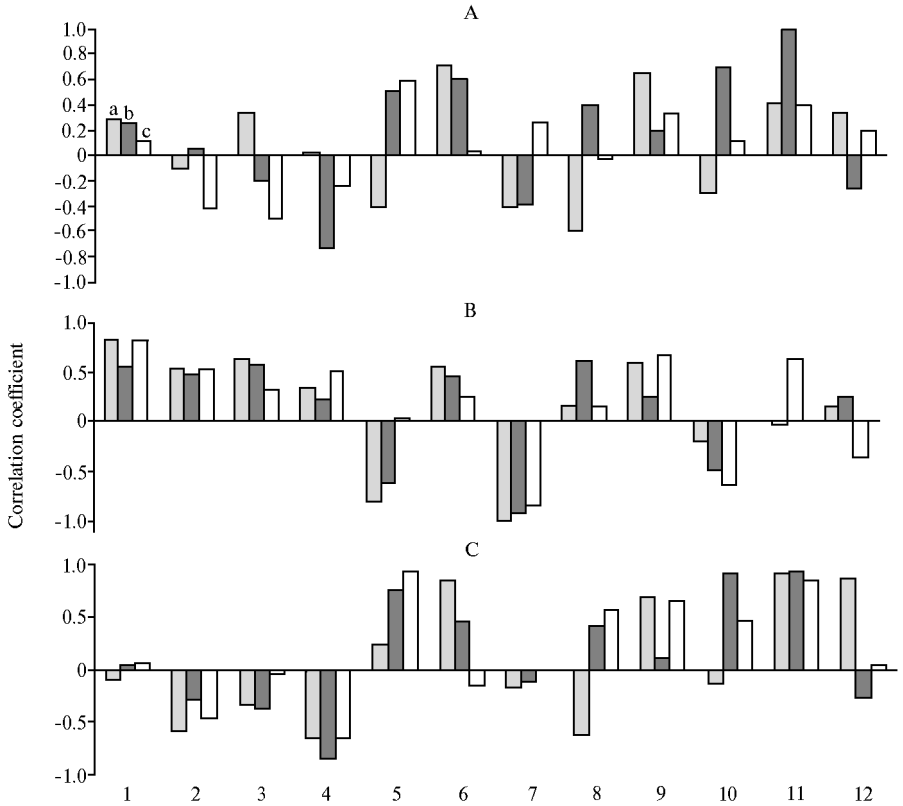
In the initial period of postembryonic development in hybrids, an increase in amylase activity by 49.1 % and 93.1 % was recorded on day 17 and day 14, respectively. Chicks of the parental lines did not show any significant chang-

es in blood amylase at that time, and its activity remained high until the age of 14 days. This may be due to the intensive growth of the pancreas and the deficit of newly synthesized enzymes, thus, their transcytosis occurred from blood to the acinar cells of the gland to meet the growing body requirement in digestive enzymes. From the 21-day-old age and until the end of the growing period, the blood amylase activity decreased, becoming by the day 35 more than 2 times lower than that of a day old chicks. In the parental lines, the age-related dynamics of amylase activity was different: by the three week age, it declined, and then elevated by the age of 35 days. The proteolytic enzymes showed an undulating decrease in activity: at 35 days old age, the index was 42.9 % lower for the hybrids, 35.7 % lower for the Plymouth Rocks, and 28.6 % lower for the Cornishes, if compared to that of one-day old chicks. This can be explained by the involvement of blood proteases in the regulation of blood pressure. With advancing age, this indicator becomes higher, and the intensity of assimilation in the body vice versa decreases. There was a negative correlation in hybrids found between the activities of proteases in pancreatic tissue and in blood ($r = -0.75$).

In the first 2 weeks of life, the intensive development of the intestinal microflora occurs [22]. According to our data, the count of cellulosolytic bacteria in meat-type chicks reached high values by the age of 14 days, the chicks of the parent lines and hybrids at this age did not show significant differences. By the age of 21 days, the hybrids had a decrease in the ratio of cellulolytic microorganisms by 26.4 % (from 50.8±1.84 to 37.4±1.25 %). A similar trend was observed in Cornish chicks (a decrease by 32.0 %, from 53.2±2.47 to 36.2±1.57 %). In the hybrids, the number of cellulolytic bacteria decreased with advancing age, and at the age of 35 days they amounted to 20.3±0.85 % of the count. In the chicks of the parent lines, an undulating change in the amount of cellulolytic bacteria occurred in course of growing up: at the age of 35 days, 41.0±1.87 % of these bacteria was recorded in the Plymouth Rocks, and 44.8±2.27 % in the Cornishes. Therefore, the state of the intestinal microflora depends on many factors, moreover, nutrition and diet do not always play a major part.

The interaction of digestive enzymes with microbiota of the intestine is of particular relevance, since a large number of biological additives are used to stimulate digestion. It is known that the compounds that are not digested in the gastrointestinal tract become food for intestinal microorganisms, and also undergo oxidative deamination and other catabolic processes in liver. It leads to a decrease in the efficiency of food use and the accumulation of intermediate toxic byproducts in the body [23]. The calculation of Pearson linear correlation coefficients between the count of microorganisms and the enzyme activity indices allows us to establish direct relationships between the variables by their absolute values. This work studied such correlations for tissue organ homogenate (Fig.) and blood (data not shown). The resulting coefficients reflected the negative interdependence between the activity of the proteolytic enzymes of the pancreas and the number of cellulolytic bacteria, which indicates a competitive relationship between digestive enzymes and microorganisms. The reason is that the microflora is involved in the final part of the digestive processes (inactivation of intestinal and a number of pancreatic enzymes) and performs a protective function, preventing the development of pathogens in the intestine. The number of lactobacilli required for feed fermentation positively correlated with the activity of all pancreatic enzymes in hybrids, as well as with amylases and proteases in the parental lines. Hybrids had negative correlation between the activity of pancreatic proteases and the presence of Clostridia microorganisms ($r = -0.76$). This negative correlation may be explained by the fact that the state of commensal

microflora largely depends on the hydrolysis of the protein in the intestine: the worse the protein is digested, the more favorable the conditions (due to putrefactive processes) for pathogenic microorganisms and the development of inflammatory processes are.



Pearson correlation coefficients between the presence of different groups of bacteria in cecum and the activity of pancreatic amylase (A)**, lipase** (B) **and proteases** (C) **in liver tissue homogenate in hybrid broiler chicks** (a) **and their parental breed lines Plymouth Rock** (b) **and Cornish** (c) **at the age of 35 days:** 1 — family *Eubacteriaceae*, 2 — family *Ruminococcaceae*, 3 — family *Lachnospiraceae*, 4 — family *Clostridiaceae*, 5 — phylum *Bacteroidetes*, 6 — order *Lactobacillales*, 7 — order *Bacillaceae*, 8 — order *Selenomonadales*, 9 — family *Enterobacteriaceae*, 10 — *Staphylococcus* sp., 11 — *Fusobacterium* sp., 12 — *Campylobacterium* sp. (experimental farm).

Commensal microorganisms also include enterobacteria. However, in this case, there was a positive correlation of their count and activity of all pancreatic enzymes in hybrids and chicks of the Cornish breed. A similar trend was noted for pathogenic bacteria of the genera *Staphylococcus*, *Campylobacter*, *Fusobacterium*, many of which serve as causative agents of infectious diseases in poultry. Consequently, the microbes of these groups participate in digestion in the intestine, and their count is due to the activity of pancreatic enzymes. In studies on fistulled pigs, the results indicated an increase in the amount of *E. coli* after feed intake [24].

So, in meat-type hens during the embryonic period, hybrids have more developed digestion than the parental lines, that is manifested by the presence of amylolytic and lipolytic activities in the embryonic homogenates, and in the intestine and pancreas homogenates. At the age of 1 day, the activity of enzymes in the pancreas is high and does not differ significantly between hybrids and parental lines. The physiological maturation of the pancreas in chicks ends by day 14 of life, and in hybrids it occurs in shorter time. The highest correlation between the activity of proteolytic enzymes of the pancreas and the number of cel-

lulolytic bacteria was noted for the Clostridia microorganisms. In hybrids and the Plymouth Rocks, the number of lactobacilli positively correlates with the activity of all pancreatic enzymes. The development of commensal and pathogenic microflora in hybrids and chicks of the paternal and maternal lines also positively correlates with the activity of pancreatic enzymes. Therefore, during the embryogenesis, the intensive development of individuals is determined by the activity of digestive enzymes which is higher in hybrids, and during the post-embryogenesis it is due to its correlation to the composition of the intestinal microbiota.

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BROODINESS INSTINCT IN FARM POULTRY: REALIZATION AND METHODS OF SUPPRESSION

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Abstract

Many hens of gene pool populations along with their breed features are characterized by the manifestation of such a trait as broodiness which has been lost in commercial lines and breeds. This trait has some negative impact at commercial farming, because during the nesting period the hens stop egg laying that leads to decreasing in egg performance and the number of progeny. Predisposition to the broodiness is an inherited trait and its eradication can be achieved by use of selection methods. But elimination of the families with high levels of nesting will lead to the destroying of the genetic structure in the gene pool population. Hormonal methods and technological shocks (electric current, high-intensity light and transfer to the new environmental conditions) may also be used to suppress broodiness instinct. But some of them could be not available technically and other ones should not be applicable ethically. In our research, the Chinese silky chickens (*Gallus gallus*) from the gene pool collection of All-Russian Research Institute of Farm Animal Genetics and Breeding which are characterized by highly developed broodiness served as a model. In individual cage batteries the occurrence of broodiness instinct resulted in specific behavioral reactions and refusing in the majority of cocks to give sperm to massage. In the group keeping of birds on the floor, the presence of cocks to some extent stimulated the hens to lay eggs. But during the entire productive period about 70-75 % of hens demonstrated nesting behavior and did not lay eggs. Most part of cocks also showed specific behavior and did not mate with hens. This led not only to the low yield of hatching eggs from a pen, but also to the loss of a large number of genotypes of both hens and cocks in the reproduction of the flock. In order to suppress the broodiness instinct in Chinese silky hens, contained in individual cages, we used injection of native mixed sperm of cocks into the oviduct or cloaca of the hens. The insemination of the hens (three inseminations with 4-day intervals) started four weeks prior to the breeding season. During two experiments about 70 % of all hens responded to the stimulation by restarting of oviposition or increasing of egg performance. The frequency of occurrence of individuals with a high laying intensity (51-60 % and higher) increased, while frequency of hens with low egg laying rate went down. This method of broodiness suppression is more preferable and more physiologically acceptable in comparison to the traditional shock methods, but with the same efficiency.

Keywords: broodiness, artificial insemination, gene pool preservation, egg performance

One of the important tasks of modern poultry farming is the preservation of gene pool breeds. Programs for the conservation of genetic resources of local, rare and endangered species operate in many countries. Gene pool breeds not only serve as a reservoir of potentially valuable alleles and genes for breeding, but also have a number of advantages over commercial poultry: high viability in an extensive breeding system without strict vaccination regulations; high taste and nutritional properties of meat and eggs; an anything goes attitude to the diet and the ability to assimilate local poultry feed production; attractive coloring of plumage, etc. [1, 2].

Many gene pool breeds, along with other features, possess such a property

as an instinct of broodiness lost by the commercial poultry. In modern conditions, due to the widespread distribution of artificial incubation, this sign has lost its practical importance for the reproduction and extension of the species, being more likely a factor that damages commercial poultry farming. This is especially true for poultry with low egg productivity, as during broodiness, hens stop egg laying, which significantly reduces the yield of commercial and hatching eggs and, accordingly, the chickens for replacement stock. However, there is evidence that within the breed, a poultry inclined to broodiness has a higher vitality and survival compared to species that have lost such an instinct.

Predisposition to broodiness is a hereditary trait. Thus, modern breeds of chickens of Mediterranean origin (egg type) almost do not show the instinct of broodiness. However, among chickens of Asian origin (meat type), brooding hens are often found. The fact that in equal conditions in some breeds (Rhode Island, Russian White) this instinct is almost completely lost and almost does not manifest itself but in others (Chinese silky, Bentam) is preserved to some extent, also points to its hereditary nature. In early genetic studies, it was suggested that there are factors in the sex Z chromosome of the hen that determine the behavior of the female broodiness [3, 4]. So, back in 1930, D.C. Warren [3], carrying out reciprocal crosses of the White Leghorn breed, with a weak instinct of broodiness (in 5 % of hens), with the Rhode Island Red breed, in which this instinct is observed in 83 % of hens, reported sex-linked heritability of the studied trait. At the same time similar results were obtained by E. Roberts and L. Card [4]. However, when testing the hypothesis by hybridologic analysis in special crosses between White Leghorn chickens without the instinct of broodiness and Bentam chickens that hatch eggs, it was showed that the broodiness behavior is apparently not controlled by one main gene (or several genes) in the Z chromosome. If such a gene exists, it participates in activation of the broodiness process in addition to two interacting autosomal genes, one of which represses the other [5-7].

As is known, hypothalamus, pituitary gland and genital glands are involved in regulation of poultry sexual function. Under appropriate conditions, a prolactin-releasing factor is formed in the hypothalamus of females. Under its influence in the anterior lobe of the pituitary gland the main hormone of broodiness, prolactin, is produced. In the broodiness period, in the pituitary gland of females the basophilic cells disappear, and oxyphilic cells appear in large quantities (lactotropocytes) which are even figuratively called “broodiness cells”. The injection of prolactin or the blood of hatching chickens to males and egg-laying hens leads to the development of a characteristic broodiness behavior [8]. Prolactin suppresses the secretion of gonadotropins in female poultry, i.e. follicle-stimulating and luteinizing hormones, and also has a depressing effect on the secretion of sex hormones, reduces the steroid activity of the ovary. Therefore, during the broodiness and young-stock breeding, sexual behavior is absent, and the hens do not mate [9-12].

A 24 bp insertion (nucleotides in positions 377-354) was found in the promoter region of the prolactin gene (–358 PRL, Chr2, GenBank AB011438). The presence of the insertion has been studied in detail by various research groups, and it has been found that it positively correlates with the rate of oviposition in poultry and negatively correlates with the instinct of broodiness. The individuals with heterozygous genotype In/Del have the highest prolactin mRNA level [13-16]. Genetic changes in the prolactin-receptor prolactin regulation system, which lead to inhibition of their activity, can be used as genetic markers for rearing hens with a decreased instinct of broodiness and, as a consequence, with increased egg productivity [17-19].

Thus, using selective genetic methods, it is possible to rid the population of individuals with a pronounced instinct of broodiness. In commercial poultry farming, in particular, in the turkey farming, in which this instinct still manifests to a larger extent, such methods are widely used along with hormonal [20-22] and technological approaches, involving various types of shock effects on the poultry (electric current, high intensity light, transfer to the new environmental conditions), that is not always possible for technical and ethical reasons [23, 24]. In small gene pool breeds, the elimination of families that show the instinct of broodiness will inevitably lead to a narrowing genetic diversity, a disturbance of the breed genetic structure and will increase breed extinction risk. Therefore, other methods are needed for formation control of the instinct of broodiness that do not adversely affect the breed genetic structure and are easy to apply.

In this paper, we are the first to propose the use of natural biological mechanisms that allow the activation of ovulation and egg formation in laying hens due to a special factor contained in the male sperm, in order to suppress the instinct of hatching. We have not found reports about such experiments on poultry in the world's scholarly publications.

The purpose of the research was to develop a technique that would suppress the instinct of broodiness in hens of the gene pool breeds to stimulate the process of ovulation and successful reproduction.

Techniques. Chinese silky chickens (*Gallus gallus*) were chosen as a model. Farm experiments were carried out in 2015-2016 (experiment I) and in 2016-2017 (experiment II) (Genetic Collection of Rare and Endangered Chicken Breeds, the Genofond Branch VNIIGRZh, Leningrad Province). The age of the poultry was 24-45 and 28-53 weeks (the difference in the chicken age by years is due to differences in technological schedules in the farm). The poultry of the experimental groups (n = 17 in experiment I and n = 25 in experiment II) were kept in individual cages up to weeks 56-60 of life with artificial insemination and individual daily egg-laying record. Broodiness instinct was controlled and stimulation was performed. In the control groups (n = 25 in experiment I, n = 17 in experiment II) chickens up to the same age were kept in flooring sections together with the cocks for natural mating. The group productivity was recorded daily, sand the hatching hens were under control. Feeding and housing technologies were as generally accepted for gene pool breeds. There were no differences in the origin of the poultry between the groups. The laying hens were fed with a complete fodder PK-1-1 (16.4 % of crude protein, 258 kcal/100 g of metabolic energy). After the end of the poultry use, the data was summarized to determine the final productivity of the hens.

Intensity of egg laying (I, %) was calculating as $I = (B \times 100)/(D \times P)$, with B as the total number of laid eggs over a period, D as the number of days in the period, and P as the number of laying hens in the group.

To suppress the instinct of broodiness and to stimulate ovulation, native mixed sperm of cocks, obtained by massage, was injected into the oviduct or cloaca (in the case of insemination when the oviduct cannot be removed) at a dose of 0.05 cm^3 [26, 27]. The insemination of chickens began 4 weeks before the breeding season (3 inseminations with a 4-day interval). The influence of stimulation was traced in dynamics, for which the count of the number of laid eggs and the intensity of egg laying were estimated in three conventional time intervals, i.e. i) from the beginning of the accounting period of oviposition to insemination (44-67 days, depending on the technological schedule); ii) during the insemination period (4 weeks before the breeding season and during the breeding season), and also within 3 weeks after it, as the sperm of the cocks can remain in the genital tracts of the hens within 3 weeks without losing fertility [26] (62-

74 days); iii) 3 weeks after the final insemination and until the end of the accounting period (35-46 days).

In the statistical processing of the intensity of oviposition per group, with regard to the period of the experimental treatment, the mean (*M*) and standard error of the mean (±SEM) were calculated. The significance of differences was evaluated by Student's *t*-test. The differences were considered statistically significant at $P < 0.05$.

Results. The theoretical background for the study was the known data that in animals, for which induced ovulation is characteristic, the corresponding mechanism is triggered by a special factor contained in the sperm of males, the protein β-NGF (β-growth factor of nerve tissue) which can be found in almost all mammals, including humans. It has been established that β-NGF provokes ovulation in many mammalian species that are characterized by induced ovulation. In animals with regular ovulation, β-NGF does not induce it, but has a positive effect on the fertility of the female and behaves like a female sex hormone [25].

In the available publications, information on similar studies carried out on poultry was not found, but it was suggested that such a mechanism belongs to general biological ones, including birds.

The Chinese silky breed used as a model refers to the decorative one. The weight is 1.0-1.4 kg for the hens, and 1.2-1.7 kg for the cocks. Hens can lay up to 80 light brown eggs per year weighing 40-41 g each. The choice of this breed for research is due to their strong instinct of broodiness. The hens of this breed are often used for hatching quail and pheasant eggs and taking care of the young poultry. We do not consider it permissible to eradicate the instinct of broodiness, as a breed specific feature, in this breed. However, it is necessary to suppress this instinct for a certain period of time to successfully reproduce the population and preserve genetic diversity in the breed.

In our experiments, due to the battery cage for housing used, the poultry did not contact the litter and the laid eggs, which to some extent hindered the instinct of broodiness, but did not completely suppress it, and the behavior characteristic of the early stages of natural hatching is still developed (clucking, appearing of brood-patches on the chest and abdomen). In the Chinese silky breed poultry, even under cage housing, there was a mass manifestation of the broodiness instinct, which additionally led to characteristic behavioral response in a large number of cocks who at that time refused to give sperm to massage.

It was found that the intensity of oviposition varied within 30-39 % in the control group when flooring housing used. Apparently, the presence of cocks to some extent stimulated chickens to oviposition, however, during the entire productive period, about 70-75 % of the population actively hatched and did not lay eggs. Most cocks also showed characteristic behavior, being in nests with hens and not mating. This led not only to a low yield of hatching eggs from the poultry housed in the sections, but also to the loss of a large number of genotypes of both hens and cocks during flock reproduction, which is critical for the preservation of gene pool in breeds with low egg-laying. For good reproduction of such populations, commercial breeders are forced to store additional egg set formed during breeding season, which is economically, veterinaryly and zootechnically inexpedient. Moreover, there is no guarantee that additional egg sets will reproduce all genotypes and preserve the genetic structure of the breed.

Obviously, the presence of cocks in the flock did not sufficiently stimulate the hens to oviposition and did not diminish the manifestations of broodiness. For that matter, we have used the method developed by us to stimulate ovulation by introducing the mixed native sperm of cocks into the chicken oviduct.

Stimulation started 4 weeks before the beginning of the breeding season established in the farm, so that hens could adapt to the restoration of egg laying; at the beginning of the breeding season in the genital tracts of the female there were no viable spermatozoa, and hens were ready for a monosperm insemination by certain cocks.

Egg laying in Chinese silky breed chicken (*Gallus gallus*) hens depending on the response to stimulation of ovulation by native cock sperm during different periods (*M*±SEM, Genofond VNIIGRZh, 2015-2017, on-farm experiments)

Parameter	Egg-laying intensity, %		
	prior to insemination	at insemination and during 3 weeks after	3-week post insemination period
Experiment I			
Accounting period	44 сут	62 сут	46 сут
Whole group (17 birds)	39.0±3.3	59.0±3.3	42.0±2.0
Responded hens (12 birds, 71 %)	33.0±2.5[a]	66.0±3.2[b]	51.0±2.7[c]
Non-responded hens (5 birds, 29 %)	52.0±1.6[d]	44.0±2.6[e]	22.0±2.2[f]
Control I (25 birds)	35.0±2.3[a]	42.0±2.4[a]	39.0±2.4[a]
Experiment II			
Accounting period	67 сут	74 сут	35 сут
Whole group (25 birds)	33.0±2.2	42.0±2.5	37.0±2.0
Responded hens (17 birds, 68 %)	31.0±2.7[a]	47.0±2.7[b]	40.0±2.6[c]
Non-responded hens (8 birds, 32 %)	36.0±4.2	32.0±4.5[d]	30.0±3.0[a]
Control II (17 birds)	31.0±2.1	33.0±2.3[a]	36.0±2.1

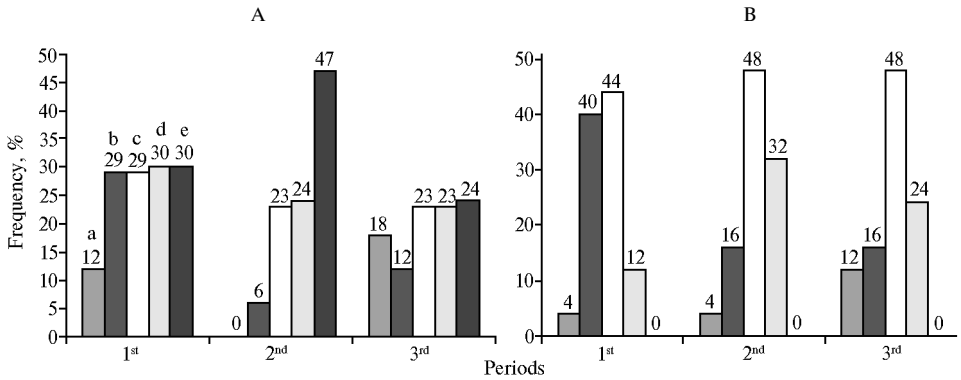
In the experiment I, differences are statistically significant at $P < 0.001$ for [ab, ac, ad, be, df, ef, cf], at $P < 0.01$ for [bc], and at $P < 0.05$ for [de]. In the experiment II, differences are statistically significant at $P < 0.001$ for [ab], at $P < 0.01$ for [bd], and at $P < 0.05$ for [ac].

Stimulation of restored oviposition or increase in its intensity, as influenced by insemination, resulted in a positive effect in more than $^2/_3$ of the population. So, 71 and 68 % of hens responded to stimulation in the experiment I and experiment II, respectively (Table). Basically, there were those hens whose instinct of broodiness is clearly pronounced and the periods of alternation of oviposition and broodiness are clearly traced. The other chickens did not respond to artificial insemination with native mixed sperm of cocks. Since the beginning of the first egg laying and until the end of commercial use, these hens showed almost no tendency to hatch and had a smooth curve of egg laying.

In the experiment I, the oviposition has doubled in responding hens after insemination, from 33 to 66 %; during the third period, a regular decline in egg-laying began. But, as we believe, due to the prolonged effect of stimulation, the decline was quite smooth, from 66 to 51 %. At the same time, the egg laying reduced from 52 to 22 % (see Table) for the same 15 weeks in hens which did not respond to stimulation and showed the peak of egg laying intensity at the beginning of the survey. The results of the experiment II confirmed the regularity observed in the experiment I, i.e. a significant increase in the intensity of oviposition with a smooth decrease in the hens, responded to stimulation, and a steady decrease in egg laying in non-responded hens (see Table). The mixed sperm was used in the stimulation experiments to exclude the individual immunological incompatibility of hens and cocks and to level differences in the quality of sperm form different cocks.

As a result of both experiments, it was also found that insemination of hens led to an increase in the frequency of individuals with high oviposition. In the experiment I, before stimulation, the hens with egg production of 0-10 % averaged 12 % of the total population, reaching 29 % for 11-30 % egg laying and 30 % for 61-80 % egg laying. After insemination, the number of hatching hens with egg laying intensity of 61-80 % increased up to 47 % (+17 %), whereas the frequency of those with 11-30 % egg laying decreased by half, to 6 %, and no hens with egg laying of 0-10 % were observed (Fig., A). After 3 weeks from

the last insemination, the frequency of hens with a rather high productivity for the breed decreased again. The number of hens with 0-10 % egg laying increased to 18 %, the individuals with 11-30 % egg laying reached the initial level of 12 % observed in the population, and the ammount of hens with 61-80 % egg laying declined by half, to 24 %. At the same time, the portion of hens with an average productivity index of 31-60 % remained unchanged. In the experiment II, the frequency of hens with 0-10 % egg laying remained within 4 % of the population both before and after insemination. For 11-30 % egg laying, the hen number decreased from 40 to 16 % after insemination and remained the same 3 weeks after. The number of hens with 31-50 % egg production increased insignificantly (+4 %) after stimulation and remained equal to 48 % of the population during the next 15 weeks. The frequency of highly productive hens with 51-60 % oviposition in the population increased after stimulation almost threefold, from 12 to 32 %, and then, 3 weeks after the last insemination, decreased to 24 % which, however, is twice as high as the similar indicator before insemination (see Fig., B).



Occurrence of hens with different egg laying ability in the Chinese silky breed chicken (*Gallus gallus*) population under the influence of insemination by mixed sperm of cocks: A — experiment I (n = 17), B — experiment II (n = 25); 1st, 2nd and 3rd periods are before insemination, during insemination and during 3 weeks after insemination, and 3 weeks after insemination; intensity of egg laying of 10 % (a), 11-3 0% (b), 31-50 % (c), 51-60 % (d), and 61-80 % (e) (Genofond VNIIGRZh, 2015-2017, on-farm experiments).

Probably, the effect of hens' insemination is prolonged, which allows us not only to increase egg production in the whole flock, but also to increase the frequency of hens with higher values of this index. Thus, the proposed procedure of suppressing the instinct of broodiness in chickens by artificial introduction of the sperm of cocks in the genital tracts of the female is simple enough in execution, physiological in its nature and no less effective than the used hormonal methods and technological shocks.

Thus, it has been found out that in poultry, as in mammals, the introduction of native mixed sperm into the genital tracts of the female through artificial insemination serves as a factor that stimulates ovulation. The β-NGF protein present in the sperm of mammals is presumably also present in the sperm of poultry, particularly in cocks. Approximately 70 % of hens respond to the stimulation of ovulation by the resumption of oviposition or an increase in egg laying, The frequency of individuals with a high percentage of egg laying (51-60 % or more) increases with a simultaneous decrease in the proportion of hens with a low egg laying. So stimulation of ovulation by 3-fold artificial insemination of hens, using native mixed sperm of cocks at a dose of 0.05 cm^3 and 4 day intervals for 4 weeks prior to gathering of eggs for incubation, may be recommended to suppress the instinct of broodiness in hens before the breeding season.

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BOVINE OOCYTE ABILITY TO EMBRYONIC DEVELOPMENT WHEN MATURING IN DIFFERENT TWO-PHASE CULTURE SYSTEMS

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Abstract

Existing approaches to a two-phase method of culture of cattle oocytes do not take into account their specific demands during maturation from metaphase I to metaphase II including the need for normalization of profiles of sex steroid hormones in a culture medium. The aim of the presented research was to compare the developmental competence of bovine (*Bos taurus taurus*) oocytes matured in conventional single-phase and different two-phase systems. We have studied for the first time the ovum ability to develop to the blastocyst stage and the quality of embryos produced when replacing the standard medium at the second stage of culture with a medium free of follicle-stimulating hormone (FSH) as well as by a granulosa cell culture. When using the single-phase system, cumulus-oocyte complexes (COCs) were cultured for 24 h in the medium TCM 199 containing 10 % fetal bovine serum (FBS), 10 μg/ml FSH, and 10 μg/ml luteinizing hormone (LH). In the two-phase system, oocytes matured in the same conditions for first 12 h. Then COCs were transferred to a new medium (TCM 199 containing 10% FBS or the same medium supplemented with 5 μg/ml LH) and cultured for 12 h in the presence or in the absence of granulosa cells. After 24 h of maturation, oocyte fertilization was performed. In embryos that developed to the stages of late morula and blastocyst on day 7 of culture, the degree of apoptosis and the total number of nuclei were assessed (by the TUNEL method and DAPI staining, respectively). In media collected after oocyte maturation, levels of progesterone and 17β-estradiol were determined by enzyme immunoassay. The proportions of oocytes entered the first cleavage division (57.6-68.1 %) or developed to the stages of late morula/blastocyst (16.7-20.7 %) were not associated with the culture method or medium. The transfer of oocytes after 12 h of maturation to the medium free of gonadotropic hormones resulted in 1.3-1.4-fold increase in the total number of nuclei in the late morula/blastocysts both in the absence and in the presence of granulosa cells, whereas this number decreased to the level of single-phase control on addition of LH. At the same time the proportion of apoptotic nuclei (2.3-6.9 %) did not depend on the system of oocyte maturation or the effect of LH. In the two-phase system, the level of 17β-estradiol in the maturation medium (with and without LH) was 1.3-1.4-fold lower ($p < 0.01$) as compared to the single-phase system. On the contrary, co-culture of COCs with granulosa cells led to a rise in the concentration of 17β-estradiol in the culture medium containing LH (from 418±16 to 496±26 pg/ml, $p < 0.05$). Meanwhile, no differences in the progesterone level in the culture medium of COCs were found between all the systems studied. The findings suggest that the two-phase system of maturation of bovine oocytes may be used as an alternative to the conventional IVM protocol. The transfer of oocytes after 12 h of culture to the medium free of gonadotropic hormones causes an increase in the quality of the late morula/blastocysts produced as well as leads to normalization of the 17β-estradiol profile in the culture medium at the terminal step of the ovum maturation.

Keywords: bovine oocytes, two-phase system of in vitro maturation, embryonic

The use of in vitro fertilization with embryo transplantation (IVF-ET) is considered as one of the reliable and effective methods of breeding highly productive and valuable species, as well as preserving the genetic potential of rare and endangered species [1]. Nowadays, the developed protocols for the in vitro culture and in vitro fertilization of bovine oocytes (*Bos taurus taurus*) make it possible to obtain a sufficiently high yield of morulas and blastocysts, but the potential for embryonic growth in ova that mature outside the body is still much lower than in ova in vivo [2].

In the animal body, the development of oocytes occurs in the follicles of ovaries, where they naturally and consistently acquire the capacity for nuclear maturation from the diplonema stage to metaphase II stage. The process of nuclear maturation of oocytes is accompanied by a complex of cytoplasmic transformations, encompassing changes in the organization of individual organelles and molecular transformations that are necessary for ova competence to the fertilization and subsequent embryonic growth [3]. When immature oocytes are isolated from follicles and cultured in vitro, meiosis resumes in them spontaneously and prematurely, which leads to incomplete cytoplasmic maturation and decrement of the ability to develop [4]. At the same time, environmental conditions in culture become one of the key factors determining the quality of mature oocytes [2].

At this moment, there are various approaches to the increment of the usefulness of oocytes during their in vitro maturation (IVM). The attempts to simulate events in natural conditions are the most interesting. One of such approaches focuses on the analysis of metabolic processes in maturing oocyte-cumulus complexes (OCCs) [5, 6]. Based on the analysis data, it is proposed to modify the culture medium in accordance with the energy needs of the oocytes and their metabolic cooperation with the surrounding somatic cells. Attempts in the works of other researchers have been made to use additives in the medium of paracrine factors, which are produced by oocytes and can support a certain degree of differentiation of cumulus cells [7, 8].

A differentiated approach involving the use of a two-phase culture system looks more promising, since there is a temporary inconsistency (asynchrony) between nuclear and cytoplasmic transformations in the in vitro maturation period of mammalian oocytes. The applicability of using a two-phase protocol for in vitro maturation of bovine oocytes (bovine cattle) has been actively studied in the last few years. The search for optimal methods of inhibiting the spontaneous resumption of meiosis [9, 10] is in progress. It is believed that the inhibition of meiosis can allow more oocytes to complete cytoplasmic transformations and acquire competence for development. Oocytes isolated from follicles are cultured for a while in a maturation medium with addition of meiosis regulators, mainly, inhibitors of cdk-kinases, capable of blocking the transition from diplotenes to metaphase I, and then during the absence of these substances. Nevertheless, the obtained results are quite contradictory and suggest the need to search for specific molecules for oocyte stimulation during blocking of meiosis [11-15].

It should be noted that the main cytoplasmic transformations, which ultimately determine the oocytes ability to embryonic growth, occur only at the final stage of maturation, which shows the importance of studying the specific needs of female gametes precisely in this period. According to several authors, with the in vitro maturation of bovine oocytes, as in vivo, there is an increase in the progesterone concentration and a decrease in the estrogen concentration in the culture medium. The most significant changes in the kinetics of these

processes occur 8-10 hours after the onset of IVM, which corresponds to a peri-period from metaphase I to metaphase II [16-18]. At the same time, in many conventional single-phase systems for the bovine oocytes culture that include follicle-stimulating (FSH) and luteinizing (LH) hormones or their analogs, the nature of the changes in the 17β-estradiol and progesterone ratio differs from that in vivo [19].

Therefore, it seems advisable to use a new approach to the two-phase culture. It is based on the ova maturation of bovine to the stage of metaphase I in a standard system and subsequent transfer to a new medium modified by the application of physiologically relevant substances that have a luteotropic effect. Other variant is based on granulosis cells possessing greater steroid activity than cumulus cells [20].

We have studied for the first time the ability of ova to develop to the blastocyst stage and the quality of the obtained embryos by replacing the standard medium in the second stage of culture medium free from FSH (an estrogen synthesis stimulant), as well as the culture of granulosa cells.

The aim of the presented study was to compare competence to growth in bovine oocytes that matured in the generally accepted single-phase and various two-phase systems.

Techniques. In all the experiments, except for indicated selected cases, reagents of the company Sigma-Aldrich (USA) were used.

The object of the study was oocyte-cumulus complexes (OCCs) from antrum follicles of bovine ovaries (*Bos taurus taurus*) and bulling heifers. Ovaries selected after slaughter were delivered to the laboratory in 3-5 hours at 30-35 °C and were repeatedly washed in sterile saline solution with antibiotics (penicillin 100 IU/ml, streptomycin 50 μg/ml). The OCCs were isolated by dissecting the follicle walls with a blade and were washed 3 times in TC-199 medium containing 5 % fetal bovine serum (FBS), 10 μg/ml heparin, 0.2 mM sodium pyruvate and 50 μg/ml gentamicin. Round shape oocytes with homogeneous cytoplasm and uniform width pellucid area surrounded by multilayer compact cumulus were collected for the experiments. All oocyte manipulations were performed with a stereomicroscope SMZ (Nikon, Japan) on a warm stage MATS-OZ (Tokai Hit, Japan) at 37 °C.

The OCCs were cultured in 4-well plates in groups of 30-35 pieces in 500 μl of medium at 38.5 °C in an atmosphere with 5 % CO_2 and 90 % humidity. To produce maturing oocytes, a standard single-phase (control) and two-phase culture systems were used. Using a single-phase system, the OCCs were cultured for 24 hours in a TC-199 medium containing 10 % FBS, 1 mM sodium pyruvate, 50 μg/ml gentamicin, 10 μg/ml FSH and 10 μg/ml LH. In a two-phase system, the oocytes matured under the same conditions for the first 12 hours. The OCCs were then transplanted into a new medium and cultured for a further 12 hours in the presence and absence of granulosa cells. At the second stage of two-phased culture, a TC-199 medium containing 10 % FBS, 1 mM sodium pyruvate and 50 μg/ml gentamicin (internal control) or the same medium supplemented with LH (5 μg/ml) was used.

Granulose cells were obtained by aspiration of fluid from follicles 3-5 mm in diameter and subsequent centrifugation of the material at 250 g for 10 min. After removal of the supernatant, the cells were washed twice in TS-199 medium containing 5 % FBS and 50 μg/ml gentamicin. A finite number of cells were counted in Goryaev's chamber, the proportion of living cells was determined with a 0.1 % trypan blue solution. The granulosa cells (1×10^6/ml) were pre-cultured in 4-well plates in 500 μl of TC-199 medium containing 10% FBS, 1 mM sodium pyruvate, and 50 μg/ml gentamicin. After 12 hours, 250 μl of medium was

replaced with fresh medium, then added to OCC wells. Co-culture of oocytes and granulosa cells was carried out for 12 hours.

At the end of culture, the medium was collected, frozen and stored at –20 °C. The content of progesterone and 17β-estradiol in media samples was determined by the method of enzyme immunoassay. The tests were carried out using a Uniplan (Pikon, Russia) plate-type spectrophotometer and commercial sets of NVO Immunotech reagents (Russia) according to the instructions of the proprietor companies. The sensitivity of the method was 0.4 nmol/L for progesterone and 25 pmol/l for 17β-estradiol. All analyzes were performed in duplicate, the coefficient of variation in the assay did not exceed 13 %.

Oocytes were fertilized in vitro, as described previously [21]. The OCCs matured in a single-phase or two-phase system were washed once in a Fert-TALP medium modified with 10 μg/ml heparin, 20 μM penicillamine, 10 μM hypothurine and 1 μM epinephrine, and transferred to 4-well plates (Nunc, Denmark), which contained 400 μl of the same medium, covered with an equal volume of mineral oil. Active spermatozoa obtained by the swim-up method [22] were added to the wells with mature oocytes at a final concentration of 1×10^6 spermatozoa/ml. In all experiments, frozen-thawed semen of one bull was used for oocyte fertilization. Oocytes fertilization, and embryo culture were carried out at a temperature of 38.5 °C in the atmosphere with 5 % CO_2 and 90 % humidity.

After 18-20 hours of incubation with sperm the oocytes were gently pipetted and washed in Fert-TALP medium to release cumulus cells and adherent spermatozoa. Intended zygotes were transferred to the CR1aa medium [23] and cultured for 4 days, after which the developing embryos were placed in the same medium containing 5 % FBS. On day 2 after oocytes fertilization a morphological evaluation of the divided zygotes was carried out, on day 7 the number of embryos matured to the stages of the late morula and blastocyst was determined. The evaluation was performed with a stereomicroscope SMZ (Nikon, Japan) with a ×40 magnification.

The resulting morulas and blastocysts were fixed with a 4 % solution of paraformaldehyde in sodium phosphate buffer for 60 min at the room temperature. After fixation, the embryos were permeabilized for 30 minutes in 0.1 % sodium citrate solution containing 0.5 % Triton X-100. The degree of apoptotic changes in nuclear material in embryos was determined by the TUNEL method using the In Situ Cell Death Detection Kit, fluorescein (Roche Diagnostics, Switzerland) according to the manufacturer's instructions. The embryos were then stained for 20 minutes with a DAPI solution (1 μg/ml) to localize the nuclei, transferred to dry skim glass and enclosed in Vectashield medium (Vector Laboratories, UK). Microphotography and evaluation of the preparations were performed under the motorised microscope Axio Imager.M2 (Carl Zeiss, Germany) equipped with a fluorescent attachment using the ZEN 2 pro software (Carl Zeiss, Germany). The degree of apoptosis in blastocysts was estimated by the fraction of TUNEL-positive nuclei (green color) from the total number of nuclei (blue color).

Experiments on the culture of oocytes were repeated at least 6 times. Obtained data was processed using the one-way ANOVA method and the two-way ANOVA variance analysis using SigmaStat software (Systat Software, Inc., USA). The results are presented as mean values (X) and standard error (±SEM). The significance of differences of the mean values compared was assessed using Tukey's test.

Results. Extracorporeal maturation of oocytes is an important stage in the technology of in vitro production of bovine embryos, the modeling of which can significantly improve its efficiency [7]. In the present work, along with the

standard single-phase IVM protocol, we first used the two-phase culture of bo-bovine oocytes, which assumed their maturation within the first 12 hours in a conventional system containing gonadotropic hormones, and the next 12 hours in a new medium without hormones. Besides, an attempt was made to modify the second phase by registration into the LH system and/or culture the oocytes on the monolayer of granulosa cells.

At the first stage of the work, we investigated the quantitative and qualitative characteristics of bovine oocytes during embryonic growth in vitro (Table). The ability of oocytes to enter the first cleavage division and mature to late morula/blastocyst stages (Fig. 1, A) did not depend on the method of culture. The presence of granulosa cells in the second phase of oocyte maturation did not significantly change the studied parameters, however, when LH were added to this system, there was a tendency ($p < 0.1$) to decrease the embryo yield at the advanced stages of growth in comparison with that obtained in a single-phase system.

Ability of bovine oocytes (*Bos taurus taurus*) to embryonic growth in different in vitro maturation systems (X±SEM, n = 6-7)

System (group)	GC	Number of oocytes, pcs.	Cleavage, %	Matured to late morula/blastocyst stage			
				% rate		number of nuclei	
				of oocytes	of embryos	total, pcs.	apoptopic, %
Without GC							
SPS	–	224	68.1±3.1	19.9±2.2	28.9±2.2	40.4±2.6[a]	5.4±2.2
TPS (C)	–	217	66.3±3.9	17.5±2.6	27.1±4.1	56.2±5.8[b]	2.3±0.8
TPS (LH)	–	220	66.1±4.0	16.8±2.6	25.6±3.7	41.3±3.9	5.1±0.8
In the presence of GC							
SPS	–	196	67.1±3.9	20.7±2.2	30.6±1.7	42.0±4.0[c]	6.4±1.3
TPS (C)	+	189	57.6±5.6	16.7±2.3	28.6±1.7	54.9±4.0[d]	5.1±1.1
TPS (LH)	+	195	58.2±3.1	13.9±2.6	23.8±4.0	42.4±6.9	6.9±2.7

Note. SPS — single-phase system (comparison group), TPS (C) — two-phase system, without luteinizing hormone (control), TPS (LH) — two-phase system with luteinizing hormone (5 ug/ml); GC — granulosa cells.
[a, b] $p < 0.05$, [c, d] $p < 0.05$ — the reliability of the differences between the comparison groups.

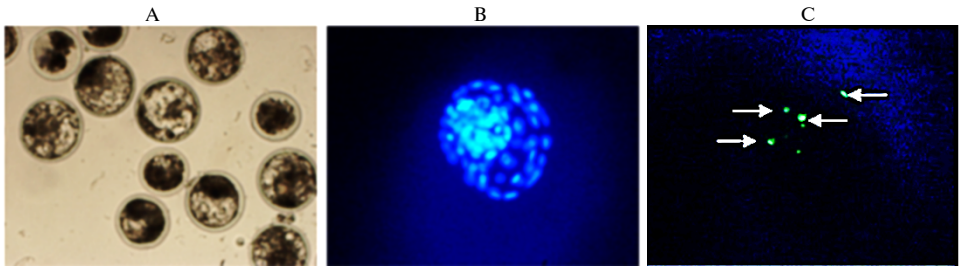

Fig. 1. Representative microphotographies of bovine embryos (*Bos taurus taurus*) on day 7 of in vitro culture and detection of apoptosis: A — morphology of morula and blastocyst (×100 magnification), B — staining of nuclei in blastocyst with DAPI (blue), cytological preparation (×200 magnification), B — staining of apoptotic nuclei in blastocyst by TUNEL method (green), cytological preparation (TUNEL-positive nuclei are marked by white arrows, ×200 magnification); microscope Axio Imager.M2, Carl Zeiss, Germany.

The quality of the embryos, which was estimated from the number of nuclei contained in the late morulas/blastocysts on day 7 after fertilization (see Fig. 1, B), was higher during the two-phase culture in the control group. The transfer of oocytes after 12 hours of maturation into a medium, gonadotropic hormones-free, led to an increase in the number of nuclei by a factor of 1.3-1.4 ($p < 0.05$), both in the absence and in the presence of granulosa cells, while adding LH. This number was reduced to the indications in a single-phase control (comparison group). At the same time, the proportion of apoptotic nuclei in the late morulas/blastocysts (see Fig. 1, C) was independent of the oocyte maturing system or the effect of LH.

As is known, the transition to a two-phase system for the culture of swine oocytes, which involves the exclusion of gonadotropic hormones in the second phase of maturing, radically changed the efficiency of the entire in vitro embryo production technology for this species of animals [24]. The data of our work are to a certain extent similar to the results observed in the maturation of swine oocytes. The absence of FSH and LH in the medium at the second stage of culture of bovine oocytes had a positive effect on the development of late morulas/blastocysts.

Next step of the work we determined the concentrations of 17β-estradiol and progesterone in culture media conditioned with maturing OCCs, depending on the culture system used, considering the importance of steroid hormones for nuclear and cytoplasmic maturation of bovine cattle oocytes [25]. At the end of the culture, the content of 17β-estradiol in the two-phase system without hormones (448±25 pg/ml) was less than in the single-phase system (583±27 pg/ml) 1.3 times ($p < 0.01$) (Fig. 2, A). Registration of LH into the two-phase system led to a more significant decrease in this indicator (up to 418±16 pg/ml, $p < 0.001$). In contrast, co-culture of OCCs with granulosa cells resulted in an increase in the concentration of 17β-estradiol in the culture medium, especially in the presence of LH (up to 496±26 pg/ml, $p < 0.05$). At the same time, we did not find any differences between all the investigated variants in the progesterone content in the culture medium of OCCs (see Fig. 2, B).

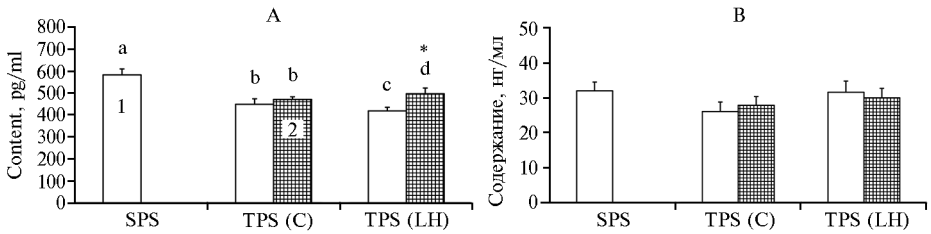

Fig. 2. The content of 17β-estradiol (A) **and progesterone** (B) **in the culture medium after in vitro maturing of bovine oocytes (*Bos taurus taurus*) in different systems:** SPS — single-phase system (comparison group), TPS (C) — two-phase system, without luteinizing hormone (control), TPS (LH) — two-phase system with luteinizing hormone (5 μg/ml); 1 — oocyte-cumulus complexes (OCCs), 2 — oocyte-cumulus complexes + granulosa cells (OCCs + GC). Vertical segments are standard errors of means (±SEM). The number of independent experiments — $n = 6$ (single-phase system), $n = 5$ (two-phase system).
$^{a, b}$ $p < 0.01$, $^{a, c}$ $p < 0.001$, $^{a, d}$ $p < 0.05$ — the reliability of the differences between the comparison groups.
* $p < 0.05$ — the reliability of the differences between OCC and OCC + GC.

The observed variations in the content of 17β-estradiol in the medium with the constant production of progesterone by the follicular cells in the second phase of oocyte maturation did not significantly affect the ability of the latter to mature to the late morula/blastocyst stage. When oocytes matured in the control two-phase system, the quality of embryos at the pre-implantation stage of growth could be associated with a decrease in the content of 17β-estradiol in the culture medium to a certain level, since the number of nuclei in them decreased both with a further decrease and with an increase in the concentration of 17β-estradiol in the presence of luteinizing hormone.

Thus, a two-phase maturation system for bovine oocytes can be used as an alternative to the conventional IVM protocol (in vitro maturation). Oocytes transfer to fresh medium without gonadotropic hormones after 12 hours of culture in a standard medium does not impair their ability to further embryonic growth and improves the quality of the obtained late morulas/blastocysts. Besides, the concentration of 17β-estradiol in the culture medium at the final stage of oocytes

maturation is reduced, which may also have some positive effect on the oocytes. Under the studied conditions, the absence of an increase in the progesterone content in the medium indicates the need to search for physiologically relevant factors that stimulate the production of progesterone in oocyte-cumulus complexes, with a view to their further application in the second phase of oocyte culture.

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EXPRESSION of *NK-lysin*, *blvr*, *ifn*-α AND BLOOD CELL POPULATIONS IN COWS INFECTED BY BOVINE LEUKEMIA VIRUS

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Abstract

At now, it is impossible to prevent or control the spread of retroviral infections, in particular, bovine leukemia virus (BLV). The existing methods of vaccination and detection of infected animals remain insufficient (G. Gutiérrez et al., 2014; M. Nishiike et al., 2016) which necessitates further investigations of the interactions between pathogen and host organism. Previously, we obtained evidence that a common characteristic for BLV infected animals with moderate and high leukocytosis was the increase in platelets, and in cows with a pronounced leukocytosis the decrease in the number of neutrophils (G.Yu. Kosovovskii et al. 2017). In order to assess the relationship between BLV infection in animals, the ratio of cell populations in the peripheral blood and the expression of the genes, encoding the BLV receptor (*blvr* gene), a comparative analysis of antivirus protection protein, interferon alpha (INFA), and effector protein of innate immunity, NK-lysine, was carried out in two groups of cows that differed in origin and farm conditions. In result, the evidences were obtained that cows from two farms differed mainly in the amount of peripheral blood neutrophils and platelets. However, in both farms the BLV infected animals had the reduced gene expression of NK-lysine and an increased number of platelets compared to cows free from infection. Relatively increased expression of *blvr* was observed in BLV infected cows, reflecting, apparently, the increase in the proportion of the young forms of B-lymphocytes (M. Lavanya et al., 2008). On the basis of the own obtained findings and literature data the scheme is proposed of the influence of BLV on the expression of NK-lysine and the suppression of apoptosis. As per the scheme, viral protein TAX (a transcription activator) induces the expression of host *tnf*-α gene (M. Arainga et al., 2012) which, in turn, activates the Treg regulators of immune homeostasis (L.Y. Chang et al., 2015); Treg produces TGF-β (transforming growth factor beta), TGF-β inhibits the proliferation and activity of T-killers and NK-cells, the producers of NK-lysine, and increases the number and activity of platelets which inhibits the apoptosis (K. Ohira et al., 2016; S.C. Tao et al., 2016). The proposed scheme suggested that the key event in pathogenesis, induced by BLV, is the effect on the innate immune system.

Keywords: bovine leukemia virus, granulocytes, agranulocytes, NK-lysine, the receptor of bovine leukemia virus, interferon-alpha, gene expression, innate immunity

Infections are the main cause of economic losses in dairy and beef cattle husbandry. A significant contribution to these is made by enzootic bovine leukosis (EBL) of cattle caused by the Bovine Leukemia Virus (BLV) [1, 2]. Among animals infected by BLV, 60-70 % remain asymptotic carriers of the pathogen; over time, lymphocytosis develops approximately in 30 % of these carriers, while leukemia and lymphoma occur only in 5-10 % of infected animals. Health improvement of herds

is difficult because antibodies to the virus proteins can be found in animals even in the absence of virus-producing B-lymphocyte clones, and the detection (or non-detection) of the integrated proviral DNA of BLV does not always provide a reliable prognosis for the development of the disease, therefore, there is a need to search for additional characteristics of infectious process induced by BLV [3].

Most often, the BLV proviral DNA is carried by about 1 % of peripheral blood cells [3]. There are two subpopulations of B-lymphocytes infected by BLV, the first subpopulation consists of active producers of mature viral particles and IgM immunoglobulin, and the second one consists of "silent" B-lymphocytes with low expression of BLV proviral DNA and IgM, which differ from the cells of the first subpopulation in higher expression of several oncogenes and in an increased rate of cell death. Both cell subpopulations have the same increased expression of the BLV receptor on B-lymphocytes as compared to that of uninfected animals. That is, the BLV susceptibility does not differ between these two subpopulations [4]. It was found that the number of virus-producing B-lymphocytes is significantly higher in the ex vivo system than in vivo, which indicates the presence of intensive "clearing" selection against BLV-producing B-lymphocytes with involvement of immune system factors [5].

Our previous study of profiles of peripheral blood cell populations in groups of Black-and-White Holstein breed infected with BLV and free from infection indicated a link between pronounced leukocytosis, an increment in the number of platelets, a decrease in neutrophils, and a relatively high neutrophil set enrichment in animals with antibodies to BLV (RID^+), but without the presence in their genomes of BLV proviral DNA [6].

This paper is the first to report the analysis of gene expression for effector protein NK-lysine (one of the main proteins of cytotoxic T-killer and NK-cell granules) [7], a BLV receptor (BLVR, delta subunit of the AP3 transport system of lymphocyte cytolemma) [8] and monocyte interferon alpha (IFNA) [9], and to suggest a scheme of the BLV impact on the *NK-lysin* expression and suppression of apoptosis.

The aim of the work was to identify factors that could be associated with changes in the functional activity of the immune system in animals infected by BLV.

Techniques. The study included animals aged 2 to 5 years; 57 Black-and-White Holstein cows (ZAO Mozhayskoe, Moscow Province), and 60 crossbreeds of Simmentals × Holsteins obtained by recurrent backcrossing for Holstein breed domination (Zarya farm, Penza Province). Blood samples for analysis were collected from the tail vein.

Erythrocyte and leukocyte profiles and erythrocyte characteristics were determined on an automatic hematological analyzer Abacus junior Vet5 (Diatron, Austria, the principle of operation is based on the Coulter method). Individual samples of fresh whole peripheral blood of each animal (4 ml) stabilized with EDTA were used. Carriers of BLV proviral DNA were detected using Mancini radial immunodiffusion (RID) and PCR protocol developed by us earlier [10]. DNA was extracted from 100 μl of whole blood using a set of M-Sorb reagents for DNA isolation from clinical samples (Synthol, Russia) according to the manufacturer's inctruction. Based on this, animals were separated into those free of infection and carrying in BLV proviral DNA the genome. Total RNA was isolated from 1 ml of whole blood sample using ExtractRNA reagents (Eurogen, Russia) in accordance with the manufacturer's recommendations. RNA treatment with DNase I (Thermo Fisher Scientific, USA) and synthesis of the first cDNA chain using the MMLV RT kit (Eurogen, Russia) was performed according to the manufacturers' instruction.

NK-lysin, *blvr*, and *ifn*-α gene expressions was compared by quantitative polymerase chain reaction (RT-PCR) in a LightCycler 96 device (Roche, Swit-

zerland) with the use of SYBR Green intercalating dye and PCR qPCRmix-HS SYBR kit (Eurogen, Russia). The ribosomal protein gene RPLPO expression was the reference. The amplification was carried out in 20 μl of the reaction mixture containing 0.2 μmol of the forward and reverse primers and 2 μl of cDNA. Following primers were used: for *NK-lysin* — 5′-CCTCGGTGCTCCTGGTYGC-3′, 5′-GGTCACCCTGGGGATCCTC-3′; for *blvr* — 5′-CTATCGGACCAGTATGTGAAG-3′, 5′-CTCCTCGGTGACGATGTCC-3′; for *ifn*-α — 5′-GATGAGAGCCTCCTGGACAA-3′, 5′-GAAGTATTTCCTCACAGCCAG-3′, and for *RPLPO* — 5′-CAACCCTGAAGTGCTTGACAT-3′, 5′-CAGATGGATCAGCCAAGAAG-3′. PCR protocol was as follows: denaturation at 95 °C for 15 s, primer annealing at 60 °C for 15 s, elongation at 72 °C for 15 s (40 cycles). The fluorescence recorded in the SYBR fluorescent channel. The reaction specificity was verified based on the temperature dissociation curves of the amplicons obtained.

Quantitative analysis of gene expression was carried out using a LightCycler 96 SW1.1 software (Roche, Switzerland).

Statistical data processing was performed in Statistica 6.0 software (StatSoft Inc., USA). Differences were considered significant at $P < 0.05$. The tables show the arithmetic mean (X) and the errors of the arithmetic mean ($\pm x$).

Results. In our previous studies, evidence has been obtained that cows infected with BLV differ from those free from infection not only by leukocytosis, but also by a reduced counts of neutrophils, as well as an increased amount of platelets and monocytes [6]. Infected animals were divided into two groups by the number of leukocytes, i.e. the cows close to the typical for uninfected animals and those with high leukocytosis (more than 17×10^9/l).

1. Comparative analysis of peripheral blood erythrocyte and leukocyte profiles in cows free from infection (control) **and infected by bovine leukemia virus, in two farms in the Moscow and Penza regions** ($X\pm x$)

Cell population, characterization	Cows without BLV proviral DNA (RID^-, BLV^-, control)	Cows infected by BLV (RID^+, BLV^+)	
		without high leukocytosis	with max leukocytosis
Erythrocytes:			
№ 1	6.86±0.22* (*n* = 7)	6.15±0.17 (*n* = 16)	6.34±0.36* (*n* = 6)
№ 2	5.79±0.29* (*n* = 10)	5.60±0.29 (*n* = 7)	5.66±0.27* (*n* = 8)
Leucocytes:			
№ 1	10.21±0.70 (*n* = 7)	11.73±0.88 (*n* = 16)	25.49±1.01** (*n* = 6)
№ 2	7.75±1.08 (*n* = 10)	9.00±1.44 (*n* = 7)	34.21±6.18** (*n* = 8)
Lymphocytes:			
№ 1	4.85±0.48 (n = 7)	6.12±0.73 (*n* = 16)	21.70±1.08 (*n* = 6)
№ 2	4.36±0.94 (n = 10)	5.40±1.27 (*n* = 7)	29.68±5.71 (*n* = 8)
Monocytes:			
№ 1	0.52±0.15 (n = 7)	0.28±0.09 (*n* = 16)	1.61±0.24* (*n* = 6)
№ 2	0.36±0.11 (n = 10)	0.27±0.10 (*n* = 7)	0.61±0.21* (*n* = 8)
Neutrophils:			
№ 1	4.54±0.50* (*n* = 7)	4.63±0.83* (*n* = 16)	1.66±0.35* (*n* = 6)
№ 2	2.39±0.65* (*n* = 10)	2.76±0.38* (*n* = 7)	3.31±0.59* (*n* = 8)
Eosinophils:			
№ 1	0.45±0.16 (n = 7)	0.59±0.09 (*n* = 16)	0.51±0.18* (*n* = 6)
№ 2	0.63±0.12 (n = 10)	0.55±0.12 (*n* = 7)	0.93±0.20* (*n* = 8)
Basophils:			
№ 1	0.0114±0.0014* (*n* = 7)	0.0088±0.0027 (*n* = 16)	0.0067±0.00211* (*n* = 6)
№ 2	0.0050±0.0022* (*n* = 10)	0.0143±0.0043 (*n* = 7)	0.0175±0.0059* (*n* = 8)
Platelets:			
№ 1	12.90±9.91* (*n* = 7)	160.81±47.55 (*n* = 16)	122.33±78.28 (*n* = 6)
№ 2	126.50±35.15* (*n* = 10)	318.14±107.92 (*n* = 7)	252.62±43.35 (*n* = 8)
The average volume of erythrocytes, μm^3:			
№ 1	44.86±2.11** (*n* = 7)	46.12±0.86** (*n* = 16)	46.50±1.09 (*n* = 6)
№ 2	55.30±1.58** (*n* = 10)	52.86±1.12** (*n* = 7)	49.75±1.42 (*n* = 8)
Variability of erythrocytes in diameter, %:			
№ 1	20.83±0.52 (*n* = 7)	18.55±0.34 (n = 16)	18.83±0.31 (n = 6)
№ 2	27.43±9.17 (*n* = 10)	17.69±0.48 (n = 7)	17.09±0.36 (n = 8)

N o t e. BLV^- and BLV^+ — the presence or absence of bovine leukemia virus, RID^- and RID^+ — negative and positive reaction of immunodiffusion. Erythrocytes — $\times10^{12}$/l, leucocytes — $\times10^9$/l; № 1 and № 2 — the farms surveyed, *n* — number of animals tested.

* $P < 0.05$, ** $P < 0.01$ (reliability of differences from control).

In the present study, we analyzed the profiles of peripheral blood cells in crossbred cows resulted from recurrent backcrossings of Simmentals with Holsteins (Table 1) in order to clarify the reproducibility of the differences we previously repealed.

The animals of these two farms differed noticeably by the average erythrocyte counts and volume, as well as by the abundance of the populations of neutrophils, basophils and platelets. Interestingly, a relatively larger number of erythrocytes in cows from ZAO Mozhaiskoe (hereinafter, referred to as № 1) coincided with a smaller individual volume of erythrocytes compared to that in animals from the Zarya farm (hereinafter, № 2) (see Table 1). In this, the cows from both farms showed a statistically significant ($P < 0.05$) negative correlation between the number of erythrocytes and their average volume ($r = –0.90$ for № 1 and $r = –0.86$ for № 2). So, apparently, the total functional potential of the erythroid cell series can differ not significantly.

Blood leukocytes, lymphocytes, monocytes and eosinophils in the animal subgroups free from infection and with moderate leukocytosis did not differ between both farms. But in the farm № 2, the number of leukocytes and lymphocytes was statistically significantly higher ($P < 0.05$), and monocytes were lower ($P < 0.05$) in the subgroup with high leukocytosis, when compared to the farm № 1 (see Table 1). Cows from farm № 1 showed some thrombocytopenia in comparison to the animals from farm № 2, with the same differences between subgroups for platelets, i.e. the smallest counts were in animals free from infection, and the largest counts were observed in the animals infected by BLV and moderate leukocytosis (see Table 1).

In cows from farm № 1 which were free from infection (conditionally control subgroup), blood basophils were statistically significantly higher ($P < 0.05$) than that in farm № 2, and if in farm № 1 in animals infected by BLV the number of basophils decreased compared to the conditional control, then in the farm № 2, on the contrary, this index increased (see Table 1).

Different subgroups from farm № 2 did not differ significantly in blood neutrophils, whereas in the farm № 1 in the subgroup with pronounced leukocytosis, the number of neutrophils was the lowest. At the same time, as to neutrophils in the subgroups free from infection and with moderate leukocytosis, the figures in the farm № 1 were almost twice as high as in the farm № 2 (see Table 1). In the scholarly publications, there is conflicting data about the number of neutrophils as impacted by BLV in the carrier animals. Some researchers did not found differences between cows infected and non-infected by BLV [11], while the others report about neutrophilia observed in some animals [12]; also the pronounced neutrocytosis was found in the milk of BLV infected cows [13].

Thus, in cows of different origin, kept in different farms, the leukocyte component, when compared peripheral blood agranulocytes and eosinophils, was similar in the animal free from BLV infection and with moderate leukocytosisof, but differed significantly when compared neutrophils and basophils, which were higher in the farm № 1, and platelets, which were lower in the farm № 1. Another manifestation of differences was that in the farm № 1 in subgroups with pronounced leukocytosis, the counts of neutrophils and basophils significantly decreased, if compared to the cows from the farm № 2, at lower scores of leukocytes and lymphocytes (see Table 1). The obtained data indicates that cows from farm № 2 have a definite suppression of cell populations associated with the function of innate immunity (neutrophils, basophils) in comparison to the animals from farm № 1.

In order to evaluate the activity of key genes involved in pathogenesis induced by BLV, we compared expression of *NK-lysin*, *blvr*, and *ifn*-α genes

(Table 2). The *NK-lysin* expression was significantly reduced, more than 2-fold, in animals free from infection from farm № 2, when compared to the same subgroup of cows from farm № 1. This is consistent with the data that in the animals from farm № 2 there is a certain suppression of blood cell populations associated with the innate immunity (see Table 1). Nevertheless, BLV infected animals from farm № 2, alike cows from farm № 1, showed a marked decrease in *NK-lysin* expression (see Table 2). In other words, BLV-infected animals showed further suppression of natural immunity effectors, namely NK-lysine, despite the differences between the farms in patterns of peripheral blood cell populations associated with natural immunity of animals. It should be noted that in both farms the subgroups were identified that had antibodies to BLV (RID^+), but no cell clones carrying BLV proviral DNA in the genome. There were 7 such cows among 34 RID^+ animals tested (20.6 %) in the farm № 1, and 11 ones out of 38 tested (28.9 %) in the farm № 2. This is slightly more than that reported [12, 14], but, nevertheless, reproducible in different farms and testifies to the regularity of false-positive results in the detection of animals infected with BLV when using only RID.

2. Expression of genes *NK-lysin, blvr, ifn-α* in peripheral blood of cows free from infection (control) **and infected by bovine leukemia virus** (with BLV proviral DNA) ($X \pm x$, two farms in Moscow and Penza regions)

Farm, gene	RID^- cows (n) without BLV proviral DNA (control)	RID^+ cows (n) with inserted BLV DNA	
		without high leukocytosis	with max leukocytosis
№ 1, *NK-lysin*	64.30±18.74 (n = 7)	54.49±8.98 (n = 16)	36.15±11.04 (n = 6)
№ 2, *NK-lysin*	27.43±9.17 (n = 10)	9.17±4.09 (n = 7)	3.76±1.22 (n = 8)
№ 1, *blvr*	7.10±1.50 (n = 7)	11.26±2.17 (n = 15)	12.18±2.37 (n = 6)
№ 1, *ifn-α*	40.90±21.67 (n = 10)	68.42±48.26 (n = 11)	1.90±0.84 (n = 6)
№ 2, *ifn-α*	–	–	21.80±12.00 (n = 7)

N o t e. BLV — bovine leukemia virus, RID^- and RID^+ — negative and positive reaction of immunodiffusion, n — number of animals tested. Gene expression was normalized using ribosomal protein RPLPO reference gene. Gaps mean the absence of data.

Note that in both subgroups of RID^+ cows without the insertion of BLV proviral DNA (BLV^-) from two farms, there was an increment in the characteristics associated with innate immunity, compared to those recorded in animals carrying BLV proviral DNA. Thus, in the farm № 1 in RID^+BLV^- cows, the blood neutrophil count reached $(3.59 \pm 0.61) \times 10^9$/l, whereas in animals with high leukocytosis was $1.66 \pm 0.35) \times 10^9$/l. Also, in the farm № 2 in RID^+BLV^- cows, the *NK-lysin* expression was 13.62±4.03 compared to 9.17±4.09 for animals with moderate leukocytosis and 3.76±1.22 for animals with the maximum leukocytosis (see Table 2). Apparently, RID^+BLV^- cows in these two farms can have relatively increased activity of innate immunity and, accordingly, are able to eliminate BLV-infected cell clones more successfully.

Expression of the *blvr* gene in the farm from the Moscow region was higher in infected cows (see Table 2), but that did not correlate with an increase in the lymphocyte population. It was reported that *blvr* expression is detected in young B lymphocytes and decreases as they age [8]. Our results suggest a relatively increased contribution of young B lymphocytes to the lymphocyte cell population in animals infected by BLV.

Ifn-α expression was characterized by high individual variability (see Table 2). Apparently, this is due to the involvement of the *ifn-α* gene product in a wide range of signaling pathways and network interactions [15]. Nevertheless, in both farms, a tendency of decreased expression of *ifn-α* was observed in cows with high leukocytosis, compared to those free from BLV infection and with

moderate leukocytosis (see Table 2). In $RID^{+}BLV^{-}$ animals, a tendency towards a relatively increased *ifn*-α expression (155.26±106.17) appeared in the farm from the Penza region, which may also support the assumption of a relatively increased activity of natural antiviral protection systems in these animals.

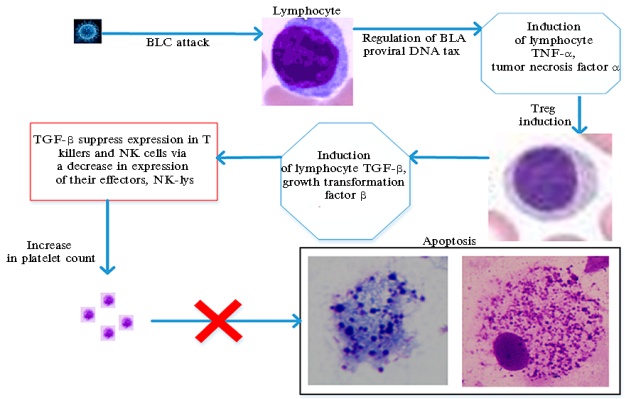
In general, the obtained data suggests that, regardless of the severity of leukocytosis in BLV-infected animals, a decrease in *NK-lysin* expression, an increase in the population of young B lymphocytes (as the growth of *blvr* gene expression shows) and a certain tendency to a decline in interferon alpha gene (*ifn*-α) expression are detected in pronounced leukocytosis. It seems that the most reliable indicator of weakening protection against BLV, irrespectively of the individual features of peripheral blood ratio of granulo- and agranulocytes, can be a significant restriction *of NK-lysin* expression in BLV-infected animals, as identified by us in both farms (see Table 2). Interestingly, in our earlier studies, the sequencing of DNA fragments flanked by inverted repeat of (GAG)6C microstatellite showed that in BLV-infected animals, unlike those free from the infection, this sequence is integrated into a structural gene encoding NK-lysine [16]. However, at this moment, there is no way to differentiate the presence of mutations that reduce the *NK-lysin* expression in BLV-infected cows from limiting the expression or reducing the proliferation of competent cells due to functional changes in the status of the immune system.

It is known that the initial stages of any infectious process are associated with activation of innate immune system and close network interactions between agranulocytes and granulocytes, whereas the final result of such interactions critically depends on the initial state of populations of white blood cells, the leukocytes [17]. It is likely that it is the insufficient knowledge of these relationships that causes the lack of success in the development of vaccination methods against such delta-retroviruses as BLV and T-cell lymphotropic virus type 1 (HTLV-1) [18].

The populations of regulatory T-cells (Treg) [19] are the leading factor of immunological homeostasis. Treg cells interfere with the activation of effector T lymphocytes involved in autoimmune and allergic processes, and, at the same time, reduce antitumor immunity. It was found that in cattle infected by BLV, activation of Treg cells is detected, which is accompanied, in particular, by a decrease in the cytotoxic activity of natural killers (NK-cells) [20, 21]. The proportion of Treg cells positively correlates with the number of lymphocytes, the virus titer and viral load in BLV infected cattle. There is experimental data showing that suppression of antiviral activity and cytotoxicity of NK-cells is due to activation of the secretion of growth-transforming beta factor (TGF-β) by Treg cells [22]. It should be emphasized that one of the effects of BLV proviral gene *tax* on host genes is an increase in the expression of tumor necrosis factor TNF-α [23, 24], which, in turn, activates Treg cells [25, 26]. In other words, the expression of BLV proviral DNA is accompanied by activation of TNF-α expression in the host. This induces Treg cells, the activation marker of which is the expression of TGF-β, which suppresses the proliferation and expression of cytotoxins in NK-cells, that corresponds to our data about the restriction of *NK-lysin* expression in BLV-infected cows in both farms.

The functional activity of NK cells directly depends on neutrophils, forming the axis of interaction between neutrophils and NK cells [27-29]. In recent years, accumulated experimental data showed that neutrophils, as an important source of cytokines and chemokines that activate other cells of the immune system, play a special role in regulating interactions in leukocyte populations during adaptive and innate immune response [18]. At the same time, the dependence of survival of neutrophils on interaction with platelets is reported [30]. Platelets,

among other properties, have a blocking effect on apoptosis [31].



The effect of BLV proviral DNA transcription resulted in a decrease in activity of T killers and NK cells and an increased population of platelets, the apoptosis blockers: BLV *tax* — activator of BLV proviral DNA transcription which influences the expression of a number of host genes, in particular TNF-α gene; TNF-α — tumor necrosis factor α, produced by lymphocytes; TGF-β — growth transformation factor β; Treg — thymus-dependent population of regulatory cells.

The accumulated data allow us to propose a scheme for the effects of transcription of proviral DNA BLV after its insertion into the host genome: the transcriptional activator of the BLV proviral DNA Tax induces an increase in the amount of TNF-α in the host, which activates Treg cells that suppress, in particular, the cytotoxic function of NK cells (Fig.). This scheme corresponds to the experimental results obtained by us and gives grounds to assume that the key factors for prognosis of BLV retrovirus resistance, as well as possible targets in developing methods for its enhancement, lay in the metabolic pathways associated with activation of mechanisms of innate immunity and, in particular, NK lysin expression.

So, this study allows us to make the following conclusion. The number of peripheral blood erythrocytes in cows of different origins and from different farms may not be the same, but negative correlation of these differences with an average erythrocyte volume is statistically significant. In animals from different farms, the blood neutrophil level can differ substantially, which is accompanied by corresponding differences in the expression of the effector factor of innate immunity, the NK-lysin (i.e. the less neutrophils, the lower the expression of *NK-lysin* genes). Despite pronounced differences, in both surveyed farms the BLV infection of cows is realized against the background of a decreased NK-lysin expression compared to that of animals free from infection. One of the biomarkers of inflammatory processes is the increase in the counts of platelets, and according to this indicator, cows from different farms can also vary significantly. However, in animals infected by BLV, in comparison to those free from infection, a significant increase in the population of platelets exhibiting an anti-apoptotic function is detected, that, apparently, can provoke neoplastic transformation of cells.

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A STUDY OF THE OPTICAL PROPERTIES OF PERIPHERAL BLOOD NEUTROPHILS OF HORSES EXPOSED TO TOTAL EXTERNAL γ-RADIATION

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Abstract

An important diagnostic test to reveal diseases in agricultural animals is to define the content of formed elements, including neutrophils. Qualitative and quantitative neutrophil characteristics are essential in health assessment and in case of radiation damage of animals due to the radiation induced death of granulocyte progenitors in the bone marrow. Worth noting is a small number of publications on the state of neutrophils in exposed animals, not only in agricultural but also in the laboratory ones. In particular, the literature practically lacks data on the optical properties of neutrophils and the effects on them of the radiation factor. Therefore, the aim of the paper was to study the optical properties of these cells isolated from the peripheral blood of horses exposed to total external γ-radiation. The absorbancy of horse neutrophil suspension was determined on a spectrophotometer CF-26 (Russia) at 600 nm and optical path length of 1 cm. The animal blood was taken from the jugular vein prior to irradiation and then on days 1, 3, 5, 7, 10, 15, 20, 25 and 30 after irradiation. The neutrophil suspension was obtained from the peripheral blood by urografin density gradient centrifugation with a specific density of 1.077 g/cm^3. A group of four horses served as control, these were kept in the same conditions as 4 groups of experimental animals, 5 horses each. The latter were exposed to total external γ-radiation at doses of 2, 3, 4 and 5 Gy. The value of the optical density of neutrophil suspension calculated for 10^5 cells per 1 ml, which averages 0.572±0.023 relative units, was found to be practically unchangeable in horses exposed to external γ-radiation in a wide dose range. This is the evidence of stability of the optical properties of neutrophil granulocytes in animals with a radiation damage, which allows photometric investigations of these cells in irradiated horses. The photometric analysis of the suspension of the isolated neutrophils has revealed the following regularities. The optical density of cells in intact horses was 0.235+0.032 relative units. In the early period of radiation pathology in all groups of the treated animals the value of the neutrophil optical density increased 2.1-2.8 times, then sequentially decreased and increased in the latent period on days 5-10 and fell drastically (up to 10-12 times) in the main phase of radiation sickness on days 20-30. The horses exposed to γ-radiation at 5 Gy showed noticeable reduction in the parameter (18 times lower) on day 10. The investigations have shown that total exposure to γ-radiation in a wide dose range results in changes of optical properties of suspension of the isolated neutrophils which is consistent with the dynamics of the neutrophil content in the peripheral blood of horses.

Keywords: horses, total external exposure to γ-radiation, peripheral blood, neutrophills, optical density, neutrophil content

It is known that an important diagnostic test to reveal diseases in agricultural animals is to define the content of formed elements, including neutrophils [1-4]. Neutrophils (neutrophilic granulocytes) are a factor of nonspecific resistance, providing the first stage of an immune response to bacterial and other pathogens and preceded by a more specific lymphocytic pathogene [5, 6]. These cells are capable of phagocytosis [7-9], but they are also recognized as universal effectors of homeostasis [10, 11]. Neutrophil is considered as a unique unicellular secretory gland that realizes the effector potential, including secreting soluble products - pro- and anti-inflammatory cytokines, anaphylotoxins and other bactericidal and cytotoxic substances, depending on the performed functions [12-

14]. Neutrophil granulocytes have receptors for a huge number of endogenous mediators (signal molecules) that trigger certain signal systems that affect specific biochemical processes in the cell [15-17]. They are able to respond to the cytokines exposure by increasing the expression of genes involved in the realization of the immune response and the appearance of receptors inherent in antigen-presenting cells [18, 19]. Besides, secreting biologically active substances, neutrophilic granulocytes can have a regulatory effect on the function of other immune-competent cells [20-22]. All this gives neutrophils the ability to quickly respond to the slightest changes in the constancy of the internal environment of the body, which allows us to consider these cells as a kind of "homeostasis mirror".

Neutrophils and their qualitative and quantitative characteristics are important for assessing the health status during radiation damage to animals and radiation-induced death of granulocyte cell precursors in the bone marrow [23]. Of particular interest is the study of these polymorph-nuclear leukocytes in horses - animals with specific physiology and metabolism, as well as a special ratio of blood elements with a large number of neutrophils (45-62 % against 20-35 %, respectively, and 35-45 % in bovine cattle and sheep) [2]. At the same time, there are few studies of the state of neutrophils under radiation effects on the organism of farm animals [24, 25] and other species of mammals [26-31].

The data presented in this study on the evaluation of the neutrophilic pool of peripheral blood of horses and the change in the optical density of the neutrophil suspension in the radiation injury of animals in semi-lethal and sub-lethal doses at various stages of pathology development significantly supplement the knowledge in this field, since such information is practically absent in the literature.

The aim of the work was to study the optical properties of neutrophils isolated from peripheral blood of horses after the general external action of γ–radiation in a wide range of doses.

Techniques. Experiments were performed on 24 horses (outbred mares) at the age of 3-5 years with a body weight of 394±0.32 kg, which were kept in the vivarium of the All-Russian Research Institute of Radiology and Agroecology (ARRIRA). The ration was balanced according to the norms of the ARRIAH (All-Russian Research Institute of Animal Husbandry, Moscow Province). Control was a group of 4 horses, which were kept under the same conditions as the experimental ones. The animals of four test groups (I, II, III and IV, of 5 heads each) were subjected to a general external action of γ-radiation at doses of 2, 3, 4 and 5 Gy, respectively, at a dose rate of 1 Gy/h at the GUZH-24 unit (Russia) (the radiation source was ^{137}Cs with γ-quant energy of 0.67 MeV). Uniformity of irradiation was monitored using a VAJ-18 dosimeter (Germany) equipped with a spherical ionization chamber VAK-253 (Germany). The non-uniformity of the γ-field did not exceed 15 %.

General state, behavior, response to external stressors, appetite, body temperature, pulse and respiratory rate, gastrointestinal tract functionы, body weight dynamics, life expectancy and hematologic indices of animals were controlled by conventional methods.

Peripheral blood were sampled from the jugular vein before and on day 1, 3, 5, 7, 10, 15, 20, 25 and 30 after the total external exposure of γ-radiation. Anticoagulant was sodium citrate at a final concentration of 0.38 %. Neutrophils were isolated by the method of blood sample centrifugation in the gradient of an isodensity substance with d = 1.077 g/cm^3 we developed earlier [32]. The isolated cells were washed by single centrifugation at 200 g and 20 °C for 10 minutes in a medium containing NaCl, KCl, K_2HPO_4, $MgCl_2$, glucose and 5-N-2-(hydroxyethyl) piperazine-N′-2-ethanesulfonic acid (145; 5; 0.5; 1; 3 and 10

mmol/l, pH 7.4, respectively) (Merck, Germany).

The total number of white blood cells was counted under the Biolar-2 microscope (Poland) in whole peripheral blood samples, followed by the determination of the percentage of neutrophils in smears [1]. The viability of the isolated neutrophils was controlled in a test with trypan blue (Sigma-Aldrich, USA). Photometric analysis of neutrophil suspensions was performed on a spectrophotometer SF-26 (Russia) at λ = 600 nm and optical path length 1 cm [31].

The mean (X) and standard errors of the mean ($\pm x$) are presented. Statistical analysis was performed using Student's t-test. Differences between control and test values were considered statistically significant at $p < 0.05$.

Results. The viability of the isolated cell populations of neutrophils, estimated in the trypan blue test, was 92-97 %. Neutrophils are large cells 9-15 μm in size, the surface of which has small roughnesses, small folds, vesicular and rod-shaped evaginations [5]. There is evidence that some physical and chemical factors can modify the adhesiveness of neutrophils [24, 33, 34]. Therefore, it was necessary to find out whether the exposure to radiation with a wide range of doses affects the optical properties of neutrophils, since a change in the shape and surface of cells, as well as the formation of cell conglomerates, can alter the light absorption. To do this, the optical density (OD) of the neutrophil suspension was calculated at 10^5/ml in horses irradiated at doses of 2, 3, and 4 Gy and in control animals. As it turned out, this indicator was not significantly different in control and test (Table 1). The average OD_{600} indicators for non-irradiated horses were 0.567±0.021 with an average value of 0.572±0.023 for all measurements. That is, the corrected value of the optical density of the 10^5/ml neutrophil suspension, which practically corresponds to the optical density of one cell, was virtually constant at various times after horse irradiation at doses of 2, 3 and 4 Gy making 0.570-0,600 in most cases (see Table 1).

1. Optical density (OD_{600} per 10^5 cells/ml) **of suspension of equine neutrophils isolated from whole peripheral blood as dependent on the dose of external γ-radiation at different periods after exposure** ($X \pm x$, vivarium of the All-Russian Research Institute of Radiology and agroecology, Obninsk)

Period, days	Dose of external γ-radiation, Gy			Non-irradiated horses (n = 4)
	2 (n = 5)	3 (n = 5)	4 (n = 5)	
Before exposure (initial index))	0.565±0.027	0.573±0.019	0.582±0.023	0.567±0.021
After exposure:				
1	0.593±0.023	0.571±0.018	0.579±0.021	0.573±0.023
3	0.598±0.019	0.592±0.021	0.568±0.017	0.572±0.018
7	0.614±0.035	0.597±0.026	0.592±0.024	0.578±0.022
10	0.595±0.022	0.603±0.025	0.571±0.032	0.587±0.027
15	0.603±0.027	0.564±0.017	0.567±0.026	0.575±0.019
20	0.562±0.018	0.581±0.026	0.585±0.025	0.588±0.024

Thence, the optical properties measured as light absorption at λ= 600 nm do not undergo significant changes in the development of acute radiation pathology of mild and moderate severity, which allows the photometric analysis of these cells.

The study of the optical density of a suspension of neutrophils isolated from peripheral blood of the horses exposed to γ-radiation over a wide dose range revealed the following features. The OD_{600} of the aliquot of the suspension in all non-irradiated horses was 0.235+0.032 (Table 2).

The exposure of γ-radiation which causes acute radiation lesion from mild to moderate severity, at the initial period led to a significant increment in optical density of the neutrophil suspension, reaching a 2.1-2.8-fold and 1.1-1.5-fold increase on day 1 and day 3, respectively, when compared to non-irradiated animals. Form day 5 to day 7, when irradiation in doses 2, 3 and 4 Gy, the index

was a little bit lower then initial one. The dose of 5 Gy caused a 1.6-fold decrease in the index on day 5 and a 1.4-fodl increase on day 7. At 2, 3 and 4 Gy, the optical density, after an increase up to control values and even higher (at 3 Gy) on day 10, then declined steadily and repeatedly, being 3.7, 2.9 and 4.5 times lower on day 20, 4.6, 8.9 and 10.5 times lower on day 25, and 7.6, 12.1 and 11.4 times lower on day 30, respectively. In the horses irradiated at 5 Gy, a sharp decrease in the index (by 18 times) on day 10 was marked, after which all the animals died. In this, we did not find a relationship between the optical density of the neutrophil suspension and the dose of γ-radiation.

2. Optical density (OD_{600}) of an aliquot of suspension of equine neutrophils isolated from whole peripheral blood as dependent on the dose of external γ-radiation at different periods after exposure ($X \pm x$, vivarium of the All-Russian Research Institute of Radiology and Agroecology, Obninsk)

Period, days	Control	Dose of external γ-radiation, Gy			
		2 (n = 5)	3 (n = 5)	4 (n = 5)	5 (n = 5)
Before exposure (initial index))	0.232±0.017	0.228±0.047	0.230±0.019	0.251±0.039	0.234±0.022
After exposure:					
1	0.227±0.028	0.615±0.047*	0.650±0.099*	0.534±0.480*	0.585±0.058*
3	0.234±0.025	0.253±0.055	0.334±0.055*	0.296±0.052	0.323±0.037*
5	0.222±0.018	0.169±0.034	–	0.132±0.024*	0.150±0.056*
7	0.247±0.019	0.174±0.030	0.202±0.063	0.171±0.018*	0.318±0.057*
10	0.235±0.022	0.221±0.035	0.339±0.060	0.200±0.041	0.013±0.004*
15	0.228±0.017	0.146±0.025*	0.173±0.048	0.166±0.048*	–
20	0.218±0.014	0.061±0.012*	0.079±0.023*	0.056±0.014*	–
25	0.229±0.013	0.050±0.006*	0.026±0.007*	0.024±0.004*	–
30	0.228±0.016	0.030±0.004*	0.019±0.004*	0.022±0.003*	–

N o t e. Gap means the absence of data.
* Differences with initial index are statistically significant at $p < 0.05$.

Thus, the total external exposure of horses to γ-radiation in sub-lethal and semi-lethal doses led to radiation lesion of mild and moderate severity, which was accompanied by an increase in the optical density of the neutrophil suspension during initial period, then this index sequentially decreased and increased in the latent period and sharply decreased in the main phase of radiation sickness from day 20 to day 30.

In the photometric study of neutrophils, it has been suggested that the optical density of the cell suspension reflects their quantity in peripheral blood of animals. For clarification, we compared the data on the optical density of the aliquot of the neutrophil suspension and the neutrophil count in the peripheral blood of irradiated and non-irradiated horses, estimated by the traditional hematological method.

3. The neutrophil count in blood and optical density of the aliquot of suspension of equine neutrophils isolated from blood of irradiated (3 Gy) **and non-irradiated horses** ($X \pm x$, vivarium of the All-Russian Research Institute of Radiology and Agroecology, Obninsk)

Period, days	Optical density, OD_{600}		Neutrophils, $\times 10^9$/l	
	control (n = 4)	experimental (n = 5)	control (n = 4)	experimental (n = 5)
Before exposure (initial index))	0.230±0.19		12.9±0.8	
After exposure:				
1	0.226±0.11	0.650±0.099*	12.7±1.3	41.1±7.2*
3	0.243±0.08	0.334±0.155	13.4±2.2	12.1±5.3
7	0.235±0.09	0.202±0.063	11.8±2.6	10.3±3.2
10	0.244±0.13	0.339±0.060	12.9±1.8	13.7±6.4
20	0.238±0.23	0.079±0.023*	13.3±1.9	4.6±0.9*
25	0.231±0.29	0.026±0.007*	12.8±2.1	1.5±0.7*

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

In non-irradiated horses, the optical density of the aliquot of the suspen-

sion was 0.230±0.190 (Table 3). After a general external exposure to γ-radiation at a dose of 3 Gy, radiation lesion was developed, which was accompanied by a 2.8-fold increment in the optical density of the aliquot of the neutrophil suspension on day 1 after irradiation, a decrement almost to the control values on days 3-10, and a 2.9-fold decrease on day 20, if compared to the initial indexes. The corresponding index in the control animals remained within the initial values throughout the study. At the same time, the peripheral blood neutrophil counts in irradiated horses changed in a similar manner (see Table 3). In non-irradiated horses, the counts averaged $(12.9\pm0.8)\times10^9$/l and remained practically unchanged for 20 days. In horses subjected to a general external exposure to γ-radiation at 3 Gy, the neutrophil count in peripheral blood increased 3.2 times on day 1 compared to the initial index, almost decreasing to the values noted in non-irradiated animals from day 3 to day 10 (see Table 3). On days 20 and 25, the number of these cells in the blood decreased 2.8 and 8.6 times, respectively, compared to the initial level. Hence, the results of a hematological assessment of peripheral blood neutrophils in irradiated horses indicated neutrophilia on day 1, caused by abortive cell ejection from the bone marrow, and neutropenia on days 20-25 with radiation-induced depletion of the granulocyte precursor pool in bone marrow.

Thus, the number of neutrophils in the peripheral blood counted directly under the microscope, and the optical density of the isolated neutrophil suspension changed after animal irradiating in a similar manner. The values of both parameters increased 3.2 and 2.8 times, respectively, on day 1, did not differ from the initial values on days 7-10, decreased 2.8 and 2.9 times on day 20, and lowered 8.6 and 8.9 times on day 25.

It should be noted that good correlation was observed between the recorded parameters at all periods after irradiation. Proceeding from such a single-type dynamics, it can be concluded that the use of photometric analysis of isolated neutrophil suspensions can be admissible when studying the changes in their count in horse blood.

Neutrophils, more precisely, the precursors of granulocytes, of mammals normally mature in the bone marrow for 8-12 days. Then they leave in the peripheral blood, where they stay 8-10 hours, then they enter different tissues and, after 3-5 days, undergo spontaneous apoptosis (programmed cell death) [35-38] or netosis [39-43]. External exposure to γ-radiation in sub-lethal and semi-lethal doses causes the death of granulocyte precursors in bone marrow, which determines the decrease in the peripheral blood neutrophil count after day 12 [23]. Our studies showed a sharp decrease in the optical density of the neutrophil suspension in horses of groups I, II and III, and on day 10 in group IV during this period (form day 20 to day 30). In other words, the main phase of radiation pathology in horses subjected to external γ-radiation at doses from sub-lethal to semi-lethal is characterized by a significant 2.9-18.0-fold decrease in the optical density of the neutrophil suspensions. The maximum decrease was noted on day 10 in the animals exposed to a semi-lethal dose (5 Gy). It should be noted that at sub-lethal doses from 2 to 4 Gy, the optical density of neutrophil suspensions did not actually differ for the period from day 7 to day 30. When exposed to a semi-lethal dose, the dynamics was completely different, showing an increase on days 1-3, a decrease below control on day 5, a 1.4-fold increase on day 7, and a sharp 18-fold decrease on day 10. That is, during the main phase of mild and moderate radiation pathology, which is accompanied by a significant decrease in the optical density of isolated blood neutrophils, practically corresponding to a decrease in their counts in the peripheral blood, it is possible to predict with confidence the emergence and strengthening of existing infectious processes in

the body. Additionally, there is reason to expect that a 10-12-fold reduction in pool of blood neutrophilic granulocytes in irradiated animals in this period will cause a change of neutrophils, which are the main links of the immune response. This will lead to violation of their regulatory influence on the functions of other immune competent cells due to a significantly reduced secretion of cytokines and other bioactive substances, even at sub-lethal γ-radiation.

So this research has shown that general external exposure of horses to γ-radiation under the studied doses leads to a change in the optical density of the suspension of isolated peripheral blood neutrophils, which is consistent with the dynamics of the count of these cells in the peripheral blood. The optical density of the neutrophil suspensions can be used in veterinary practice for express evaluation of the pool of neutrophilic granulocytes in peripheral blood of animals.

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REPRODUCTIVE AND ADAPTATION QUALITIES OF CANADIAN BREEDING HOGS COMMERCIALLY REARED IN LOWER VOLGA REGION

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Abstract

A purchase of foreign genetic material, including Canadian Yorkshire, Landrace and Duroc pigs, in order to increase the productivity of dpmestic pig breeds has recently been a steady trend in Russia. The adaptive ability of pigs is the most important feature determining their availability for industrial growing technology. The purpose of our work was to study the adaptiveness and performance of purebred Yorkshire, Landrace and Duroc pigs of Canadian selection under the conditions of OAO Cossack Holding Krasnodonskoe (Ilovlinskii Region, Volgograd Province), a large regional commercial farm for growing and fattening pigs. The study was carried out in 2013-2014. In assessing natural resistance, the auto microflora of deep skin layers was estimated. The number of erythrocytes, leukocytes, the percentage of leukocytes, the phagocytic index, total blood proteins, protein fractions, and the concentration of immunoglobulins (Ig) G-, M- and A-isotypes in blood serum were also determined as parameters of natural resistance. The reproductive performance of pigs was assessed according to the data of evaluation and livestock records. On fattening, the early maturity, overall, average per day and relative live weight gain were recorded. Sperm production in boar-producers was estimated using conventional methods, based on the volume of ejaculate and sperm concentration and motility. The economic efficiency of different pig breeds was assessed on the basis of analysis of actual and on-farm annual activities. In 2014, the analysis of populations of different breeds showed that there were 832 Yorkshire breeding sows, 50 Landrace breeding sows and 58 Duroc breeding sows. The Yorkshire sows produced 2028 piglets, their livability by weaning reached 90.83 %; for the Landrace breed, there were 85 piglets and 85.88 %, respectively (4.95 % less compared to that in Yorkshire piglets), and for the Duroc breed, these figures were 88 and 85.30 % (5.53 % less compared to that in Yorkshire piglets). Among the five families of the Yorkshire sows, the largest part of the population belonged to the families of Volga (42.17 %), Soya (17.07 %) and Oka (15.86 %). The Landrace breed sows herd was concentrated in the families of Loga (23.81 %) and Luna (23.81 %). The greatest part of the Duroc sows was related to Dakota (28.00 %) and Dama (28.00 %) families. The Yorkshire boars had a fat thickness above 6-7th thoracic vertebrae equal to 14.8 mm, which was 6.61 % less compared to the Landrace breed, and 5.40 % less compared to Duroc breed. The prolificacy of Yorkshire boars was 12.16 heads, which was 0.23 and 1.46 heads more than in Landrace and Duroc breeds, respectively. In assessing the exterior indices, the Yorkshire boars were superior to the boars of Landrace and Duroc breeds by 0.33 and 0.20 points, respectively. The natural resistance characteristics have shown a higher potential for adaptability of the Yorkshire breed to new climatic conditions and technologies. Calculation of economic efficiency of pork production has shown that the income for the Yorkshire piglet sale by live weight was 1,520 and 10,640 rubles higher compared to that for the Landrace and Duroc piglets, and the profitability was 0.86 and 4.99 % higher, respectively; for the carcasses the income exceeding amounted to 0.92 and 5.30 %, respectively.

Keywords: pig breeding, purebred Yorkshire, Landrace and Duroc pigs, Canadian selec-

tion, families, line, sows, boars, reproduction

Pig husbandry is one of the successfully developing sectors of livestock husbandry. Currently, the largest pork producers are China, the EU countries, Brazil, Russia, Vietnam, Canada, Japan, the Philippines, Mexico, and South Korea. Pig husbandry in Canada possesses unique industrial technologies and traditions that allow exporting products to 40 countries. In the structure of pig population in Canada, the Yorkshire breed makes 42 %, Landrace makes 32 %, and Duroc makes 25 %. The main exporter of pigs is Canada Pork International (CPI) agency [1-4]. In order to increase the productivity of domestic pig breeds in recent years, Russia has established a stable trend in buying foreign genetic material. In this, focus is made on specific systems used in breeding, and on the degree of hybridity compared to regional genotypes. Experts conduct a system monitoring of domestic and imported pig breeds which allows breeders to optimize crossings and facilitates an increase in breeding efficiency, particularly, with emphasis on gain of productivity and improved adaptiveness of animals. Adaptation is a necessary precondition for the realization of the genetic potential of productivity, and an important factor in increasing animal resistance and preventing against various diseases [5, 6]. Adaptive abilities are the features of animal vital activity in unaccustomed conditions, behavioral responses, anatomy, natural resistance, responses to external stimuli, associated with a set of conditioned and unconditioned reflexes [5-7].

Long inbred breeding leads to a decrease in genetic diversity within the isolated groups and causes a decrease in productivity. At the same time, traditional breeding techniques are ineffective for the improvement of poorly heritable features, such as fecundity, milk producing ability of sows, livability of piglets [6-9]. In the provinces of Alberta and Ontario, Canadian scientists are working towards preserving the genetic diversity of Duroc, Hampshire, Yorkshire, and Landrace pigs through a system approach to selection. It is established that the average productive longevity of the breeding stock is 6 years. At the same time, some researchers note the high quality of selection and breeding work carried out in specialized farms in Canada. This work can be controlled through the purposeful recording of inbreeding within the breeds [10, 11]. Innovative methods of are needed to produce pigs capable of inheriting the characteristics of several breeds.

Pig stock of Canadian selection is being imported for exploitation in the Volgograd Province. Note, in Canada, the Yorkshire, Landrace and Duroc pigs were intercrossed to obtain a highly productive commercial hybrid with optimal quality indicators, which is crucially important for commercial domestic pig husbandry (10-12).

In 2008, the OAO Cossack Holding Krasnodonskoe (Volgograd Province) imported Yorkshire, Landrace and Duroc pigs of Canadian breeding aged 3-4 months. After an adaptation period, these animals showed early maturity and productivity [12]. The purchase of pedigree material from Canada continued in order to increase the consolidation of the genes of the best Yorkshire, Landrace and Duroc lines [7].

The adaptive abilities of pigs are the most important feature that determines their suitability for commercial rearing. Criteria of the degree of animal adaptation in changing environmental conditions are indicators of the reproductive capacity and natural resistance of sows, as well as the productivity of boars [13-15]. Another approach to studying the adaptive abilities of pigs is markers associated with a number of genes encoding target traits found both in domestic pig breeds [16-20] and in their evolutionary relatives, the wild boars [21].

This research for the first time reported a set of indicators that allowed

exhaustively estimating the physiological state and reproductive capacity of Yorkshire, Landrace and Duroc Canadian breeds while adapting to new climatic and technological factors in the conditions of the large Russian commercial pig breeding farm. The obtained results confirmed the high genetic potential and stable heritability of productive qualities in all three breeds. At the same time, a number of parameters (yield and livability of piglets, natural resistance) revealed the advantage of Yorkshire pigs.

The aim of this work was a comparative assessment of the adaptiveness of different pig breeds of Canadian selection and the realization of their genetic potential of growth, development, productivity and reproductive qualities in the conditions of the Lower Volga region.

Techniques. The research was carried out in 2013-2014 in the commercial pig husbandry complex (108 thousand heads) of OAO Cossack Holding Krasnodonskoe (Ilovlinskii Region, Volgograd Province) on Yorkshire, Landras and Duroc pigs. Animal feeding and housing corresponded to those accepted in the farm.

Morphological, biochemical and immunological analyses of blood were carried out on 6 analogue individuals from each group of pigs. The auto microflora of deep skin layers was estimated [22]. The number of blood erythrocytes, leukocytes, percentage of leukocytes, and phagocytic index was determined by conventional methods. The concentration of immunoglobulins (Ig) G, M and A isotypes in blood serum was evaluated by the classical method of radial immunodiffusion with monospecific antisera and monoclonal antibodies to each isotype of immunoglobulins, as well as with the standard blood serum of a pig with a known level of each immunoglobulin isotype [23-25].

To compare the reproductive ability of pigs, the data of valuation and zootechnical records were used in the order established for the pedigree reproducer. The physiological state of the animals was established according to blood indices. When fattening, early maturity (days), absolute (kg), average per day (g), and relative (%) live weight gain were recorded. Sperm production in boars was estimated using conventional methods based on the volume of ejaculate and sperm concentration and motility.

The economic efficiency of growing different pig breeds was assessed based on the actual and on-farm annual activities.

The experimental data was processed in Statistica 6.0 software (StatSoft, Inc., USA) using variation statistics methods and determining the reliability criterion for the Student-Fisher difference at three probability levels. The arithmetical mean (M), error of mean ($\pm m$), Student-Fisher reliability t-criterion, and validity coefficient (p) were calculated.

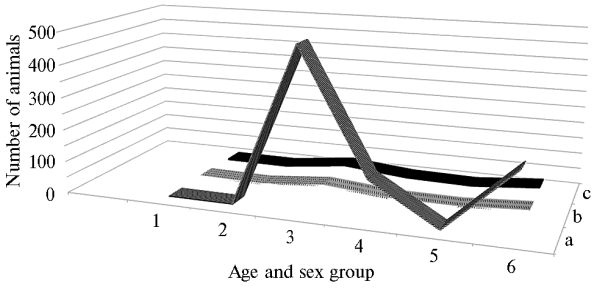

Fig. 1. Age and sex composition of Yorkshire (a)**, Landrace** (b) **and Duroc** (c) **pigs of Canadian selection in OAO Cossack Holding Krasnodonskoe:** 1 — main boars, 2 — test boars, 3 — main sows, 4 — test sows, 5 — replacement boar pigs, 6 — replacement sow pigs (Ilovlinskii Region, Volgograd Province, 2013).

Results. In OAO Cossack Holding Krasnodonskoe, the number of pedigree animals was 498 for Yorkshire, 50 for Landras, and 58 for Duroc (Fig. 1). The Yorkshire sows were the largest part of the livestock (88.51 %), and the Landrace (50 %) was the leading breed in the number of the main boars served as producers (see Fig. 1).

Bacterial seeding for auto microflora from the deep layers of the skin revealed high-

er values of the analyzed parameter in Landrace and Duroc animals compared to the Yorkshire breed analogues. In Yorkshires, when a decrease of auto microflora count to 3.34±0.31 CFU/cm^2, the blood erythrocytes, lymphocytes and albumins increased significantly ($P \geq 0.05$) by 5.6 %, 6.9 % and 22,0 %, respectively. The phagocytic index was 20 % higher than that in Landrace and Duroc animals, in which the auto microflora counts were 5.2±0.29 and 6.6±0.39 CFU/cm^2, respectively.

In Yorkshire sow pigs, the blood concentration of all immunoglobulins was higher than that of Landrace and Duroc animals, i.e. IgG was 11.69 % and 16.13 % higher ($P \leq 0.05$), IgM was 17.76 % ($P \leq 0.01$) and 31.78 % ($P \leq 0.001$) higher, and IgA was 25.76 % ($P \leq 0.01$) and 43.94 % ($P \leq 0.001$) higher (Table 1).

1. Blood immunoglobulin concentration (mg/ml) **in pigs of Canadian origin under the conditions of OAO Cossack Holding Krasnodonskoe** (*M*±*m*, Ilovlinskii Region, Volgograd Province, 2013)

Breed	IgG	IgM	IgA
Yorkshire (n = 6)	24.80±1.39	3.21±0.09	1.32±0.08
Landras (n = 6)	21.90±0.89	2.64±0.13**	0.98±0.06**
Duroc (n = 6)	20.80±0.92*	2.19±0.11***	0.74±0.05***

*, **, *** Differences with indexes in Yorkshire pigs are statistically significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0{,}001$, respectively.

The most important stage in studying adaptive capabilities of sows is the evaluation of their productivity, including such parameters as prolificacy, size of piglets, their livability, milk producing capacity estimated by the weight of 21-day old piglets in a litter. According to the records of 2014, Yorkshire sows produced 2028 piglets, their number by weaning reduced to 1842, i.e. livability by weaning was 90.83 %. As for the Landrace sows, there were 85 piglets, 73 of them left alive by weaning with livability of 85.88 % which was respectively 4.95 % less compared to that in Yorkshire piglets. The Duroc sows produced 88 piglets, 75 of them surviving by weaning with livability of 85.30 % which was 5.53 % less compared to that in Yorkshire piglets. The milk producing capacity of sows at weaning estimated by weight of 21-day old piglets was 87.5 kg in the Yorkshire breed, which exceeded the index of the Landrace and Duroc animals by 1.2 and 9.5 kg, respectively. Former studies reported on the impact of adaptive abilities of animals on their productive indicators [6, 13, 14, 17].

Analysis of the data on the main sows showed that of 16,728 piglets derived from the Yorkshire animals in 2014, 15,195 survived by weaning, the livability being 90.84 %. These figures for the Landrace sows were 678 and 610 pigments, respectively, the livability of 89.8 % or 1.04 % less than in the Yorkshire piglets. As for the Duroc breed, the figures were 755 and 646, the livability of 85.56 %, being 5.28 % compared to that for the Yorkshire breed. The data obtained indicates that Yorkshire sows showed higher reproducing ability compared to the Landrace and Duroc peers, which characterizes their pedigree features.

Figure 2 shows the division of the OAO Cossack Holding Krasnodonskoe the sow population in 2013 into families (Fig. 2). Among the five families of Yorkshire sows, the largest part of the population belonged to the families of Volga (42.17 %), Soya (17.07 %) and Oka (15.86 %) sows. The Landrace breed sow herd was concentrated in the families of Loga (23.81 %) and Luna (23.81 %) sows. The greatest part of the Duroc sows was related to Dakota (28.00 %) and Dama (28.00 %) families.

In general, Yorkshire sows surpassed the Landrace and Duroc analogues according to their reproductive qualities. However, the fattening indicators in Duroc and Landrace pigs were higher than those of Yorkshire ones. Such differences are explained by the fact that the Yorkshire breed has a com-

bined type of productivity, the Landrace breed has a bacon type, and the Duroc breed is of a meat type.

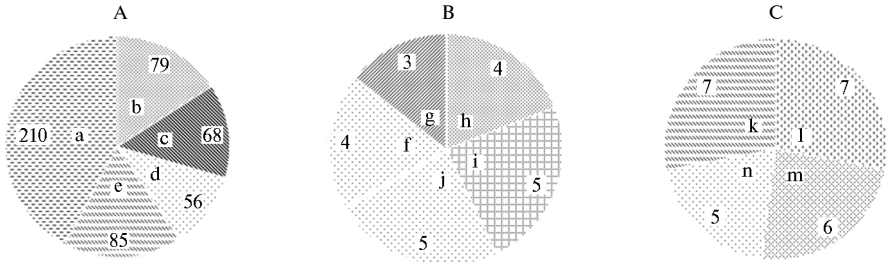

Fig. 2. The structure of sow populations (heads) **of Yorkshire** (A)**, Landrace** (B) **and Duroc** (C) **breeds of Canadian origin in OAO Cossack Holding Krasnodonskoe as divided into families of sows:** a — Volga, b — Oka, c — Rona, d — Sena, e — Soya; f — Lavla, g — Leya, h — Lipa, i — Loga, j —Luna; k — Dakota, l — Dama, m — Dvina, n — Diva (Ilovlinskii Region, Volgograd Province, 2013).

Pedigree boar pigs of Yorkshire, Landrace and Duroc breeds belonged to the genealogical lines 4, 4 and 3, respectively. Yorkshire boars reached a body weight of 100 kg at the age of 149.8 days, and their maturity rate was 1.02 % higher than that of Landrace boars, and 4.14 % higher than that of Duroc boars. In this, Yorkshire boars needed 0.06 and 0.06 less feed units, or by 2.36 and 2.36 %, respectively, per 100 kg body weight compared to Landrace and Duroc peers. The Yorkshire boars had a fat thickness above thoracic vertebras 6-7 equal to 14.8 mm. It was 6.61 % less compared to the Landrace breed, and 5.40 % less compared to Duroc breed. In assessing the exterior indices, the Yorkshire boars were superior to the Landrace and Duroc boars by 0.33 and 0.20 points, respectively, and had the prolificacy index of 12.16 heads, which was 0.23 and 1.46 heads more than in Landrace and Duroc breeds, respectively. Thus, the Yorkshire boars of the combined productivity stand out with their high growth rate, which was characterized by a faster achievement of 100 kg body weight at a relatively low feed cost, and also had a high prolificacy index compared to the Landrace and Duroc boars.

Breeding and intensification of pork production needs the best producers, for which the young boars from highly productive breed lines are purchased. It is necessary to take into account the reproductive ability and sexual activity of animals, as well as their own productivity [13, 14].

In our studies, Yorkshire boars, as compared to the Landrace and Duroc analogues, had higher ejaculate volume (by 13.64 and 12.44 %, respectively); the sperm concentration was 7.69 and 7.69 % higher; the motility of the sperm was 3.22 and 5.0 % higher; the total number of spermatozoa with linear motion was 8.95 and 8.33 % higher (Table 2). That is, as for sperm production, Yorkshire boars exceeded the Landrace and Duroc peers.

2. Sperm production in boars of Canadian origin under the conditions of OAO Cossack Holding Krasnodonskoe (*M*±*m*, Ilovlinskii Region, Volgograd Province, 2013)

Boar name and number	Ejaculate volume, ml	Sperm concentration, bln/ml	Sperm motility, %	Spermatozoa with linear motion	
				bln	%
Yorkshire (*n*=6)					
Reyn 61834	165.00±7.40	0.26±0.03	85.00	28.00±0.63	84.20
Don 67305	170.00±6.40	0.28±0.02	90.00	26.00±0.58	85.40
Khoper 67455	128.00±5.90	0.25±0.01	80.00	31.00±0.62	85.60
Khoper 72030	155.00±6.20	0.26±0.02	90.00	29.00±0.56	82.60
Nil 72031	170.00±7.30	0.25±0.03	85.00	30.00±0.59	81.20
Averaged	157.60±7.89	0.26±0.01	86.00	28.80±0.86	83.80

Continued Table 2

Landrace (n=6)					
Lev 2705	150.00±5.40	0.25±0.06	85.00	28.00±0.54	85.00
Lev 3381	120.00±4.80	0.23±0.04	80.00	24.00±0.49	84.00
Lev 3384	150.00±5.50	0.22±0.05	80.00	24.00±0.56	80.00
Lider 2986	135.00±4.60	0.25±0.07	85.00	28.00±0.62	84.00
Lider 3377	150.00±5.60	0.23±0.08	80.00	24.00±0.54	80.00
Lot 2690	110.00±4.70	0.25±0.04	85.00	28.00±0.61	85.00
Lot 1004	150.00±4.90	0.25±0.06	85.00	28.00±0.59	85.00
Lot 1006	130.00±4.70	0.23±0.03	80.00	24.00±0.62	80.00
Lir 1007	130.00±4.60	0.25±0.05	85.00	28.00±0.61	85.00
Averaged	136.11±4.98	0.24±0.00	82.78	26.22±0.70	83.11
Duroc (n=6)					
Dok 3444	150.00±5.60	0.26±0.03	85.00	26.00±0.64	80.00
Dok 4023	150.00±5.40	0.25±0.05	80.00	24.00±0.56	85.00
Dinar 445809	130.00±4.60	0.23±0.04	75.00	28.00±0.63	85.00
Dinar 4022	150.00±4.90	0.23±0.02	80.00	28.00±0.61	80.00
Denver 246	110.00±3.90	0.25±0.06	85.00	26.00±0.58	85.00
Averaged	138.00±8.00	0.24±0.01	81.00	26.40±0.75	83.00

In Yorkshire breed, when fattening piglets from one sow (10.9 heads) not heavier than 100 kg, a yield of 1090.00 kg body weight and 843.66 kg for carcass can be averagely produced. The production costs when growing Landrace and Duroc pigs is higher than that of the Yorkshires, since their fattening to the same body weight takes 1 to 6 days more. The income for the Yorkshire piglet sale by body weight was 1,520 and 10,640 rubles higher compared to that for the Landrace and Duroc piglets, and the profitability was 0.86 and 4.99 % higher, respectively; for the carcasses, the income exceeding amounted to 0.92 and 5.30 %, respectively.

Thus, in the Lower Volga region in pig breeding commercial complex, pigs of Yorkshire, Landrace and Duroc Canadian breeds as a whole had high productivity. It is shown that animals of these breeds have high genetic potential with a stable inheritance of productive qualities by descendants. At the same time, the Yorkshires produced more piglets with higher livability, which ensured a greater yield of products per sow. The natural resistance characteristics of this breed have shown a higher potential for their adaptability to new environment and technologies. In order to increase biodiversity and improve the domestic production of pork, it is advisable to use pedigree animals of Yorkshire, Landrace and Duroc breeds from both already existing, and new lines.

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HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN EDILBAI LAMBS SUBJECTED TO PHARMACOLOGICAL CORRECTION OF HYPOMICROELEMENTOSIS DUE TO BIOGEOCHEMICAL CONDITIONS OF THE LOWER VOLGA REGION

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Abstract

In recent years, latent hypomicroelementoses are increasingly diagnosed in a variety of farm animals, including sheep, in Russia, EU, India and other countries (V.T. Samokhin, 2008). The deficit of selenium, iodine and other vital elements leads to a decrease in hematological parameters and a change in the activity of free radical oxidation and oxidant protection which is a fundamental molecular and cellular mechanism of pathogenesis at various hypomicroelementoses (T.N. Rodionova et al., 2010). The aim of the paper was to comprehensively survey the biogeochemical situation in the Lower Volga region and to evaluate the influence of Se and I deficiency on the physiological status of Edilbai lamb not subjected to pharmacological correction of hypomicroelementoses and after administration of Sedimine® (Russia), a metabolism-regulating organic composition containing Se, I and Fe (III)—dextran stabilizing complex. The probes of soil and water, plants, sheep organs and tissues were sampled according to V.V. Kowalski (1974). Trace elements were detected using atomic absorption spectrometry (M.E. Britske, 1982), selenium was assayed fluorimetrically according to V.V. Yermakov (1975), and iodine was analyzed in thiocyanate-nitrate test. To quantitate the products of lipid peroxidation in lamb blood, we estimated diene conjugates by UV absorption spectra at 233 nm (Z. Platzer, 1970), malondialdehyde — according to V.S. Buzlama et al. (1997), catalase activity — as per M.L. Korolyuk (1980) and glutathione peroxidase — as described by R. Paglia et al. (1967). Hematological parameters were evaluated according to I.P. Kondrakhin et al. (2004). It was shown that the basic components of terrestrial ecosystems in the Lower Volga region are low in selenium, iodine and cobalt relative to the Black Earth region (V. Kowalski, 1974). In the intact lambs, negative balances of iodine and selenium were found during the observation. Also, hematological parameters and antioxidant defense were low. Blood and metabolic parameters together with the balance of studied microelements indicated a development of latent combined selenium and iodine hypomicroelementosis in the lambs. The use of organic product Sedimine resulted in a significant ($P < 0.05$) correction of the syndrome of selenium and iodine deficiency in Edilbai lambs. Indeed, the blood levels of selenium increased by 84.0 % and iodine — by 92.7 %, the number of erythrocytes was 15.6 % higher, hemoglobin was 7.8 % higher, Se, I, globulins were 8.76 % higher, while the amount of glucose reduced by 33.9 %. The diene conjugates diminished by 28 %, the malonaldehyde — by 8.2 %, the catalase activity increased by 43.3 %, and glutathione peroxidase activity was 39.4 % higher. The four month-aged lambs which received Sedimine showed the improvement in all the studied metabolic parameters and the weight gain which exceeded that in the control by 14.4 % ($P < 0.05$). Additionally, after the Sedimine application the lamb meat was superior to that of the control animals in accumulation of physiologically important I, Se, Mn, Cu, and Co.

Keywords: Edilbai sheep, lambs, biogeochemical provinces, microelements, Se, I, hypomicroelementosis, lipid peroxidation, antioxidant defense, hematological indexes

In recent years, latent complex hypomicroelementoses [5-9], induced by oxidative stress and lack of microelements in the environment [10-13], are increasingly diagnosed in a variety of farm animals in Russia [1-4], EU, India and other countries. This is associated with changes in the activity of free radical oxidation and antioxidant protection [8, 9], which serve as fundamental molecular

and cellular mechanisms of pathogenesis in various endemic diseases [10, 14-16]. As a result, growth and development of animals slows down, integrative functions deteriorate, productivity decreases [1, 17-19]. To prevent and correct the latent forms of hypomicroelementoses, it is necessary to carry out complex studies that include monitoring the biogeochemical situation in the region, assessing the balance of chemical elements in the organism, analyzing the physiological status of animals (including the metabolism of proteins, lipids, carbohydrates, vitamins and minerals) products of lipid peroxidation (LPO) and the activity of antioxidant enzymes.

We are developing a fundamentally novel physiological and biogeochemical concept for the theoretically substantiated selection of deficient microelements necessary for the organism of farm animals [3, 4, 20-22], methods for their application and calculation of dosages using a mathematical analysis of the pharmacokinetics of medications [4, 21]. This approach offers a possibility to prevent and correct the latent forms of combined hypomicroelementoses, to improve general state, reproductive function and productivity of animals.

In the present paper, we for the first time discovered the latent form of combined hypomicroelementosis in Edilbai lambs in the Lower Volga region and showed that the intramuscular injection of the Sedimin® preparation has a positive effect on their physiological state, metabolic processes, hematologic indices, antioxidant system.

The aim of the work was to make a comprehensive study of the biogeochemical situation in the Lower Volga region in connection with the effect of the deficiency of essential microelements on such integrative functions of the Edilbai lambs as growth and development, and also to evaluate the pharmacological correction possibilities of physiological and biochemical status of lambs during hypomicroelementoses.

Tecnjiques. Samples of soils (0-25 cm), water, plants species, feed, organs and tissues of animals for atomic absorption analysis for the content of trace elements were selected according to V.V. Kovalsky [22] in different areas of the Volgograd and Astrakhan regions.

Tests on 4-month-old Edilbai lambs were carried out in the SPKh Krasnoyarets (Krasnoyarsk Region, Astrakhan Province) in 2014-2015. For the test, two flocks of lambs analogues were selected, 252 heads as control animals, and 268 heads as test animals. Animals were housed in sheepfold sheds and grazed on pasture. In addition to milk, lambs of both groups from the age of 20 days received an equal amount of barley groats. After weaning from the ewes, the lambs ate plant forage with the addition of concentrates according to the norms established in the farm.

Balance test was performed on five control and five test gimmers according to the methodology of the All-Russian Research Institute of Animal Husbandry [23]. Lambs from the test group were injected intramuscularly with the Sedimin® preparation (OOO A-BIO, Russia). Sedimin is certified by the Russian Pharmaceutical Committee (GOSTR № ROSS-RU.PO-96.N10771, preparation No. PVR-2-3.6101651) and is an aqueous mixture of iodine and selenium compounds on the stabilizing basis of the dextran complex. Sedimin was administered at a dose of 1 ml at 20-day age, 2 ml at a 2-month age, 3 ml at a 3-month age and 4 ml at the last month of growing.

Before the test and at the end of survey, animals were weighed. Blood samples were collected before feeding, hematologic indices (number of blood formed elements, hemoglobin, leukoformula), content of Ca, P and carotene were determined by I.P. Kondrakhyn [24]. Diene conjugates (DC) were studied by UV absorption spectra at $\lambda = 233$ nm [25], the malonic dialdehyde (MDA)

was assayed by the method of V.S. Buzlama [26]. The catalase activity was studied by the method of M.L. Korolyuk [27], the activity of glutathione peroxidase (GPO) was assessed according to R. Paglia et al. [28]. Microelements in biological samples stored in desiccators were determined by the atomic absorption method [29] using a Hitachi 180-50 spectrophotometer (Japan). The content of selenium was determined fluorimetrically [30], iodine was measured by the rhodanide-nitrite method (GOST 28-548-90) [31].

During statistical processing of the obtained data, the mean (M), the mean error ($\pm m$), and the correlation coefficients (Cv) were calculated. The significance of differences was assessed by the Student's t-test at significance level of 0.01-0.05.

Results. In the soil samples from Astrakhan and Volgograd regions, the content of cobalt, selenium, copper, zinc and manganese were on average 8.0±1.03, 0.03±0.022, 15.8±1.27, 45.4±2.10 and 142.8±10.60 mg/kg, respectively. The indicators in water were as follows (μg/l): Co — 0.7±0.02, Ni — 0.5±0.01, Se — 0.029±0.002, Mn — 9.8±0.70, Zn — 32.1±2.60, Cu — 4.5±0.70, I — 1.7±0.13. Much more selenium (0.058±0.012 mg/kg) was detected in summer (0.058±0.012 mg/kg) than in October (0.021±0.004 mg/kg).

The amount of microelements in plants correlatively depended on the content in the soil ($r = +0.68$), the species and physiological characteristics of plants, the climate of a specific region (data not shown), and fluctuated at wide ranges. The iodine content varied from 0.01 to 1.99±0.06 mg/kg. Attention was drawn to the low amount of cobalt (from traces to 2.2±0.09 mg/kg) and selenium (from 0.01 to 0.07±0.004 mg/kg) in plants, with the exception of astragalus (Co — 9.6±0.17, Se — 12.6±2.18 mg/kg). In the spring and summer months, the accumulation of selenium in plants, similar to the effect observed in soils, was somewhat higher than in the autumn. This is explained by the largest migration of selenium from soil to plants during growing.

Manganese was found in optimal amounts in plants (from 47±3.12 to 98±6.4 mg/kg) compared to the content in similar macrophytes from the so-called reference dark fertile soil region [22]. The content of selenium, iodine and cobalt in soils and plants was significantly less than in their analogues from the reference region. The copper in soils and plants (from 2.3±0.07 to 11.3±1.4 mg/kg) was at the lower limit of normal [22, 32]. The optimum concentration of selenium in drinking water used by animals and birds has not yet been established. In the reservoirs water of the Volga River delta, the selenium index was 0.019±0.0006 mg/l.

The lack of a number of chemical elements (Se, I and Co) in soil and plant forages negatively affects the realization of animal genetic program and reduces the physiological capabilities of integrative functions of growth and development in lambs [2-4]. The quantity of selenium in different organs of lambs decreased sequentially in the following order: liver > muscles > spleen > bone tissue > lungs > wool > blood > kidneys > small intestinal wall. The cobalt order looked like this: liver > bone tissue > spleen > intestinal wall > wool > kidneys > blood > muscles. Considering the large volume of muscles in sheep, muscle tissue, as well as liver, can be considered a depot of cobalt and other studied trace elements. The decreasing series of manganese, copper, zinc and iodine were similar to each other with very little difference. For example, amount of copper (9.5±0.07 mg/kg) in small intestine wall was almost the same as in spleen, wool, lungs and kidneys. According to the zinc content, the intestinal wall (155±13 mg/kg) was close to the wool and liver. Most of the zinc was found in the lungs (156±8.4 mg/kg), where this element is concentrated, entering the carbonic anhydrase which regulates respiratory function in animals.

Comparing the data on the accumulation of trace elements in organs and tissues of Edilbai lambs with similar characteristics in young sheep in the reported of other authors [1-3, 33-35], it can be noted that the level of I, Co and Se in our experiments was relatively low and correlated with a low content of these trace elements in plants of the Lower Volga region ($r = +0.74$).

After weaning from the ewes, the average daily balance of iodine and selenium in lambs, completely transferred to plant and concentrated forage, was negative (Table 1). The balance of cobalt was of a tense nature and approaching zero balance. The balance of Mn, Cu and Zn was positive, the organism of lambs did not lack these elements, which was due to their relatively high content in forage.

1. Balance of microelements (mg) **in Edilbai lambs** ($n = 10$, $M \pm m$; SPKh Krasnoyarets, Krasnoyarsk Region, Astrakhan Province, 2014-2015)

Trace element	Input with forage	Excreted			Assimilated (balance)
		with feces	with urine	total	
Co	0,31±0,04	0,28±0,01	0,02±0,003	0,30±0,01	+0,01±0,003
Se	0,69±0,03	0,73±0,02	0,03±0,005	0,76±0,02	–0,07±0,003
I	0,10±0,08	0,14±0,04	0,04±0,006	0,18±0,04	–0,08±0,005
Cu	5,97±0,11	5,82±0,95	0,06±0,002	5,88±0,72	+0,09±0,006
Mn	68,0±1,34	63,2±3,15	0,11±0,007	63,31±2,07	+4,69±0,270
Zn	29,6±0,09	22,5±1,96	0,21±0,009	22,71±1,35	+6,83±0,050

The results of balance tests, along with biogeochemical monitoring of the environment and the content of trace elements in organs and tissues, allow us to state the deficiency of selenium and iodine in the Edilbai lambs that suggests the necessity of using Se and I preparations in the Lower Volga region. However, for the final decision necessitates estimation of physiological status of animals, including hematological indices, the degree of free radical oxidation and antioxidant protection.

The antioxidant mechanisms induce enzymatic and non-enzymatic processes. Antioxidants are involved in the regulation of peroxidation as components of an unite system that includes a number of enzymes, low molecular weight compounds, physiologically active substances of protein and lipid nature, including vitamins, Ca, Se, Fe, Zn and Cu, and possibly other macro- and microelements that are part of antioxidant enzymes or can activate enzymes [36]. It is known that cation radicals of selenium, manganese, zinc, copper, molybdenum, cobalt, and iron-sulfur clusters can participate in the initiation of free radical oxidation [3, 4, 15, 37].

Intramuscular application of iodine and selenium, lacking in the organism of growing lambs, led to an increase in hematopoiesis (Table 2). In the lambs from the test group, the erythrocytes increased by 15.6 %, hemoglobin increased by 7.8 %, selenium — by 84.0 %, iodine — by 92.7 %, total protein — by 4.44 %, globulins — by 8.76 % if compared to the same parameters in the control group ($P < 0.05$). The parameters of the carbonate blood buffer in lambs receiving Sedimin were 34.9 % above the control ($P < 0.05$). In the animals from the test group, blood glucose level was 33.9 % lower than that in the control group. The amount of total protein of the globulin fraction containing selenium was also slightly higher in lambs receiving Sedimin than in the control animals ($P < 0.05$).

At weaning, all physiological and biochemical indicators of lambs' blood, which were pharmacologically corrected for microelement deficiencies, reached a physiological norm. The number of DC decreased by 28.0 %, MDA by 8.2 %, the activity of antioxidant enzymes increased (by 43.3 % for catalase, and by 39.4 % for GPO), while the glucose content decreased by 39.9 % relatively to the beginning of the test. The obtained data testifies to the metabolism activation

in animals of the test group and disappearance of the signs of latent combined hypomicroelementosis, which agrees with the results of other studies [33].

In control lambs, the blood concentration of diene conjugates compared to that at the beginning of the test significantly increased by 17.60 %, while malonic dialdehyde was only 8.06 % higher (the blood level of MDA, as one of the final products of free radical oxidation, is not subjected to significant fluctuations).

2. Blood parameters in Edilbai lambs injected intramuscularly with the Sedimin® (*M*±*m*; SPKh Krasnoyarets, Krasnoyarsk Region, Astrakhan Province, 2014-2015)

Parameter	Test group (n = 5)		Control group (n = 5)	
	beginning of the test	end of the test	beginning of the test	end of the test
Erythrocytes, million/μl ($\times 10^{12}$/l)	7.52±0.28	8.69±0.57*	7.62±0.36	8.01±0.56
Hemoglobin, g/l	81.14±4.12	88.27±6.38*	82.9±3.19	81.5±2.75
Leukocytes, thousand/μl ($\times 10^{9}$/l)	8.45±1.96	8.76±0.71	8.15±0.78	8.26±0.78
Total protein, g/l	69.70±4.16	72.80±6.09*	68.71±3.55	67.21±3.33
Albumin, g/l	23.60±1.88	24.50±1.36	24.30±1.64	23.72±2.51
Globulin, g/l	36.50±2.35	39.70±2.15*	36.50±3.15	35.83±1.78
Glucose, μmol/l	3.01±0.08	1.99±0.07*	2.98±0.06	3.55±0.28*
Diene conjugates, μmol/l	4.11±0.17	2.96±0.17*	3.97±0.56	4.67±0.19*
Malonic dialdehyde, μmol/l	0.76±0.06	0.69±0.02	0.62±0.16	0.67±0.07
Glutathione peroxidase, mmol G-SH · l^{-1} · min^{-1} · 10^3	5.92±0.08	8.25±0.06*	5.77±0.42	4.01±0.54*
Catalase, μmol/ml	3.88±0.13	5.56±0.27*	3.86±0.29	2.88±0.02*
Alkaline reserve, vol.% CO_2	41.80±2.06	56.42±1.17*	42.90±4.15	32.30±1.43*
Selenium, μg/l	38.20±1.44	60.30±1.06*	37.90±1.06	29.50±1.16*
Iodine, mg/l	0.41±0.02	0.52±0.02*	0.44±0.05	0.39±0.01*

N o t e. Description of the groups is given in the Technique section.
* Differences with the beginning of the test are statistically significant at $P < 0.05$.

The blood catalytic activity in control lambs at the end of the test was 25.5 % less than at the beginning of the test. Blood glutathione peroxidase reduced its activity by 30.6 % ($P < 0.05$) to the 4-month age compared to the beginning of the test. This is due to the beginning of the use of plant forages, poorly provided with selenium, iodine and cobalt which are part of antioxidant enzymes (i.e. selenium is necessary for GPO activity, zinc, copper, manganese are necessary for superoxide dismutase) and can activate catalase activity [4, 36]. There was a decrease in the amount of selenium, iodine, and acid capacity of blood in lambs from the control group at the end of the test compared to its onset, indicating an increasing acidic stress which predetermines latent hypomicroelementosis. In the organism, LPO products began to accumulate while reducing the activity of antioxidant enzymes. All this led to depletion of cells antioxidant protection, development of the syndrome of latent hypomicroelementosis, and a decrease in integrative functions of growth and development of young animals.

At the 4-month age, the body weight of lambs was 35.9±2.23 kg in the control flock, and 44.1±3.11 kg in the test flock, or 14.4 % more. The organs and tissues of the lambs from the test group were better provided with physiologically important trace elements (by 15-36 %) than in the control animals, which agrees with the data of foreign authors who studied the content of trace elements in organs and tissues of the lambs of Landsherp German merino [18, 34, 35]. In 4-month-old lambs, the ratio of the meat part of the carcass, which is a functional production feature of the Edilbai sheep and serves as the breed genetic parameter and the functional norm for Edilbai sheeps [33], was 78.6 % in control and 87.0 % in the test lambs.

Thus, the low accumulation of iodine and selenium in the main components of ecosystems in the Lower Volga, along with revealed low hematological parameters, high content of lipid peroxidation products (diene conjugates, malonic dialdehyde) and low activity of antioxidant enzymes (catalase, glutathione

peroxidase) in growing Edilbai lambs in combination with the results of balance test, indicate the development of latent combined hypomicroelementosis in the animals. Intramuscular injection of Sedimin® containing selenium and iodine improves the physiological state of growing lambs, normalizes metabolism and hematological parameters, stabilizes activity of antioxidant system and provides a reliable increase in body weight by 14.4 % ($P < 0.05$) compared to control.

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HORMESIS IN BARLEY (*Hordeum vulgare* L.) PLANTS DERIVED FROM γ-IRRADIATED SEEDS UNDER CONTRASTING WEATHER CONDITIONS

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Abstract

Identification of mechanisms of adaptive response to weak external exposure is one of the most complex and urgent problems of the modern biology. Such reactions include the effect of hormesis which is the stimulating effect of moderate doses of stressors (e.g. the low doses of various physical and chemical agents) repeatedly confirmed at all levels of the organization of living matter. The dynamics of the growth and development of barley (*Hordeum vulgare* L.) plants, grown from γ-irradiated barley seeds of Nur variety, which combines high productivity potential (up to 80 centner/ha), resistance to drought, good forage and brewing qualities of grain, high resistance to lodging and serious diseases, was studied in a field trial. It was shown that irradiation of seeds significantly influenced the development of plants throughout the vegetative period. The duration of the initial stages of ontogenesis was shortened, and the phase of full ripeness came on 5-7 days earlier than in the control. The length of the stems, the weight of 1000 grains, the number of grains per ear, the number of productive stems, the weight of straw and ears increased. The dependence of economically valuable traits on the dose of γ-irradiation of seeds was statistically significantly better described by models that take into account the effect of hormesis. The manifestation of the effect of pre-sowing γ-irradiation was different in the years with contrasting weather conditions. In the dry year of 2014, the increase in yield was determined by the increase in the number of productive stems, and under optimal conditions in 2015, this was due to the increase in the number of grains per ear. In 2016, an increase in the amount of precipitation by 2.5 times relative to the climatic norm leveled the stimulating effects. The results obtained in this study indicate that pre-sowing γ-irradiation of seeds notably affects the development of barley plants throughout the growing season, significantly changing the structure of the crop. In the plants from the seeds irradiated at stimulating doses, the manifestation of economically valuable traits was statistically significantly increased when vegetation seasons were contrasting in weather conditions. Realization of the effect of hormesis specifically depends on the conditions in which the plants developed.

Keywords: barley, gamma irradiation, seeds, hormesis, growth and development stimulation, yield

Identification of mechanisms of adaptive response to weak external exposure is one of the most complex and urgent problems of the modern biology. Such reactions include the effect of hormesis which is the stimulating effect of moderate doses of stressors existence of which was repeatedly confirmed at all levels of the organization of living matter for various physical and chemical agents [1-3[. The obtained experimental data in the last decades show that γ-irradiation of seeds in stimulating doses leads to a change in gene expression, quantitative and qualitative reconstruction of the enzyme systems [4, 5], changes in the concentrations of phytohormones [6], an increase in the mitotic index in root meristems [7, 8], which causes the growth acceleration and plant development in the early stages of ontogenesis. However, up to the present moment,

there is no clear understanding of the mechanisms for the formation of stimulating effects. Moreover, in the field conditions, a number of factors influence the expression and the very possibility of hormesis manifestation [3, 9, 10], the role of which should be assessed to elucidate the mechanisms of response to low-dose irradiation and its practical use.

In our studies [11, 12], the response of barley seeds of different varieties to γ-irradiation at doses of 2-50 Gy was analyzed, and the range of γ-irradiation (10-20 Gy) was estimated in which stimulation of plant development in the early stages of ontogenesis occurs. It has been shown [4, 12] that the activity of a number of enzymes involved in metabolism and antioxidant defense in cells increases in the range of doses 16-20 Gy, stimulating the development of seedlings. Therefore, the arising question is whether the advantage obtained in the early stages of ontogenesis can be realized during the further plant development and crop formation. Keeping up this advantage by plants until the end of the growing season depends on many factors [9, 13]. In this, it is often not clear how short-term and prolonged stimulating effects of irradiation are formed.

The aim of this work was to evaluate the dynamics of growth and development of plants grown from γ-irradiated barley seeds and to clarify the role of growth conditions in the modification of these processes.

Techniques. Experiments were performed in 2014-2016 on spring barley (*Hordeum vulgare* L.) of Nur variety (1st reproduction). Barley seeds were subjected to pre-sowing irradiation using a γ-HPS 120 (60Co) device (Russia) at doses of 16-20 Gy previously established as having stimulation effect [11, 12], and also at doses of 8 and 50 Gy, the dose rate of 60 Gy/h. The moisture content of the seeds (≈ 14 %) corresponded to GOST 12041-82 [13]. The dose of radiation was estimated using a DKS-101 dosimeter (Politekhform-M, Russia, rated relative measurement error 4 %).

The seeds were sown on the date of irradiation (8.05.2014, 15.05.2015 and 8.05.2016, the experimental field of the All-Russian Research Institute of Radiology and Agroecology). Plants from irradiated and control seeds grown on the same field on 20 plots measuring 2×3 m^2 were collected from the central part of each plot (1×2 m^2); the remaining area served as the first protective circuit. A border check rows of barley plants 50 cm wide (the second protective circuit) were planted along the perimeter of the whole test plot. The distance between the plots and from the border along the perimeter of the entire plot area was 40 cm. Plots on the experimental field in 2014 and 2016 were randomized, in 2015 set in an orderly manner. Four plots were used for each dose. Before sowing irradiated and test seeds, a fertilizer mixture was added in accordance with recommendations [10]: 4 kg per 225 m^2, N, P_2O_5, K_2O ratio of 1:1.15:1.45. Hydrolytic acidity, total exchangeable bases, exchangeable calcium and exchangeable magnesium were 4.92; 3.80; 2.87 and 0.52 mg-eq/100 g, respectively, humus — 1.63 %, mobile phosphorus and exchangeable potassium — 24.3 and 66.9 mg/kg, respectively; pH = 4.10. Irradiated and control seeds were sowed according to recommendations [10] in prepared rows with a 4-5 cm depth, located at a 5 cm distance from each other**.** Weeding was carried out when needed but at least once every 14 days. The yield was harvested at a stage of complete ripeness after 95-98, 103-106 and 99-100 days of growing, respectively, in 2014, 2015 and 2016. At least 400 plants were analyzed for each dose.

The onset of ontogenesis phases was determined by dividing the plot into 24 sections of 0.25 m^2. Ten plots were selected, on which the percentage of plants belonged to one or another stage of development was determined. The ontogenesis phase was considered to have occurred if it was manifested in no less than 75 % of the plants. After achieving complete ripeness, the plant were

removed and following parameters evaluated: the height of the plants, the number of stems (tilling capacity), the average number of productive stems per plant, the number of grains per ear, the average straw weight of 100 plants, the average weight of 100 plant ears and 1000 seed weight. Straw, ears and seeds were weighted using an analytical balance OHAUS (USA). The parameters of the yield quality (proteins content, fiber, fat, dry matter and ash in grain and straw) were measured using an Infrapid-61 IR analyzer (Labor-MIM, Hungary). The weather condition data were obtained from the weather station (Obninsk, synoptic weather station index WMO ID 27606) [14].

Statistical processing was carried out in MS Excel, STATISTICA 6.0 (StatSoft Inc., USA), ORIGIN 6.0 (OriginLab Corp., USA), and R ver. 3.2.1 (R Foundation, USA) software. For the mathematical description of the results, the software environment R was used (Brain-Cousens and Cedergreen-Ritz-Streibig models, taking into account the possibility of hormesis manifestation) [15]. Samples were compared using the Mann-Whitney test. The differences were considered statistically significant at $p < 0.05$.

Results. Nur barley variety used in the study combines both high productivity potential (up to 80 centner/ha), resistance to drought, good forage and brewing qualities of grain, and high resistance to lodging and several dangerous diseases. In 2014, transit time of ontogeny phases in plants as dependent on the dose of pre-sowing γ-irradiation of seeds, was not studied. In 2015, the seedling phase was shorter in seeds irradiated with doses of 16 and 20 Gy (Table 1).

1. Deviations in ontogenesis phases (days) **as compared to control depending on the dose of γ-irradiation of barley (*Hordeum vulgare* L.) Nur variety seeds in the years with optimal and excessive moistening** (field plot trials, Kaluga Province)

Phase	2015					2016				
	control	8 Gy	16 Gy	20 Gy	50 Gy	control	8 Gy	16 Gy	20 Gy	50 Gy
Seedlings	8	0	–1	–2	–1	8	0	0	0	0
Tillering	15	–1	–1	–2	0	14	0	0	0	0
Stem elongation	15	0	0	+1	0	15	0	–2	–2	0
Heading	18	–1	0	+1	–1	17	0	0	0	0
Flowering	3	0	–1	–1	0	3	0	0	0	0
Milky ripeness	14	–1	–2	–1	0	12	0	0	0	0
Middle dough stage	15	0	0	–1	0	14	0	0	0	0
Complete ripeness	18	0	0	–2	0	17	0	0	0	0
Total, days	106	103	101	99	104	100	100	98	98	100

Apparently, the earlier appearance of complete shoots was caused by the acceleration of the root system development of plants grown from irradiated seeds, which we observed in our previous studies [4, 12]. It should be noted that the seedling phase was shorter in plants grown from seeds irradiated at a dose of 50 Gy, which does not belong to stimulants and does not improve morpho-physiological indices in laboratory conditions [12]. The rapid passing the seedling phase in the plants grown from irradiated seeds determined the earlier onset of the tillering and stem elongation. However, the heading phase in all plants occurred almost simultaneously. By reducing the time of the flowering, milky, middle dough and complete ripeness in plants grown from seeds irradiated at a dose of 20 Gy, and the phases of flowering, milky, middle dough ripeness in plants from seeds irradiated at a dose of 16 Gy, complete ripeness occurred 7 and 5 days earlier, respectively, than in the control. Similar results [16] have been described in the literature, as well as a wave-like manifestation of the stimulating effect on ontogenesis [17, 18] when control plants at flowering reached the same phase of ontogenesis as the experimental ones but then again lagged behind till the phase of complete ripeness stage. In 2016, seed irradiation almost did not affect the time of passing the main phases of ontogenesis. Only earlier stem elongation was observed in plants from irradiated seeds, which led to a faster transition to

tillering. However, this did not affect the general distribution of the ontogenesis stages in time. The lack of stimulation in passing ontogenesis phases in plants grown from irradiated seeds in 2016 was apparently associated with an extremely high amount of precipitation.

In 2014, the stem height in plants from seeds irradiated at 8 Gy statistically significantly exceeded the control (Fig. 1, A) and was characterized by the lowest variability of the studied parameter. In 2015, the height was statistically significantly higher throughout the entire dose range. In 2016, no increase in the height of productive stems compared to control was observed. A statistically significant increase in 1000 grain weight was detected in 2014 for 16 and 20 Gy and in 2015 for 20 Gy (see Fig. 1, B). In 2016, a statistically significant change in the 1000 grain weight was not found.

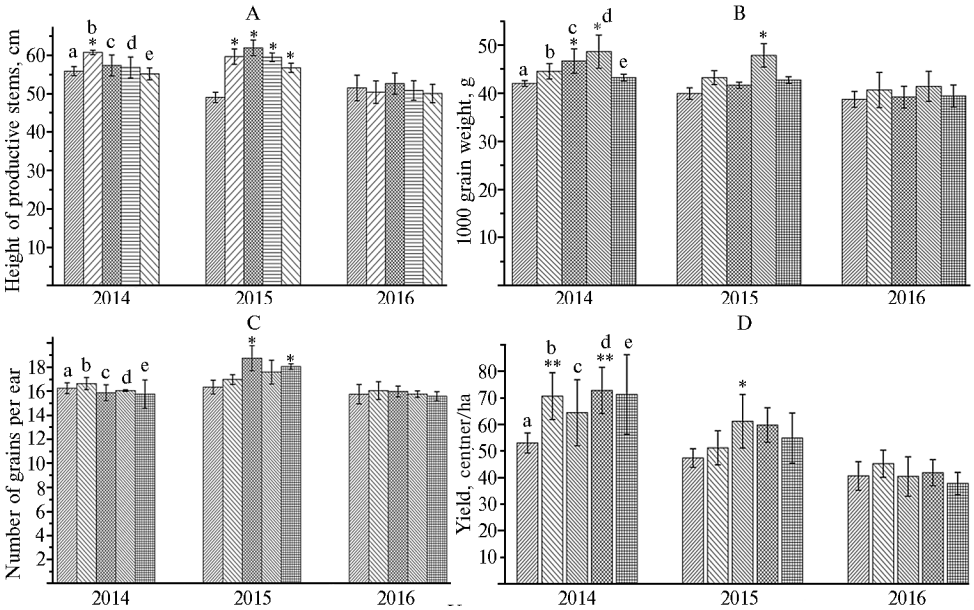

Fig. 1. Height of productive stems (A)**, 1000 grain weight** (B)**, the number of grains per ear** (C) **and the yield** (D) **depending on the dose of γ-irradiation of barley (*Hordeum vulgare* L.) Nur variety seeds in the years with precipitation deficit (2014), optimal conditions (2015) and an excess of moisture (2016):** a — control, b — 8 Gy, c — 16 Gy, d — 20 Gy, e — 50 Gy. The diagrams show the standard errors of the mean (field plot trials, Kaluga Province).
*, ** Differences with control are significant at $p < 0.05$ and $p < 0.15$, respectively.

Although the number of seed germs per ear is genetically controlled, the number of grains in the ear is strongly dependent on the interaction of the genotype and the environment [19]. Particularly strong influence on this element of the yield structure is provided by growing conditions in the zone of unstable moistening [20]. In 2014 and 2016, we found no statistically significant differences in the number of grains per ear, but in 2015 this value statistically significantly increased under the influence of doses of 16 and 50 Gy (see Fig. 1, C).

In 2014, the number of stems per plant increased statistically significantly for 16, 20 and 50 Gy (Fig. 2, A), and the number of stems with ear per plant was higher for 8, 20 and 50 Gy pre-sowing exposure of seeds. In 2015 and 2016, no statistically significant changes in the number of stems and stems with ears were detected. The ratio of productive shoots to the total shoots did not vary statistically significant for all the doses studied. Hence, the number of productive shoots increased or decreased proportionally to the total number of shoots. This is in line with the data that the ratio of productive stems to the total stem number is mostly constant for a species and does not depend on external factors [21,

22]. However, it is necessary to note the decrease in the ratio between the number of productive shoots and the total number of shoots in 2016 compared to 2014 and 2015.

In 2014, with an increase in the dose of seed irradiation, the weighs of ears and straw increased (see Fig. 2, B), but statistically significant differences were observed only for 8 Gy (straw) and 20 Gy (ears). In 2015, an increase in the ear weight was observed upon seed exposure to 16 Gy. The differences between the weight of straw and ears in test and control plants in 2016 were not statistically significant.

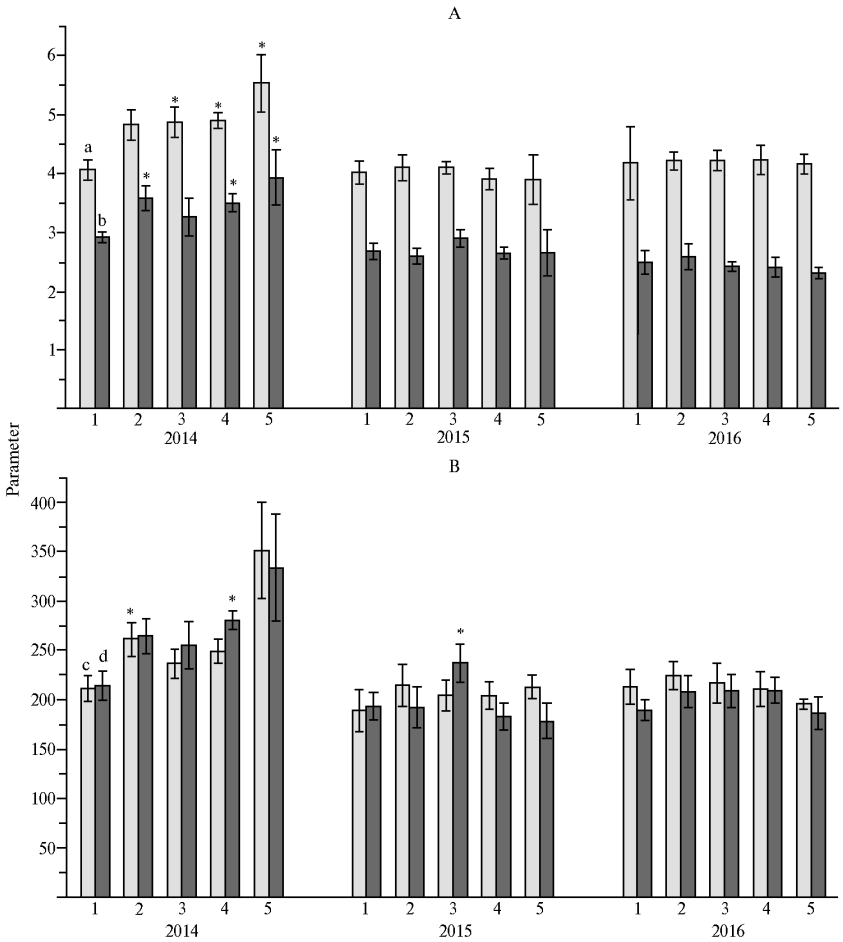

Fig. 2. Number (pcs.) **of stems** (a) **and stems with ears** (b)**, and weight** (g) **of straw** (c) **and ears** (d) **depending on the dose of γ-irradiation of barley (*Hordeum vulgare* L.) Nur variety seeds in the years with precipitation deficit (2014), optimal conditions (2015) and an excess of moisture (2016):** 1 — control, 2 — 8 Gy, 3 — 16 Gy, 4 — 20 Gy, 5 — 50 Gy (field plot trials, Kaluga Province).
*, ** Differences with control are significant at $p < 0.05$; the diagrams indicate standard errors of the mean.

The obtained results made it possible to estimate the yield of barley (see Fig. 1, D). This indicator increased by 37, 34, 38 and 37 % in 2014, and by 8, 29, 26 and 19 % in 2015 for doses of 8, 16, 20 and 50 Gy, respectively. The yield increment in 2014 in 8 and 20 Gy variants, and also in 2015 with irradiation at 16 Gy was statistically significant ($p < 0.15$). The observed tendency to increase the yield indicates the positive influence of pre-sowing seed irradiation on the growth and development of derived plants. Apparently, the absence of a positive effect of pre-sowing γ-irradiation in 2016 was due to the excessive

amount of precipitation (an almost 3-fold excess). The yield of barley in 2016 was about 40 centner/ha in the whole range of doses, which is due to a decrement in the parameters of the main elements of the crop structure (1000 seed weigh, grain number per ear and the number of productive stems) compared to those in 2014 and 2015 .

By influencing on metabolism, irradiation of seeds can lead to a change in the content of plant substances that determine quality of the products. Many researchers, in addition to the increment of the yield, noted an increase in sugar content of sugar beet, proteins in cereals, starch in potatoes, alkaloids in medicinal plants, vitamins in fruit and vegetable crops, carotenoids in carrots, ascorbic acid in cabbage, etc. [8, 16]. The biochemical analysis performed by us does not allow us to conclude that the composition of grain and straw has changed qualitatively in plants from irradiated seeds (all the studied indices were within the norm, data are not given).

The variability of weather conditions accounts for about a third of the variability in yield parameters in crops [23]. This confirms the comparison of the control values of the crop structure elements evaluated by us in the years different in weather conditions (Table 2). Therefore, weather conditions can significantly modify the effect of hormesis observed in the early stages of ontogenesis up to its complete elimination [8, 13, 23, 24].

2. Yield structure of barley (*Hordeum vulgare* L.) Nur variety plants derived from non-irradiated seeds (field plot trials, Kaluga Province)

Parameter	2014	2015	2016
Stem height, cm	55.88±1.17	49.03±1.35*	51.49±3.32
Weight of 1000 seeds, g	42.01±0.58	39.91±1.21	38.7±1.66*
Number of grains per ear, pcs.	16.24±0.45	16.34±0.57	15.57±0.81
Number of stems, pcs.	4.05±0.17	4.01±0.20	4.16±0.61
Number of stems with ears, pcs.	2.91±0.08	2.68±0.14	2.49±0.19*
Straw weight, g	214.18±14.76	193.01±13.98	189.3±10.30
Ear weight, g	211.49±13.15	188.89±21.39	213±17.60
Crop yield, centner/ha	50.01±3.77	47.34±3.52	40.56±5.40*

* Differences with the indexes recorded in 2014 are statistically significant at $p < 0.05$.

3. Weather conditions in the years of growing barley (*Hordeum vulgare* L.) Nur variety plants in the field plot trials (Kaluga Province)

Month	Sum of effective temperatures, °C			Precipitation, mm			Selyaninov's hydrothermal index		
	2014	2015	2016	2014	2015	2016	2014	2015	2016
May	423.3	347.7	339.6	19.1 (43)	70.2 (43)	117.3 (43)	0.45	2.01	3.45
June	468.7	511.6	512	78.9 (77)	80.0 (77)	268.4 (77)	0.99	1.56	5.24
July	612.6	545.8	612.7	59.0 (80)	106.7 (80)	282.7 (80)	1.04	1.95	4.61
August	302.7	470	294.9	8.2 (71)	50.0 (71)	94.0 (71)	0.91	1.06	3.18
Total	1808.2	1875.1	1759.2	165.2 (271)	306.9 (271)	762.4 (271)	0.85	1.63	4.33

N o t e. The norm of the sum of effective temperatures for barley in the Central Russia is 1200-1800 °C [24]; in parentheses, the norm of precipitation per month is indicated, mm [14]. Growing periods in 2014 — from May 8 to August 14, in 2015 — from May 15 to August 28, and in 2016 —from May 8 to August 15.

We compared the weather conditions of the growing seasons 2014-2016 and calculated the values of the Selyaninov's hydrothermal index (HTI) (Table 3) as $HTI = r/0{,}1\Sigma T_a$, where r is the sum of precipitation for a period with average daily temperatures above 10 °C, mm; ΣT_a is the sum of the effective temperatures during the growing season, °C [25]. The hydrothermal coefficient characterizes the agroclimatic conditions of cultivation [25]. The lower the HTI, the drier the growing period is. Extremely low precipitation along with the optimum sum of effective temperatures caused a very low HTI (0.45) in May 2014. Taking into account that the strongest influence of weather conditions on the results of pre-sowing seed irradiation manifests itself in the initial stages of ontogenesis [26], during insufficient moisture in May 2014, irradiated seeds gained an advantage in growth and development

which affected the yield. In general, the whole growing period of 2014 (HTI = 0.85) is estimated as drought. Unlike in 2014, May 2015 was rainy (HTI = 2.01, which indicates an excessive moistening of the soil). The HTI value was also high for the entire observation period in 2015, but on the whole HTI was approaching the norm. Despite such different weather conditions in 2014 and 2015, the yield in experimental plants derived from irradiated seeds exceeded the control values for a number of important parameters (see Fig. 1, 2). The HTI for the growing season of 2016 reached an extremely high value of 4.33. This was the result of intense and abundant rains which 2.5 times exceeded the norm of precipitation for the central part of the Russian Federation and 4.5 times exceeded the values of 2014. Excessive precipitation together with a sufficient sum of effective temperatures leveled the stimulating effect of seed irradiation.

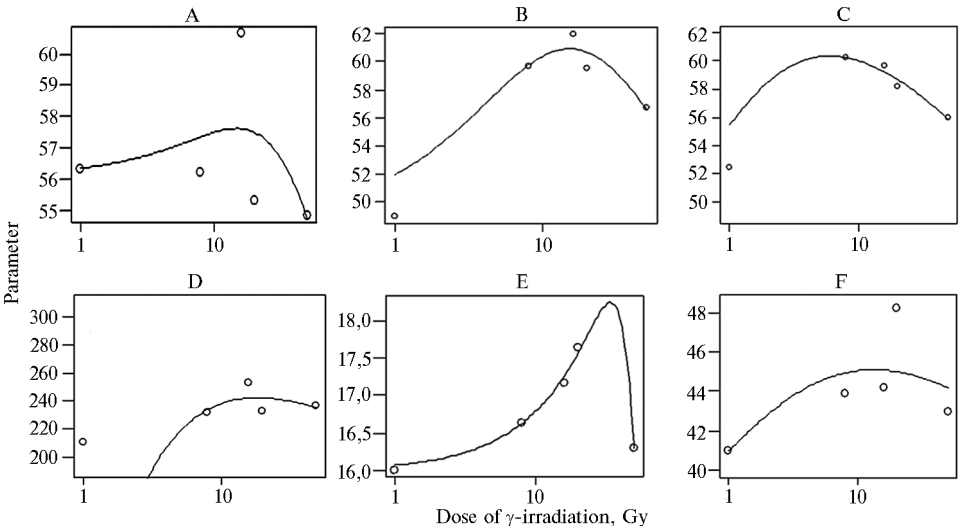

Fig. 3. Approximation of barley (*Hordeum vulgare* L.) Nur variety yielding parameters by Brain-Cousens and Cedergreen-Ritz-Streibig models taking into account the effect of hormesis (15): A — 1000 grain weight, g (2014, Brain-Cousens model, $p < 0.067$); B — stems height, cm (2015, Brain-Cousens model, $p < 0.098$); C — stems height, cm (2014-2015, Cedergreen-Ritz-Streibig model, $p < 0.013$); D — ear weight, g (2014-2015, Cedergreen-Ritz-Streibig model, $p < 0.011$); E — grain number per ear, pcs. (2014-2015, Cedergreen-Ritz-Streibig model, $p < 0.001$); F — 1000 grain weight, g (2014-2015, Cedergreen-Ritz-Streibig model, $p < 0.098$) (Kaluga Province, field plot trials; plants were grown from γ-irradiated seeds). For $p < 0.15$, the model describes the experimental data statistically significantly better than the logistic model.

In mathematical description of the obtained results (Fig. 3), it turned out that the response to irradiation in the range of stimulating doses by 1000 grain weight (see Fig. 3, A) and the stem height (see Fig. 3, B) corresponds to the Brain-Cousens model. As a matter of interest, even the combined data of field plot trials for two years with contrasting weather conditions, 2014 and 2015, for 1000 grain weight (see Fig. 3, F), stem height (see Fig. 3, C), ear weight (see Fig. 3, D) and the number of grains per ear (see Fig. 3, E) corresponded to the Cedergreen-Ritz-Streibig model. In all these cases, the effect of hormesis was statistically significant. For the remaining parameters, we did not found compliances to the Brain-Cousens and Cedergreen-Ritz-Streibi models (data not shown). It should be noted that in the present work the effect of pre-sowing seed irradiation was studied at the final stages of ontogenesis, when a number of factors actively influence the elements of crop structure. Nevertheless, the results of mathematical modeling (see Fig. 3) convincingly show that the advantage in the development of irradiated seeds in the early stages of ontogenesis can positively affect plant yielding and the yield structure in the field.

How does the obtained advantage at the onset of ontogenesis ensure an increase in the yields of barley? Formation of the plant root morphology and architecture occurs in the early stages of development and can vary greatly under the influence of external factors even within a single species [27]. In addition, in cereals the growth of the primary root system dominates during this period, and the quality of adventitious roots will determine development in the final stages of ontogenesis [28]. Thus, in drought conditions, barley plants, that have grown from irradiated seeds, due to a more developed root system in the early stages of ontogenesis have the advantage of obtaining water from the soil, which will eventually affect the subsequent stages of development. Therefore, it is not surprising that stimulation is manifested to the maximum extent in drought spring conditions, when rapidly growing seedlings were in more favorable conditions for nutrient and moisture supply [9, 16, 29] which was noted in 2014.

Despite the presence of other modifying influences, e.e. mineral deficit [9] and low soil temperature [16], the sum of effective temperatures and precipitation during the growing season remains as the key factors. Too dry or extremely humid conditions can minimize the stimulating effect of seed irradiation [16]. So, in the test of 2016 which was characterized by an excessive amount of precipitation during the entire growing season we did not record any significant changes in yields. However, weather conditions can influence the results of pre-sowing irradiation in a different way. In our experiments, the change in the water regime (2014-2015) did not lead to a leveling of the stimulating effect, but to its implementation by switching to an alternative course of ontogenesis, which ensured an increase in the yield under changed conditions. The “harvest triad” (the number of productive stems, the number of grains per ear and the 1000 grain weight), for the sake of complete realization, requires favorable conditions. Due to the self-regulation of these elements by agrocenosis under changing environmental conditions, the yield, when changing one parameter, can be maintained due to compensation by others [29]. Thus, in the drought (HTI = 0.85) 2014 year, in addition to the increment of the 1000 grain weigh, an increase in the number of productive stems was noted, and in the optimal 2015 year the yield gain was achieved due to the increased number of grains per ear. It should be noted that the control plants did not show wide variability of the studied parameters in contrasting weather conditions (the indices remained practically the same), except for the stems height in 2015, as well as the 1000 grain weight, the number of productive stems and the yield in 2016.

So, the present study indicates that pre-sowing γ-irradiation of seeds affects the development of barley plants throughout the growing season, substantially changing crop structure. Positive and statistically significant effects of stimulating doses on economically valuable traits were noted during cultivation in growing seasons with contrasting weather conditions. The realization of hormesis effect depends on the environmental factors in which plant development occurs.

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EFFECT OF INOCULATION WITH ARBUSCULAR MYCORRHIZAL FUNGUS *Rhizophagus irregularis* ON AUXIN CONTENT IN HIGHLY MYCOTROPHIC BLACK MEDICK UNDER LOW PHOSPHORUS IN SOIL

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Abstract

The investigation is focused on the elucidation of symbiotic effectiveness mechanisms of arbuscular mycorrhiza (AM) which is one of the most widespread symbiosis, developed between Glomeromycota fungi and 92 % of land plants. The role of auxin in regulation of plant development with AM fungi symbiosis might be considered as one of such mechanisms, since this plant hormone plays a key role in root development and accelerates spore germination, intensifies the infection process and subsequent growth of the hyphae after exogenous addition. This study is aimed to identify for the first time the dynamics of endogenous indole acetic acid (IAA) concentration in roots and leaves of strongly mycotrophic black medick line (*Medicago lupulina*) under conditions of low-phosphorus available for plants nutrition in the soil. Analyses were carried out in the course of plant development. On day 14 after sowing (DAS), at the stage of the first true leaf development, we observed primary infection, the formation of the first arbuscules (a, %) and vesicles in roots, and the beginning of lateral root formation and primary growth response to mycorrhization manifested in the development of above-ground plant parts. On DAS 21 (the second leaf stage) there was a significant growth response to mycorrhization and arbuscule development in roots. On DAS 35, at shooting stage, the arbuscule development and active development of vesicles in roots occurred. Finally, on DAS 50 (at flowering start) the vesicles development was more active. Analysis of mycorrhization level showed that arbuscules played a key role in the formation of effective symbiosis in black medick at the stages of the first and second leaf, and the a/(100 % – a) value increased to 8.0±0.7 and 18.5±1.3, respectively. At later stages of shooting and flowering, their role diminished as compared to the mycelium development in the roots. The efficiency of the inoculation with AM *Rhizophagus irregularis* symbiotic fungi tested by weight of aboveground parts significantly increased starting with the phase of the second leaf, and remained high until the end of the experiment (above 120 %). At the same time, the weight response was absent in roots until the phase of flowering. A 2.2-fold increase in IAA level in roots at the phase of the first leaf, i.e. at the earliest stages of AM symbiosis establishing, led to inhibition of the root growth. On the other hand, the 1.8-fold increase in endogenous IAA in leaves at the second leaf phase was preceded by the elevation of AM efficiency by weight for aboveground parts, and, therefore, a shift in IAA concentration significantly intensified

development of the aboveground parts at the shooting and flowering.

Keywords: auxin, indolilacetic acid, arbuscular mycorrhiza, symbiotic efficiency, *Rhizophagus irregularis*, *Medicago lupulina*

Arbuscular mycorrhiza (AM) is one of the most widespread natural symbiosis formed by *Glomeromycota fungi* and 92 % of land plants. AM has a significant diversity of morphotypes and plays a key role in meadow and forest ecosystems, contributing to a significant increase in the mineral (especially phosphate) nutrition of plants [1, 2]. AM can determine the productivity of plants and their adaptation to adverse environmental factors, especially to low amounts of nutrients in soil. It is shown that the availability of inorganic phosphorus (Pi) is a key factor in the development of AM symbiosis. With a lack of phosphorus, the root length can increase by more than 80 % due to the development of the fungus [3], while the increase in availability of Pi significantly reduces the colonization rate [4]. At the same time, the development of arbuscular mycorrhiza does not always affect the length of the root, i.e. AM can affect the number of lateral roots, modulating root architecture. It is assumed that such a change leads to an increase in the number of appressoria. This hypothesis is supported by data on the induction of the lateral roots formation in *Medicago truncatula* during the germination of fungal spores, as well as the increase in the number of lateral roots in the *lrt1* mutant of maize during AM formation [5, 6]. Morphological and physiological modifications in the root system of plants indicate the possibility of AM-induced changes in the activity of endogenous growth regulators. According to modern concepts, many of the phytohormones are involved in the induction of the root formation during phosphate starvation [7-11]. Since auxin plays a key role in the regulation of root development, it has been suggested that this hormone can affect AM development [12, 13].

In the past few years, the role of auxin in the development of the relationship between the host plant and the AM fungus has been identified. Exogenous treatment with auxin accelerated spore germination, intensified colonization and subsequent growth of hyphae [12]. An increase in the concentration of indole acetic acid (IAA) was observed during colonization of the leek roots by AM fungi [14] that was accompanied by changes in the structure of the roots, they became more numerous, branched and shortened [15, 16]. At the same time, AM-induced accumulation of IAA was not systemic. Inoculating one part of the roots of the plant and preserving the other to be intact (split-root system) showed that the hormone was accumulated only in the roots that were directly inoculated [17]. Data have been obtained on the increase in the content of another natural auxin, indole-3-butyric acid (IBA), in AM colonization of maize roots [18, 19]; IBA is also known as a root growth regulator [20]. Decreased AM colonization was noted in mutants with disorders of auxin biosynthesis, such as "bushy" pea mutant (*Pisum sativum*) characterized by a low amount of IAA in shoots and roots, and a diageotropic mutant ("dia-geotropica") of tomato (*Solanum lycopersicum*) resistant to auxin [13, 21]. Along with the increase in the amount of free IAA, a change in the content of its conjugates with sugars and amides was observed, which correlated with the accumulation of transcripts of genes encoding IAA-amido synthetase and GH3-like protein in mycorrhization of tomato roots [22]. Another mechanism for regulating the amount of free IAA can be associated with proteins that transmit the auxin of the PINs family, which ensure its polar transport in the axial organs and lateral redistribution. Mutants with a violation of the coding of these carriers had a weakened symbiosis with AM fungi, probably due to a change in the intensity of the lateral root insertion [23, 24].

The reported data indicates a significant dynamics of accumulation of

auxin in plant roots during the formation of a mutually beneficial AM symbiosis. The synthesis, conjugation and transport of the hormone are involved in the process. Such a complexity seems to be the reason why in a number of cases the role of auxin was not traced or manifested itself weakly [25]. The activity of the components can vary considerably depending on the species, age and stage of plant ontogenesis.

In the present study, for the first time, an increase in the auxin content in roots was shown in the strongly mycotrophic black medick line even at the early stages of AM formation, with mycorrhiza leading to inhibition of root growth, but at the same time initiating the development of the shoot. For the first time, it was suggested that AM indirectly, through a significant increase in the amount of IAA, has a beneficial effect on the development of assimilation apparatus.

The aim of our work was to analyze the effectiveness of mycorrhiza and to evaluate the dynamics of the endogenous concentration of indole acetic acid in roots and leaves of black medick under low phosphorus in the soil.

Techniques. As a model plant, black medick (*Medicago lupulina* L. var. *vulgaris* Koch) cultivar VIC32 (MlS-1 line) was chosen. For inoculation, we used strain RCAM00320 AM of the fungus *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schuessler 2010 (formerly known as *Glomus intraradices* N.C. Schenck & G.S. Sm. 1982, strain CIAM8) from the collection of the All-Russian Scientific Research Institute of Agricultural Microbiology, laboratory maintenance on *Plectranthus australis* R. Br. [26].

In pot trials [27], we used substrates with a low content of available phosphorus (Pa). In sod-podzol light loamy soil, P_2O_5 and K_2O contents according to Kirsanov were 3.9 and 7.0 mg per 100 g, respectively, with organic matter of 3 % and a pH_{KCl} (after liming) of 6.1. The substrate was an air-dry soil-sand mixture in a ratio of 2:1, which was autoclaved twice (at 2 day intervals) for 1 hour at 134 °C and 2 atm (no toxicity after treatment). Four plants per pot were planted in pots filled with 210 g of soil-sand mixture, and cultivated with slow ventilation in a light box that had previously been subjected to UV sterilization. The day/night regime was 18 h/6 h, the air temperature was 24-26 °C, and the luminous flux was 1500-1700 lm. In the test variant, inoculation was carried out with the roots of the *Plectranthus* mycorrhizated by RCAM00320, and non-mycorrhizated roots of the Plectranthus were used in control.

To quantify the mycorrhiza (arbuscular abundance a, %), maceration and staining roots of plants with AM [28] and light microscopy [29] were performed according to the description [30]. The method was improved using the advanced computer program in the Microsoft Excel shell [31]. The symbiotic efficacy of AM was determined by the gain of raw biomass of the aboveground parts and roots [32].

The auxin (IAA) level was determined in roots and aboveground parts during each phases of growth. Cotyledons were collected (day 1 after sowing), rounded and the 1st true leaf (day 14, 1st true leaf stage), rounded, 1st and 2nd true leaves (day 21, 2nd true leaf stage), 2nd, 3rd and 4th leaves (day 35, shooting stage), and three top leaves (3rd, 4th and 5th leaves) below the first inflorescence (day 50, flowering stage). The samples (lyophilized roots and leaves) were homogenized and extracted with 80 % ethanol (1:10 w/v) for 16-20 hours at 4 °C. The alcohol extract was separated by centrifugation and then evaporated to an aqueous residue. Auxins extraction into diethyl ether from the acidified aqueous residue of the alcohol extract followed by extraction into a solution of sodium carbonate and re-extraction into diethyl ether (after acidification of sodium carbonate) was carried out with a decrease in volume in each next cycle of

extraction and re-extraction [33]. After methylation, the amount of auxins was determined by solid-phase enzyme-linked immunosorbent assay [33].

The figures show the mean (M) and standard errors of the mean (±SEM). Differences assessed according to Student's t-test were considered statistically significant at $P < 0.05$.

Results. Pot test allows us to provide optimal conditions for the development of AM and to avoid spontaneous infection with rhizobia and other symbiotic microorganisms [27]. Black medick ($2n = 16$) is one of the most widespread species of the genus *Medicago*, the subgenus *Lupularia* (Ser.) Grossh., family *Leguminosae* Endl. Black medick is capable of a significant response to mycorrhiza with fungus *R. irregularis* with a low Pa content in soil [28], seed productivity is up to 2500 pcs. per plant and even more when grown in a greenhouse for reproduction. This plant species is autogamous with a genome size of ~ 500 MB. The plants of the MIS-1 line used in the study showed signs of dwarfism in the absence of AM inoculation and at a low soil Pa. Strain RCAM00320 is an obligate symbiont [26].

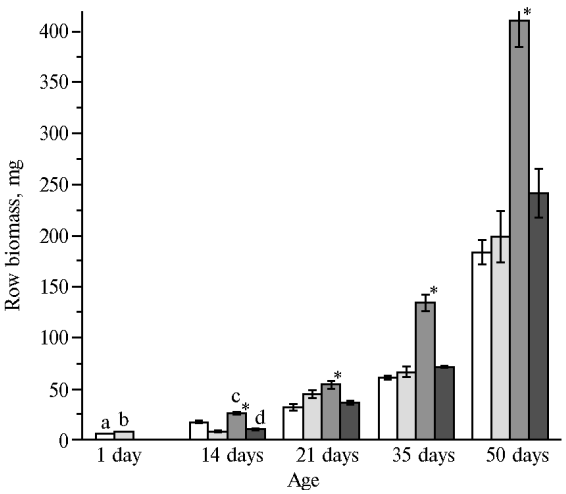

Fig. 1. The dynamics of raw biomass accumulation in the aboveground part (a, c) **and roots** (b, d) **of black medick (*Medicago lupulina*) plants without inoculation** (a, b) **in inoculation with *Rhizophagus irregularis*** (c, d) **under low available phosphorus in soil.** The asterisk indicates variants in which differences with plants without inoculation (control) are statistically significant at $P < 0.05$.

Development of black medick includes 1st and 2nd true leaves stage, shooting and flowering. It was previously established that the AM primary colonization, the formation of the first arbuscules and intra-root vesicles, the formation of lateral roots, and the primary growth response to mycorrhiza occur at the 1st true leaf stage (on day 14). In the next stage (on day 21), when the 2nd true leaf is formed, a significant growth response to mycorrhization is revealed, the arbuscules develop in the root. In the phase of shooting (on day 35), symbiotic structures (arbuscules and vesicles) are actively formed. At the beginning of flowering (on day 50), the development of vesicles is intensified [26]. Thus, these periods are most important for plant and AM formation.

At low phosphorus in soil, plants very slowly increased biomass of roots and aboveground parts in the control (Fig. 1). The dynamics of weight change was similar for both analyzed parts of plants. A significant accumulation of biomass was noted only during flowering. Inoculation of plants practically did not affect growth of the roots, but induced a multiple increase in the aboveground part biomass. The first response to mycorrhization was observed in shoots from day 14, the most significant increment of the aboveground biomass occurred during shooting.

At early stages of the plant ontogenesis, the development of *R. irregularis* was sharply intensified, primarily the formation of arbuscules. The main transporters responsible for the transition of phosphate, water and other substances are located on the branches of periarbuscular membrane. Arbuscules rate can significantly change as the plant develops, which is probably due to the limited

life time (4-5 days) of arbuscules which degradation can take only 2.5-5.5 hours [34]. In the opinion of some authors, the plant controls the number of arbuscules by redistributing the flow of carbon-containing compounds [35]. Earlier it was shown [26] that arbuscules develop intensively at later periods. The results obtained by us, apparently, reflect the intensive development of the mycelium (Fig. 2).

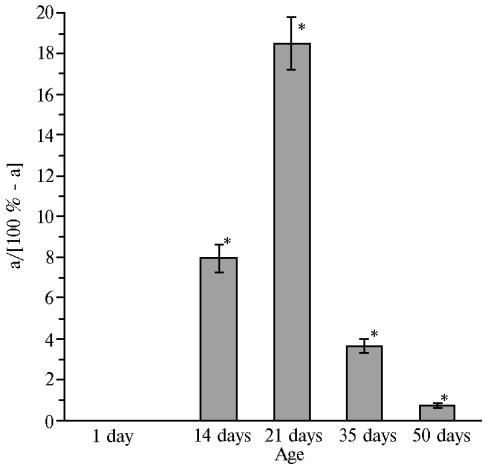

Fig. 2. The ratio a/(100 % – a), characterizing the multiplicity of the excess of the arbuscular abundance a (%) over the abundance of the intra-root mycelium of *Rhizophagus irregularis* 100 % – a (%) in black medick (*Medicago lupulina*) under low available phosphorus in soil. The asterisk indicates variants, the differences between which are statistically significant at $P < 0.05$.

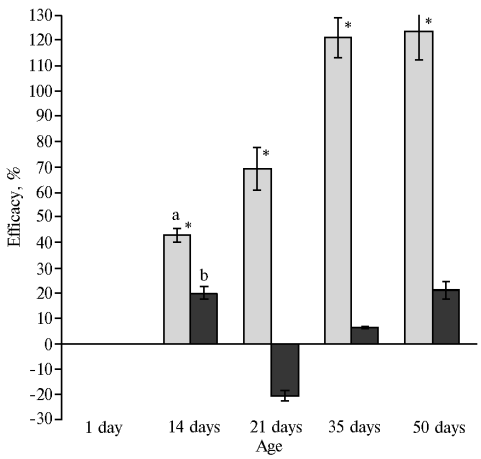

Fig. 3. Arbuscular mycorrhiza symbiotic efficacy calculated based on raw biomass of the aboveground parts (a) **and roots** (b)**, in black medick (*Medicago lupulina*) in inoculation with *Rhizophagus irregularis*** (c, d) **under low available phosphorus in soil.** The asterisk indicates variants in which differences with plants without inoculation (control) are statistically significant at $P < 0.05$.

On day 21, the effectiveness of root mycorrhization became negative (Fig. 3) and the increase in the number of arbuscules was not resulted from the intensive root growth. In the later stages of plant development, the efficiency of mycorrhization had small positive values. On the contrary, the symbiotic effectiveness of the aboveground part has always been positive. This index reached 120 % by shooting and did not change further.

These facts are in general consistent with generally accepted beliefs about the dynamic of AM development. It is known that during mycorrhization the ratio of root and shoot biomass decreases [1, 36, 37], since the increased consumption of organic substances by roots initiates the development of photosynthetic organs. According to some data, from 4 to 20 % of photoassimilates can be directed to the mycorrhizal root system [38-40]. The disruption of their transport significantly inhibits the development of AM, which can be considered as an effective regulatory mechanism. In turn, the development of arbuscules and intra-root mycelium ensures the requirements of the plant in mineral nutrition [41].

The proposed symbiotic model (strongly mycotrophic line MIS-1-*R. irregularis*) made it possible to characterize the dynamic processes in the fungus and host plant. Particularly interesting are the multidirectional changes in the accumulation of shoot and root biomass and in the effectiveness of symbiosis.

The content of IAA in the raw biomass of roots without AM was low in the seedling phase and gradually increased, slightly increasing to the flowering phase (1.4-fold in the aboveground parts and 1.8-fold in the roots) (Fig. 4). It should be noted that the content of IAA in the roots was lower

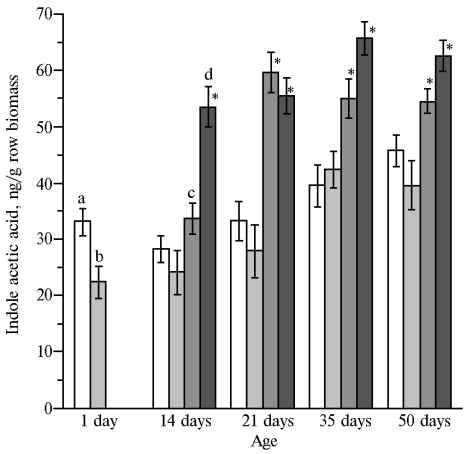

Fig. 4. Content of indole acetic acid in raw leaf biomass (a, c) **and roots** (b, d) **in black medick (*Medicago lupulina*) plant without inoculation** (a, b) **and in inoculation with *Rhizophagus irregularis*** (c, d) **under low available phosphorus in soil.** The asterisk indicates variants in which differences with plants without inoculation (control) are statistically significant at $P < 0.05$.

than in shoots, except the shooting period, which corresponds to the current understanding of the IAA balance in tissues and organs [42-44]. Despite the lack of symbiotic efficacy, calculated from root weighting, the first response to mycorrhization was the increase in the amount of IAA at the 1st true leaf (see Fig. 4). The IAA level in roots of AM plants further remained 1.6-2.2 times above the control values and reached a maximum at shooting. In the aboveground parts, the response to mycorrhization manifested in a change of IAA level was fixed at the 2nd true leaf stage, after which the amount of IAA did not increase

So, when the black medick plants were inoculated with the fungus *Rhizophagus irregularis* under low phosphorus content in soil, a significant increase in the amount of indole acetic acid in the roots and shoots was observed. An increment in the auxin level in the root occurred even at the earliest stages of arbuscular mycorrhiza formation. In this, plant inoculation practically did not affect the increase in root biomass, but led to a significant increase in biomass of the aboveground part. Probably, the attractant effect characteristic of auxin serves as a trigger mechanism for enhancing transport of carbon-containing compounds to the roots.

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