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

AGRICULTURAL BIOLOGY

Since January, 1966

ANIMAL BIOLOGY

Vol. 53, Issue 4
July-August

2018 Moscow

Covered in Scopus, Web of Science (BIOSIS Previews, Biological Abstracts, Russian Science Citation Index), Agris

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

Publisher: Agricultural Biology Editorial Office NPO

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

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

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

SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA
[AGRICULTURAL BIOLOGY], 2018, Vol. 53, № 4

CONTENTS

REVIEWS, CHALLENGES

Glazko V.I. Gene and genomic levels of domestication signature (review) 659

Galochkin V.A., Ostrenko K.S., Galochkina V.P. et al. Interrelation of nervous, immune, endocrine systems and nutritional factors in the regulation of animal resistance and productivity (review) 673

Bagno O.A., Prokhorov O.N., Shevchenko S.A. et al. Use of phytobioticts in farm animal feeding (review) 687

Sudakova N.V., Mikodina E.V., Vasilyeva L.M. Sturgeon (*Acipenseridae*) artificial reproduction paradigm changeover under conditions of natural stock deficit of sturgeon in the Volga-Caspian basin (review) 698

GENETICS OF BREEDING VALUES

Belous A.A., Sermyagin A.A., Kostyunina O.V. et al. Study of genetic and environmental factors, characterizing the feed efficiency in Duroc pigs 712

Melnikova E.E., Bardukov N.V., Fornara M.S. et al. Effects of genotypes for *IGF2*, *CCKAR* and *MC4R* on the phenotypic estimations and breeding values for productive traits in pigs 723

REPRODUCTIVE BIOTECHNOLOGIES

Tkachev A.V., Tkacheva O.L., Rossokha V.I. Associated connection of erythrocitary antigens with characteristics of stallion semen after cryoconservation 735

Abilov A.I., Mityashova O.S., Mymrin S.V. et al. Endogenous hormone level in bull sires in relation to age, autoimmune status, and production performance of maternal ancestors 743

Brigida A.V., Sorokin V.I., Kovalchuk S.N. et al. Forecasting the embryo productivity of donor cows on the basis of echographic characteristics of the ovaries 753

AQUACULTURE

Krasilnikova A.A., Tikhomirov A.M. Reproduction of Russian sturgeon (*Acipenser gueldenstaedtii*) viable juveniles using cryopreserved sperm and behavioral reactions of the cryo-progeny 762

Garlov P.E., Belik N.I., Rybalova N.B. et al. Developing the biotech method for effective reproduction of valuable fish species populations 769

ANIMAL FEED AND FEEDING

Yildirim E.A., Ilina L.A., Laishev K.A. et al. Mycotoxins diffusion in feeds of summer pasturing ration of *Rangifer tarandus* in Arctic zones of Russia 779

Kavtarashvili A.Sh., Stefanova I.L., Svitkin V.S. et al. The ration recipes developed to improve effective and safe biofortification of hen (*Gallus gallus* L.) eggs 787

Bagirov V.A., Duskaev G.K., Kazachkova N.M. et al. Addition of Quercus cortex extract to broiler diet changes slaughter indicators and biochemical composition of muscle tissue 799

Fisinin V.I., Vertiprakhov V.G., Grozina A.A. The exocrine pancreatic function in chicken (*Gallus gallus* L.) fed diets containing different ingredients 811

FUNCTIONAL BIOCHEMISTRY

Egorov I.A., Grozina A.A., Vertiprakhov V.G. et al. The age dynamics of biochemical blood indices in broiler chicken (*Gallus gallus* L.) 820

Safonov V.A., Mikhalev V.I., Chernitskiy A.E. Antioxidant status and functional condition of respiratory system of newborn calves with intrauterine growth retardation . 831

VETERINARY VIROLOGY, IMMUNOLOGY

Borodin A.M., Alekseev Ya.I., Konovalova N.V. et al. Detection of avian leukemia virus subgroup K in Russia and its molecular genetic analysis 842

Shabunin S.V., Shakhov A.G., Vostroilova G.A. et al. Porcine Biferon-C applied together with medicinal prophylaxis in commercial pig breeding provides immunostimulation of sows and an increased viability of their piglets 851

Imatdinov A.R., Dubrovskaya O.A., Morozova D.Yu. et al. The study of antigenicity, immunogenicity and protective potential of DNA constructs containing fragments of genes CP204L, E183L or EP402R of African swine fever virus strain MK-200 . . 860

CELL CULTURES

Savchenkova I.P. Multipotent mesenchymal stromal cells isolated from subcutaneous fat of mammals for the study of *Sarcoptes Scabiei/mange* in vitro 868

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 659-672
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Reviews, challenges

UDC 636.012:575

doi: 10.15389/agrobiology.2018.4.659eng
doi: 10.15389/agrobiology.2018.4.659rus

GENE AND GENOMIC LEVELS OF DOMESTICATION SIGNATURE
(review)

V.I. GLAZKO

Timiryazev Russian State Agrarian University—Moscow Agrarian Academy, 49, ul. Timiryazevskaya, Moscow, 127550 Russia e-mail vigvalery@gmail.com (✉ corresponding author)
ORCID:
Glazko V.I. orcid.org/0000-0002-8566-8717
The author declares no conflict of interests
Received April 25, 2018

Abstract

Domestication is considered as a model of microevolution, problems and traits of domestication in animal species that distinguish them from closely related wild species are discussed. Data on different levels of "signature" of domestication, such as genomic, gene, protein, metabolomic, in the key genes of formation of economically valuable traits are presented. It is noted that the main differences of domesticated species from closely related wild ones are relatively high variability not only at the phenotypic level, manifested in large numbers of breeds and wide areas, but also in the population-genetic heterogenisity, as well as functional groups of genes involved in variability. The accumulated data suggest that there is a "subgenome", the increased variability of which is a source of genetic heterogeneity of domesticated animals, necessary for effective selection on economically valuable traits and adaptive potential. Literary data on the comparative analysis of the differences between SNP and CNV markers indicate that, mostly in genomic regions, in which are localized differentiating these species types the SNP and CNV markers, localized the genes which are associated with the development of the nervous and immune systems, as well as the characteristics of animal productivity in agricultural species, and involved in these processes specific genes varies depending on species, that is, similar phenotypic solutions are achieved with the involvement of different genetic systems (F.J. Alberto et al. 2018). It is known that almost half of mammalian genomes are engaged in retrotransposons (E.V. Koonin, 2016). The comparative analysis of domesticated and closely related wild species revealed differences in the relatively high density in the domesticated species the distribution of DNA fragments flanked by inverted sequences of tandem and dispersed repeats. It is proved that there is a certain contribution of transposing elements associated with a wide range of retroviral infections in the increased genetic variability of domesticated species, which can explain the unique genetic and phenotypic variability of domesticated animals.

Keywords: domestication, signature of domestication, microsatellites, dispersed repeats, endogenous retroviruses

Domestication of plants and animals is a key event in the formation of an agricultural civilization, which has almost completely forced out the civilization of hunters and foragers.

Domestication as a model of microevolution. The generally accepted definition of domestication implies a process of historic transformation of wild animals into domestic animals specifically adapted to satisfying human needs. In a negligibly short time the evolution in domestication conditions resulted in the biggest morpho-physiological changes in animals and created the species that could not have existed in nature. It is rather difficult to describe the domestication syndrome [phenotypic characteristics uniting taxonomically remote species and distinguishing them from closely related wild animals). The domestication syndrome was rather closely studied by S.N. Bogolyubskiy [1]. The weakened natural selection and introduction of human-defined parameters during selection (ethology, productivity, reproductivity) were essential during popu-

lation-specific and genetic adaptation to reproduction in the conditions of long-term domestication and breeding of different species under continued commercial selection. At the same time it is apparent that in terms of gene pool specifics, the domesticated species differ significantly from their closely related wild species. Essentially, the domesticated species are the life sustaining basis of modern civilization. All of this requires close focus on domesticated animals because only in-depth knowledge of their specific characteristics allows us to develop efficient methods of preserving and improving new genetic resources.

The genomic signatures of domestication. A series of studies (at molecular level from an organism to a population structure of a species) are dedicated to genomic differences of domesticated species from their closely related wild species ("domestication signature") of pigs [2], large and small ruminants [3, 4], horses and donkeys [5-7]. Not all domestic species have a complete set of these characteristics; however, each species has many to a certain extent. The combination of these characteristics was called the domestication syndrome.

The complexity of genomic makeup stipulates the diversification of elements selected for analysis of genomic distinction. Usually, molecular-genetic polymorphism markers of structural gene sections are used that encode the amino acid sequences of proteins (electrophoresis protein versions), non-coding sections of structural genes and various DNA sequences, the connection of which with structural genes is, as a rule, unknown. The studies analyze genomic distribution of short repeats (RAPD, ISSR, AFLP markers), microsatellite loci (tandem repeats 2-6 nucleotides long), use the data of whole-genome sequencing, compare single-nucleotide polymorphisms (SNP) and copy number variability (CNV).

Full genetic sequencing of the following species has already been performed: chicken *Gallus gallus domesticus* and ancestral species of *G. gallus* [8], pig and wild boars [9, 10], ancestral primitive bovine cattle and modern bovine cattle breeds [11], domesticated sheep and moufflon, goats and bezoar goats [4, 12], domesticated horse and Mongolian wild horse [13-15], domesticated and common rabbit [16, 17]. The basic conclusion is that most of SNP and CNV markers differentiating the domesticated and wild species fall within gene localization areas pertaining to the development of the nervous and immune systems and productivity of domesticated animals, and these genes vary depending on species, i.e., identical phenotypic effects are achieved with involvement of different genetic systems [12]. We will review some genomic domestication signature types below.

Domestication signature for milk proteins. We have analyzed the frequency of allele versions and genotypes based on genes encoding milk proteins (κ-casein, α-IS casein, β- lactoglobulin) and two key enzymes of lipid synthesis (acyl-CoA- diacylglycerol acyltransferase 1 and stearyl-CoA-desaturase 1) in dairy (black-and-white Holstein breed and Ayrshire breed) and beef (Aberdeen-Angus and Kalmyk breed) bovine cattle. In combination with analysis of literature these studies have shown that allele versions of candidate genes involved in metabolic pathways that determine the specifics of milk productivity formation in analyzed breeds do not allow reliably forecasting the quality of milk; however, allele versions of κ-casein и stearyl-CoA-desaturase 1 [18] can be used for quality forecast (micelle size and suitability for production of hard cheese, enrichment with desaturated fatty acids).

A mutation was identified in exon 4 of κ-casein gene in bovine cattle, which results in a small size of milk micelles, which is required for quality cheese production. The study of this exon in different species showed that the ratio between nonsynonymous and synonymous substitutions significantly varied both inside the family and among families [19]. In whole protein the quantity of

nonsynonymous substitutions is noticeably higher only for *Bovinae* species (0.045 versus 0.036), whereas in the other cases there were more synonymous substitutions, as is generally accepted for the evolution of protein-coding sequences [19]. At the family level, the differences in rate of divergence of this section are statistically valid only when making general comparisons for all studied *Bovinae* species and *Caprinae* species, which is indicative of a high rate of amino acid substitution in protein after divergence of these families. Its has also been found that amino acid sequence of κ-casein that corresponds to exon 4 is identical for closely related *Bos taurus* and *B. indicus* species that diverged less than 3 million years go (with the exception of 148 position substitution of *B. taurus*) and corresponds to the allele version bovine cattle B κ-casein preferable for cheese production [18, 19]. We can assume that domestication of zeboid cattle and bovine cattle emerged in different centers: the first occurred in India, the second occurred in the Mediterranean region [20, 21]. Consequently, the allele version of κ-casein B occurred after domestication of bovine cattle in the Mediterranean region and was preserved due to selection, which was more active in the European agricultural tradition than in the Indian tradition.

K-casein of C-end domain contains all sites of posttranslational phosphorylation and glycosylation [22]. The carboxyl groups are associated via glycoside O-link with threonine and serine residue of κ-casein, whereas 50% of C-domain contains residue of Thr and Ser, a part of which can also be phosphorylated. The physical properties (dimensions, solubility) and reactivity of micellar casein significantly depend on phosphorylation and glycosylation [22, 23]. During the process of *Bovinae* family formation the evolution is fastest in the gene segment corresponding to the C-end domain [19]. In *Bovinae* family, the total quantity of Thr and Ser residue in protein remains unchanged when their positions change, in other families both the quantity and position of residue remains. This can in some way explain the increase nonsynonymous and synonymous substitutions ratio in *Bovinae* cattle that we observed. By some accounts, uneven distribution of glycosylation in κ-casein C-domain can be accompanied by differences in its inhibiting *Helicobacter pylori* causing gastrointestinal diseases [23]. It can be expected that the observed fast evolution of amino acid sequence of this κ-casein section is attributable to adaptation of closely related *Bovinae* species to different pathogens. Therefore, positive selection (fast accumulation of nonsynonymous substitutions) is observed only for one section of κ-casein molecule, its C-domain, and only for *Bovinae* species in the course of their divergence during a relatively short period of time. This can possibly be attributable to breed differences in feeding that emerged after divergence (due to domestication of most researched family representatives), which triggered a need to adapt to different pathogens of the gastrointestinal tract [19].

Metabolomic domestication signatures. We have compared polymorphism for 30 loci of different protein groups in genetic pools of domesticated and closely related wild species from two orders: *Artiodactyla* (artiodactyles) and *Perissodactyla* (perissodactyle), including wild zoo species (biosphere reserves "Askania-Nova") and bovine cattle and horses from different households in Russia and Ukraine (26 species and interspecific groups, 12 species total) [24, 25]. The analysis was supplemented with population and genetic evaluation of differentiation of 18 species of soya bean (*Glycine max*) from different countries and 3 populations of wild Ussurian soybean from different regions of the Far East: *Soja ussuriensis* Moench (the presumed ancestral species of soybean). The average degree of polymorphism for analyzed loci was somewhat higher for domesticated animal species and plants. For domesticated animals this parameter varied from 0.036 (for pigs) to 0.171 (of bovine cattle), for closely related wild

species: from 0.017 (Grant's zebra *Equus quagga boehmi*) below 0.135 (*Taurotragus oryx* eland). The groups of species were distinctly differentiated in terms of contribution of different functional genetic and biochemical systems to polymorphism. For instance, the percentage of polymorphic loci in intracellular energy metabolism enzymes scaled to the number of species analyzed for domesticated representatives, was 0.179 for hollow-horned species, 0.629 for wild species, for metabolism enzymes of exogenous substrates: 0.464 and 0.193 respectively, for transport proteins: 0.357 and 0.178 [25], which means that universal difference of domesticated species from their closely related wild species lies in the increased enzyme polymorphism: for domesticated species of the substrate metabolism (associates the animal metabolome with environment substrates), for their closely related wild species: of intracellular energy metabolism (glycolysis, pentose-phosphate pathway, Krebs cycle) [26, 27]. In other words, in one case there was adaptation to broad substrate specificity, in the other case it was optimization of intracellular energy supply with a narrow substrate range.

By analyzing the biochemical markers of total metabolism in agricultural animals we may presume that there exists some connection between the intensity of form-building interspecific processes (the quantity of breeds can reflect it) and genetic genetic variability of a species. We have compared it for the "golden five" agricultural animals (goats, sheep, bovine cattle, pigs and horses). The lowest variability evaluated according to the percentage of polymorphic loci (P) and mean heterozygosity per locus per individual (H) (maximum values are listed further) were identified in goats and pigs (P is 0.03 and 0.02, respectively, H is 0.05 and 0.07), and the highest value was typical of bovine cattle (P = 0.52; H = 0.18), which corresponds to the highest number (1500 of bovine cattle breeds. For horses these parameters were somewhat lower (P = 0.4, H = 0.16). The accumulation of data is still insufficient to assert that there is a direct connection between the degree of genetic variability of biochemical markers of total metabolism key links and potential capacity of agricultural species to create new forms; however, a certain interconnection between these faxts is apparent.

Structural genes of dairy and beef productivity. The genetic pools of autochthonous displaced bovine cattle breeds are almost completely uninvestigated for commercially valuable allele versions of structural genes, which could be used directly in the modern practical selection. We have determined the occurrence rate and distribution of allele versions of six structural genes closely connected with productivity formation [18]. These are the following genes: growth hormone (GH), pituitary-specific transcription factor of growth hormones and some milk proteins (Pit-1), leptin lipid exchange hormone (LP), myostatin, the negative regulator of myogenesis and muscle tissue regeneration, for which "dual muscle system" mutations *nt821*(*del11*) of Belgian Blue cattle and *Q204X* of Piedmontese cattle were described, κ-casein (*CSN3*), the milk micelle protein, and β-lactoglobulin (*BLG*), the basic whey protein. Polymorphism of most genes was analyzed using PCR-RFLP method (amplification of structural gene fragments limited by matched pairs of flank primers with a restriction analysis of segments obtained). We arrived at a conclusion about presence of a mutation in myostatin gene by amplification product length without restrictions [26, 27]. We compared bovine cattle breeds in different breeding regions: Gray Ukrainian cattle (Kherson region, 34 animals; the Altai Territory, 32 animals); Red Polish cattle (Teropil region, 60 animals, Poland, 87 animals); White-Headed Ukrainian cattle (Sumy region, 35 animals); Brown Carpathian cattle (Ivano-Frankivsk region, 22 animals); Yakutian cattle (Novosibirsk region, 18 animals). Polymorphism of certain genes (specifically, myostatin) was studied on beef breeds (Herefords, Aberdeen-Angus, Charolais). The analysis also included wild representa-

tives of bovine subfamily (*Bovinae*): Ankole-Watusi (*Bos taurus macrocerons*), gayals (*Bibos gaurus frontalis*), aurochs (*Bison bonasus*), bisons (*Bison bison*), a representative of spiral-horned antelope subfamily (*Tragelaphinae*) canna (*Taurotragus oryx*), which are reproduced in Askania-Nova biosphere reserve [26, 27]. It turned out that allele versions associated with commercially valuable characteristics of domesticated forms almost never occur in closely related species (for instance, according to *CSN3*); moreover, as a rule, in indigenous species the rate of occurrence of such alleles is higher than in commercial breeds. At the same time, the highly productive breeds do not have complex genotypes for desirable alleles (in different genes) in spite of distinct differences between the breeds.

The analysis of interlocus associations demonstrated that linkage disequilibrium is a very irregular characteristic, which varies in different breeds and within intraspecific groups regardless of gene synteny (colocalization in the linkage group) [28]. Earlier, we have identified statistically-valid disequilibrium of locus linkage of transferrin and κ-casein (bovine cattle chromosomes 1 and 6) and lack of such disequilibrium in syntenic transferrin and ceruloplasmin (chromosome 1); disequilibrium in locus linkage of κ-casein and growth hormone in Brown Carpathian cattle (chromosomes 6 and 19), but its absence in Grey Ukrainian cattle [18]. This implies high variability of interlocus associations regardless of synteny for domestic species [29, 30]. As we have observed, in certain cases interlocus associations can be used as additional characteristic of genetic structure of species and intraspecific groups.

Genetic signature of artificial selection for variability of a complex of genomic segments (subgenome). It is still unclear whether genetic pool specifics of domestic animals (their capacity to create a great amount of genetic ensembles underlying stable morphofunctional types) are attributable to the fact that wild and domestic species differ in terms of polymorphism of different genetic systems. This assumption was made as far back as 30 years ago [24] and received a number of confirmation [31]. We analyzed the contribution made to polymorphism of a species by polymorphism of various functional protein groups [25]. For calculations we took mean heterozygosis of a species to be equal to 1 and evaluated its percentage created by polymorphism of each group. We studied primary genetic and biochemical systems used as markers of structural genes in more than 1000 animal and plant species analyzed so far [32]. These are three protein groups with different biochemical functions: the enzymes of intracellular energy metabolism, metabolism of exogenous substrates and transport proteins. By averaging the contribution of polymorphism of each group we discovered that wild and domestic animals differ by variability predominance of various genetic and biochemical systems (as is the case with morpho-physiological parameters). Which means that in case of artificial selection (unlike natural selection) the polymorphism of enzymes, associated with intracellular energy, declines, and polymorphism of enzymes that have broad specificity and metabolizing exogenous substrates increases. The varying contribution of functional protein groups in total polymorphism that we discovered in wild and domestic mammalian species correlates well with the assumptions about a link between formation of species with reorganization of cell energy supply mechanisms [24] and the factor that artificial selection (with the exception of cross-species hybridization) usually does not result in emergence of new species.

Apparently, natural selection facilitates the formation of species by supporting enzyme polymorphism of intracellular energy metabolism, and artificial selection facilitates the emergence of new forms with a high degree of adaptation to exogenous substrates. It is possible that the scope of phenotypic variation of domesticated species is connected with a variety of metabolic rates of exogenous

substrates. The latter allows us to assume that there is a subgenome, i.e. the genes encoding the systems involved in metabolism of these substrates. Its variability determines the involvement of a species in domestication and is important for broad phenotypic variety of domestic animals and is necessary for their directed breeding.

When analyzing enzyme system polymorphism of soybean breeds, populations of wild Ussurian soybean and five other species of wild soybean, all groups displayed monomorphism for 21 loci out of 42 [25]. The genetic and biochemical systems of plants were divided into two groups: enzymes involved in the creation of adenosine triphosphate in a cell (glycolysis, Krebs cycle), i.e. those involved in glucose metabolism (G), and the rest of the enzymes not involved in metabolism (NG). The analysis covered 21 enzyme loci of each group. Seven polimorphic loci were identified in the population of wild species, including one NG locus (ESTD-1) and 6 G loci. All in all 19 loci were identified for soybean breeds (11 for G, 8 fpr NG). Up to 86% polymorphic loci of wild soybean participate in controlling the intracellular energy metabolism, which is only 58% for domestic soybean; moreover, there were 3 times more (42%) of polymorphic loci non involved in glucose metabolism than for wild species (similar was observed for domestic animals). Consequently, we can assume that plants also have a subgenome involved in regulating the links between the internal and external biochemical environments via metabolism enzymes of exogenous substrates and transport protein.

The scope of genetic variability for *G. max* is larger than for *G. soja* (the percentage of polimorphic loci P is 45 and 17%), which means that a domesticated species is more polimorphic than its closely related wild species [25-27]. The interspecific genetic distances (DN) constituted from 0.059 to 0.129 and from 0.038 to 0.264 respectively. Consequently, for soybean the interspecific differentiation of breeds is comparable with the interspecific differentiation of populations of a closely related wild species.

Therefore, domesticated breeds have a higher protein variability that determines the metabolic link with the environment, and control of intracellular energy transformation is more stable. The comparison of electrophoretic protein (enzyme) versions allows us to accentuate the domestication characteristics related to a relatively high polymorphism of genetic and biochemical systems controlling the exogenous substrate metabolism (and transportation proteins for animals).

Genomic signature of artificial selection. The transition to polylocus genome genotyping and scanning (from analysis of several hundreds markers to complete sequencing) is the primary characteristic of modern population genomics [33].

The application of RAPD markers (randomly amplificated polymorphic DNA) is restricted to the ability of PCR-amplification of DNA segments flanked by inverted decanucleotid repeats [34]. Not every nucleotide sequence in inverted in a genome with high frequency and can be used as primer. For interspecies and intraspecies studies of *Equidae* family members, UBS-85 and UBS-126E primers are suggested [35]. With these primers we identified the largest similarity of domestic horse and bovine cattle (grouped in a separate dendrogram cluster) 7 wild and 2 domesticated species of artiodactyles and perissodactyles. These data can be construed as a confirmation of a certain similarity in genome variability of domesticated species [36, 37].

The ISSR analysis (inter-simple sequence repeat) allows increasing the accuracy of annealing. The products of ISSR-amplification contain an inverted microsatellite primer sequence on their flanks, and the resulting fingerprint is usually reproduced better than in RAPD [38-42], whereas the identified polymorphism is higher. The amplification is conducted with one or several primers of 15-24 nu-

cleotides in length [38] consisting of short tandem repeats (2-4 nucleotides) and one selective nucleotide at 3′-end. The microsatellite sequences surround many genes [38] and can be used for them as anchor sequences. Both RAPD and ISSR do not require preliminary cloning and sequencing for primer selection [38-42]. When using 3 dinucleotide and 12 trinucleotide ISSR-PCR primers, 310 amplicons [25-27] were identified for 11 domesticated and wild species, and short amplicons were reliably more frequent in domesticated species.

In IRAP-PCR (inter-retrotransposon amplified polymorphism) [38], a segment between the primers is amplified, which are complementary to two adjacent retrotransposons (typically these are segments of long terminal repeats of LTR endogenous retroviruses) in alternative DNA chains with REMAP-PCR (retrotransposon-microsatellite amplified polymorphism) located between the primers for LTR retrotransposon fragment and next to the located simple microsatellite repeat acting as an anchor (SSR-primer) [43, 44]. In REMAP and IRAP, the primers for 3′- and 5′-end LTR are used. Some retrotransposons (for instance, BARE-1) are distributed along the genome length relatively evenly [43, 44], some short retrotransposons, such as MITE, are rather often localized near coding sequences [45]. The REMAP markers can be useful when studying genomic singularity of domestication: they are flanked with a microsatellite sequence, therefore it is more probable that amplified fragments are evenly distributed in chromosomes and are not clustered in the areas of retrotransposon concentrations [46].

The retrotransposons are closely connected with microsatellites, for instance, in bovine cattle genome [47]. The endogenous retroviruses are very common in genomes of main domesticated species [48, 49]. Interestingly, retrotransposons resulted in significant intragenomic differentiation of laboratory murine lines with different sources (C57BL and BALB) during a relatively short period of time (a little more than 100 years) [50, 51]. The usage of one retrotransposon (Alu) was described (due to its wide occurrence) for human genome scanning [52, 53].

The study of species-specific ISSR-PCR marker formations using ancient Altai horse breed as an example has shown [46] that genomic fragment with the size of 416 nucleotide pairs flanked by an inverted repeat $(AG)_9C$ was formed as a result of recombination between ancient mobile elements (fish DNA transposon and LTR ERV3, which is typical for many mammals) and ERV1 endogenous retrovirus sequence specific for horse genome. In Altai horse DNA the segment with the size of 235 nucleotide pairs had homology only with domestic horse ERV1, which is indicative of its apparent later origin than, for instance, homology segment with LTR ERV3. The high correlation ($r = 0.9$) between integration frequency of endogenous retrovirus sequences with the size of 235 nucleotide pairs and chromosome length points to the fact that domestic horse undergoes further transpositions, recombination and evolution of endogenous retrovirus sequences. Similar correlations between integration frequency of segments of endogenous retrovirus sequences and chromosome length were observed in bovine cattle genome [54]. These integration regions are often depleted with CG sequences and enriched with AT [54]. The relatively even distribution over horse chromosome length is also described for segments homological to a fragment of long end repeated sequence of ERV3 beta1 endogenous retrovirus [55]. It was argued that spread of retrovirus end repeated sequence (in absence of more than one full-size copy of the latter) can occur according to the following pattern: at first the endogenous retrovirus integrates in the genome on a wide scale with subsequent exposure of most formed copies leaving traces of multiple iterations in the form of small terminal sequences [55]. Significant homology has been observed between EqERV beta1 of domestic horse and unclassified endogenous retrovirus in

bovine cattle genome and MMTV murine retrovirus – the phylogenetic ancestor of viruses of hoofed mammals; therefore, we can expect that both studied species were first infected with murine virus [55]. In the course of transposition and recombination the descendants of endogenous retroviruses can cause explosive outbreaks of mutational variability. Now therefore, the assumption that the genomic elements associated with such highly variable nucleotide sequences could particularly be involved in a wide-scale phenotypic variation characteristic of domesticated species seems only logical.

The nucleotide sequence of endogenous retrovirus make a significant contribution to the families of endogenous retrovirus, fragments of which and products of recombination of which with other mobile elements constitute an almost the main part of dispersed repeats of mammal genomes [49, 54, 56]. Detailed databases have been created containing full-size endogenous retrovirus available in genomes of primary domesticated mammals [49]. The horizontal transfer of some retrotransposones that unites the genomes of taxonomically remote species [57, 58] was described and its essential role in the evolution of vertebrates is being discussed [59]. The viruses and mobile genetic elements are thought of as drivers of evolution [60]. A close link between microsatellites and retrotransposones is known [61-63]. In our studies we have shown that in genomic DNA fragments flanked by inverted repeats of microsatellite loci segments both in horses and bovine cattle the frequency of recombination predominantly among retrotransposones is high [64, 65].

Now, therefore, it is apparent that in most cases the studied phenotypic characteristics and relevant gene systems are linked with species-specific commercially valuable characteristics. It was Charles Darwin who thought of domestication processes as accelerated evolution under the influence of artificial selection [66]; however, there is still no clear definition of what domestication really means and what are its genetic mechanisms. Some researchers suggest viewing domestication as a result of interactions stable in many generations, when one species significantly affects the reproduction and survival of the other [67]. One of the conditions of transformation of a wild animal in a domestic animal is reproduction under any conditions of maintenance, feeding, space constraints, reduced motor activity and adaptation to human presence. This is due to the change of animal behavior – one of the first and brightest domestication results. In fact, domestic animals differ from wild animals primarily by its reaction to humans. In other words, domestication is a coevolution process (in essence, symbiosis), when the population adapts to anthropogenic environment by combining genetic changes.

The modern concept of phenotypic variation describes the manifestation of characteristics as a result of interaction of a genotype and factors affecting the realization of genetic information (the maintenance and reproduction conditions, microbiome, pollutants and pathogens). This process is exercised at different interdependent levels (transcriptome, proteome, metabolome, microbiome) creating nonlinear links (for instance, singular changes in transcriptome can result in multiple changes in metabolome, and vice versa); moreover, direct impact of environmental factors is possible at each level [68, 69].

The varying impact of enzymes of intracellular energy metabolism observed by us, and exogenous substrate metabolism in total polymorphism of wild and domestic mammal species correlates well with the absence of formation of species during selection and its link with reorganization of energy supply of cells during evolution. By taking into account the specificity of selection and the choice of livestock population, the similarity of protein polymorphism of wild and domestic animals is unexpected, especially since allozyme divergence in

wild species is linked to formation of species, whereas in domestic animals it is linked solely to high morpho-physiological variability. Consequently, natural and artificial selection should have different impact on polymorphism of different genetic and biochemical systems and not on overall scope of genetic variability. We have confirmed this hypothesis by comparing the contribution of protein functional group polymorphism to the total genetic variability of wild and domestic species.

As it turns out, the domestic species are significantly more uniform than wild species in terms of polymorphism of certain biochemical markers, such as transferrin, esterase, diaphorase, acid phosphatase, catalase and albumin [24]. This also supports the hypothesis about dissimilar impact of natural and artificial selection on genetic variability due to impact on different metabolic links, which results in polymorphism of various biochemical markers.

Presumably the expansion of habitat of domesticated species migrating alongside humans increased the number of contacts with retroviruses and resulted in emergence of new transposable elements in the genome. By inhibiting recurrent infections they preserved in the course of natural selection and at the same time increased genetic variability (insertion mutagenesis, recombination processes) causing mutations essential for artificial selection. Please note that a link between the emergence of allele versions essential for selection distinguishing domesticated species from closely related wild species, as well as integration of mobile genetic elements in coding sequences [31] was observed in many studies. The involvement of transposable elements in genome divergence of closely related and wild species could explain some empirical data, for instance, the accelerated rate of evolution of a number of genetic elements in genomes of domesticated species [31] and higher frequency of occurrence of short fragments of genome DNA flanked by inverted repeats in domesticated cavicornians than in closely related wild species [27].

To summarize, the findings point to the fact that animal and plant species have characteristics of domestication that differ them from their closely related wild species not only at the level of complexes of phenotypic characteristics, but also in terms of polymorphism of structural genes encoding the proteins and enzymes, and in terms of occurrence of invested repeats of microsatellite loci, mobile genetic elements and segment duplications in the genome. We can anticipate that common retroviral infections can be one of the mechanisms behind such differences. In order to describe the common and specific genetic make-ups of domestication we need to identify the source of unique genetic variability of domesticated species that display increased variability of total metabolism (with identical scale of genetic and biochemical variance) defining the link between biochemical processes in internal and external environments, genetic systems of intracellular energy transformation control are more stable (glycolysis, Krebs cycle). We can expect that the systems involved in exogenous substrate metabolism are coded by the genes of "subgenome," the variability of which is linked to phenotypic flexibility and determines the possibility of involving a species in domestication.

REFERENCES

1. Bogolyubskii S.N. *Proiskhozhdenie i preobrazovanie domashnikh zhivotnykh* [The origin and transformation of domestic animals]. Moscow, 1959 (in Russ.).
2. Wang K., Wu P., Yang Q., Chen D., Zhou J., Jiang A., Ma J., Tang Q., Xiao W., Jiang Y., Zhu L., Li X., Tang G. Detection of selection signatures in Chinese Landrace and Yorkshire pigs based on genotyping-by-sequencing data. *Front. Genet.,* 2018, 9: 119 (doi: 10.3389/fgene.2018.00119).

3. Upadhyay M., da Silva V.H., Megens H.J., Visker M.H.P.W., Ajmone-Marsan P., Bâlteanu V.A., Dunner S., Garcia J.F., Ginja C., Kantanen J., Groenen M.A.M., Crooijmans R.P.M.A. Distribution and functionality of copy number variation across European cattle populations. *Front. Genet.*, 2017, 8: 108 (doi: 10.3389/fgene.2017.00108).
4. Alberto F.J., Boyer F., Orozco-terWengel P., Streeter I., Servin B., de Villemereuil P., Benjelloun B., Librado P., Biscarini F., Colli L., Barbato M., Zamani W., Alberti A., Engelen S., Stella A., Joost S., Ajmone-Marsan P., Negrini R., Orlando L., Rezaei H.R., Naderi S., Clarke L., Flicek P., Wincker P., Coissac E., Kijas J., Tosser-Klopp G., Chikhi A., Bruford M.W., Taberlet P., Pompanon F. Convergent genomic signatures of domestication in sheep and goats. *Nat. Commun.,* 2018, 9(1): 813 (doi: 10.1038/s41467-018-03206-y).
5. Renaud G., Petersen B., Seguin-Orlando A., Bertelsen M.F., Waller A., Newton R., Paillot R., Bryant N., Vaudin M., Librado P., Orlando L. Improved de novo genomic assembly for the domestic donkey. *Sci. Adv.*, 2018, 4(4): eaaq0392 (doi: 10.1126/sciadv.aaq0392).
6. Gaunitz C., Fages A., Hanghшj K., Albrechtsen A., Khan N., Schubert M., Seguin-Orlando A., Owens I.J., Felkel S., Bignon-Lau O., de Barros Damgaard P., Mittnik A., Mohaseb A.F., Davoudi H., Alquraishi S., Alfarhan A.H., Al-Rasheid K.A.S., Crubézy E., Benecke N., Olsen S., Brown D., Anthony D., Massy K., Pitulko V., Kasparov A., Brem G., Hofreiter M., Mukhtarova G., Baimukhanov N., Lxugas L., Onar V., Stockhammer P.W., Krause J., Boldgiv B., Undrakhbold S., Erdenebaatar D., Lepetz S., Mashkour M., Ludwig A., Wallner B., Merz V., Merz I., Zaibert V., Willerslev E., Librado P., Outram A.K., Orlando L. Ancient genomes revisit the ancestry of domestic and Przewalski's horses. *Science,* 2018, 360(6384): 111-114 (doi: 10.1126/science.aao3297).
7. Librado P., Gamba C., Gaunitz C., Der Sarkissian C., Pruvost M., Albrechtsen A., Fages A., Khan N., Schubert M., Jagannathan V., Serres-Armero A., Kuderna L.F.K., Povolotskaya I.S., Seguin-Orlando A., Lepetz S., Neuditschko M., Thèves C., Alquraishi S., Alfarhan A.H., Al-Rasheid K., Rieder S., Samashev Z., Francfort H.P., Benecke N., Hofreiter M., Ludwig A., Keyser C., Marques-Bonet T., Ludes B., Crubézy E., Leeb T., Willerslev E., Orlando L. Ancient genomic changes associated with domestication of the horse. *Science*, 2017, 356(6336): 442-445 (doi: 10.1126/science.aam5298).
8. Rubin C.J., Zody M.C., Eriksson J., Meadows J.R., Sherwood E., Webster M.T., Jiang L., Ingman M., Sharpe T., Ka S., Hallböök F., Besnier F., Carlborg O., Bed'hom B., Tixier-Boichard M., Jensen P., Siegel P., Lindblad-Toh K., Andersson L. Whole-genome resequencing reveals loci under selection during chicken domestication. *Nature*, 2010, 464(7288): 587-591 (doi: 10.1038/nature08832).
9. Frantz L.A., Schraiber J.G., Madsen O., Megens H.J., Cagan A., Bosse M. Paudel Y., Crooijmans R.P., Larson G., Groenen M.A. Evidence of long-term gene flow and selection during domestication from analyses of Eurasian wild and domestic pig genomes. *Nat. Genet.*, 2015, 47(10): 1141-1148 (doi: 10.1038/ng.3394).
10. Paudel Y., Madsen O., Megens H.J., Frantz L.A., Bosse M., Bastiaansen J.W., Crooijmans R.P., Groenen M.A. Evolutionary dynamics of copy number variation in pig genomes in the context of adaptation and domestication. *BMC Genomics*, 2013, 14: 449 (doi: 10.1186/1471-2164-14-449).
11. Park S.D., Magee D.A,. McGettigan P.A., Teasdale M.D., Edwards C.J., Lohan A.J., Murphy A., Braud M., Donoghue M.T., Liu Y., Chamberlain A.T., Rue-Albrecht K., Schroeder S., Spillane C., Tai S., Bradley D.G., Sonstegard T.S., Loftus B.J., MacHugh D.E. Genome sequencing of the extinct Eurasian wild aurochs, *Bos primigenius*, illuminates the phylogeography and evolution of cattle. *Genome Biol.*, 2015, 16: 234 (doi: 10.1186/s13059-015-0790-2).
12. Zamani W., Ghasempouri S.M., Rezaei H.R., Naderi S., Hesari A.R.E., Ouhrouch A. Comparing polymorphism of 86 candidate genes putatively involved in domestication of sheep, between wild and domestic Iranian sheep. *Meta Gene*, 2018, 17: 223-231 (doi: 10.1016/j.mgene.2018.06.015).
13. Der Sarkissian C., Ermini L., Schubert M., Yang M.A., Librado P., Fumagalli M., Jynsson H., Bar-Gal G.K., Albrechtsen A., Vieira F.G., Petersen B., Ginolhac A., Seguin-Orlando A., Magnussen K., Fages A., Gamba C., Lorente-Galdos B., Polani S., Steiner C., Neuditschko M., Jagannathan V., Feh C., Greenblatt C.L., Ludwig A., Abramson N.I., Zimmermann W., Schafberg R., Tikhonov A., Sicheritz-Ponten T., Willerslev E., Marques-Bonet T., Ryder O.A., McCue M., Rieder S., Leeb T., Slatkin M., Orlando L. Evolutionary genomics and conservation of the endangered Przewalski's horse. *Curr. Biol.*, 2015, 25(19): 2577-2583 (doi: 10.1016/j.cub.2015.08.032).
14. Librado P., Fages A., Gaunitz C., Leonardi M., Wagner S., Khan N., Hanghшj K., Alquraishi S.A., Alfarhan A.H., Al-Rasheid K.A., Der Sarkissian C., Schubert M., Orlando L. The evolutionary origin and genetic makeup of domestic horses. *Genetics*, 2016, 204(2): 423-434 (doi: 10.1534/genetics.116.194860).
15. Wutke S., Sandoval-Castellanos E., Benecke N., Döhle H.J., Friederich S., Gonzalez J., Hofreiter M., Lxugas L., Magnell O., Malaspinas A.S., Morales-Muciz A., Orlando L., Reissmann M., Trinks A., Ludwig A. Decline of genetic diversity in ancient domestic stallions in Europe. *Sci. Adv.*, 2018, 4(4): eaap9691 (doi: 10.1126/sciadv.aap9691).
16. Carneiro M., Rubin C.J., Di Palma F., Albert F.W., Alföldi J., Martinez Barrio A., Pielberg G.,

Rafati N., Sayyab S., Turner-Maier J., Younis S., Afonso S., Aken B., Alves J.M., Barrell D., Bolet G., Boucher S., Burbano H.A., Campos R., Chang J.L., Duranthon V., Fontanesi ., Garreau H., Heiman D., Johnson J., Mage R.G., Peng Z., Queney G., Rogel-Gaillard C., Ruffier M., Searle S., Villafuerte R., Xiong A., Young S., Forsberg-Nilsson K., Good J.M., Lander E.S., Ferrand N., Lindblad-Toh K., Andersson L. Rabbit genome analysis reveals a polygenic basis for phenotypic change during domestication. *Science*, 2014, 345(6200): 1074-1079 (doi: 10.1126/science.1253714).

17. Irving-Pease E.K., Frantz L.A.F., Sykes N., Callou C., Larson G. Rabbits and the specious origins of domestication. *Trends Ecol. Evol.*, 2018, 33(3): 149-152 (doi: 10.1016/j.tree.2017.12.009).
18. Glazko V.I., Andreichenko I.N., Kovalchuk S.N., Glazko T.T., Kosovsky G.Yu. Candidate genes for control of cattle milk production traits. *Russian Agricultural Sciences*, 2016, 42(6): 458-464 (doi: 10.3103/S1068367416060082).
19. Glazko V., Zybaylov B., Glazko T. Domestication and genome evolution. *International Journal of Genetics and Genomics,* 2014, 2(4): 47-56 (doi: 10.11648/j.ijgg.20140204.11).
20. Msalya G., Kim E.S., Laisser E.L., Kipanyula M.J., Karimuribo E.D., Kusiluka L.J., Chenyambuga S.W., Rothschild M.F. Determination of genetic structure and signatures of selection in three strains of Tanzania shorthorn zebu, boran and friesian cattle by genome-wide SNP analyses. *PLoS ONE*, 2017, 12(1): e0171088 (doi: 10.1371/journal.pone.0171088).
21. Upadhyay M.R., Chen W., Lenstra J.A., Goderie C.R., MacHugh D.E., Park S.D., Magee D.A., Matassino D., Ciani F., Megens H.J., van Arendonk J.A., Groenen M.A., European Cattle Genetic Diversity Consortium; RPMA Crooijmans. Genetic origin, admixture and population history of aurochs (*Bos primigenius*) and primitive European cattle. *Heredity* (Edinb), 2017, 118(2): 169-176 (doi: 10.1038/hdy.2016.79).
22. Khaldi N., Shields D.C. Shift in the isoelectric-point of milk proteins as a consequence of adaptive divergence between the milks of mammalian species. *Biology Direct*, 2011, 6(1): 40-49 (doi: 10.1186/1745-6150-6-40).
23. Strömqvist M., Falk P., Bergstrцm S., Hansson L., Lцnnerdal B., Normark S., Hernell O. Human milk kappa-casein and inhibition of Helicobacter pylori adhesion to human gastric mucosa. *J. Pediatr. Gastroenterol. Nutr.*, 1995, 21(3), 288-296.
24. Glazko V.I. An attempt at understanding the genetic basis of domestication. *Animal Science Papers and Reports,* 2003, 21(2): 109-120.
25. Glazko V.I., Glazko T.T. Domestication and N.I. Vavilov's law of homologous series in hereditary variability. *Russian Agricultural Sciences*, 2013, 39(1): 8-12 (doi: 10.3103/S1068367413010072).
26. Glazko V., Zybaylov B., Glazko T. Domestication and genome evolution. *International Journal of Genetics and Genomics*, 2014, 2(4): 47-56 (doi: 10.11648/j.ijgg.20140204.11).
27. Glazko V., Zybailov B., Glazko T. Asking the right question about the genetic basis of domestication: what is the source of genetic diversity of domesticated species? *Adv. Genet. Eng.*, 2015, 4: 2 (doi: 10.4172/2169-0111.1000125).
28. Aliloo H., Pryce J.E., González-Recio O., Cocks B.G., Hayes B.J., Validation of markers with non-additive effects on milk yield and fertility in Holstein and Jersey cows. *BMC Genet.,* 2015, 16: 89 (doi: 10.1186/s12863-015-0241-9).
29. Flori L., Fritz S., Jaffrézic F., Boussaha M., Gut I., Heath S., Foulley J.L., Gautier M. The genome response to artificial selection: a case study in dairy cattle. *PLoS ONE*, 2009, 4(8): e6595 (doi: 10.1371/journal.pone.0006595).
30. Zhao F., McParland S., Kearney F., Du L., Berry D.P. Detection of selection signatures in dairy and beef cattle using high-density genomic information. *Genet. Sel. Evol.*, 2015, 47: 49 (doi: 10.1186/s12711-015-0127-3).
31. Feng X., Jiang J., Padhi A., Ning C., Fu J., Wang A., Mrode R., Liu J.-F. Characterization of genome-wide segmental duplications reveals a common genomic feature of association with immunity among domestic animals. *BMC Genomics*, 2017, 18: 293 (doi: 10.1186/s12864-017-3690-x).
32. Nevo E. Evolution of genome-phenome diversity under environmental stress. *PNAS USA*, 2001, 98(11): 6233-6240 (doi: 10.1073/pnas.101109298).
33. Nosil P., Funk D.J., Ortiz-Barrientos D. Divergent selection and heterogeneous genomic divergence. *Mol. Ecol.*, 2009, 18: 375-402 (doi: 10.1111/j.1365-294X.2008.03946.x).
34. Owuor E.D., Fahima T., Beharav A., Korol A., Nevo E. RAPD divergence caused by microsite edaphic selection in wild barley. *Genetica*, 1999, 105(2): 177-192 (doi: 10.1023/A:1003781711908).
35. Bailey E., Lear T.L. Comparison of Thoroughbred and Arabian horses using RAPD markers. *Animal Genetics*, 1994, 25(Suppl. 1): 105-108 (doi: 10.1111/j.1365-2052.1994.tb00414.x).
36. Glazko V.I., Zelenaia L.B. Differentiation of domestic horse and Przewalski's horse using various DNA sequences. *Genetika,* 1998, 34(7): 996-999.
37. Glazko V.I., Dyman' T.N., Tarasiuk S.I., Dubin A.V. The polymorphism of proteins, RAPD-PCR and ISSR-PCR markers in European and American bison and cattle. *Cytol. Genet.*, 1999, 33(6): 30-39.
38. Zietkiewicz E., Rafalski A., Labuda D. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*, 1994, 20(2): 176-183 (doi:

10.1006/geno.1994.1151).
39. Rychlik W., Spencer W.J., Rhoads R.E. Optimization of the annealing temperature for DNA amplification in vitro. *Nucleic Acids Res.*, 1990, 18(21): 6409-6412
40. Shiue Y.L., Bickel L.A., Caetano A.R., Millon L.V., Clark R.S., Eggleston M.L., Michelmore R., Bailey E., Guérin G., Godard S., Mickelson J.R., Valberg S.J., Murray J.D., Bowling A.T. A synteny map of the horse genome comprised of 240 microsatellite and RAPD markers. *Animal Genetics*, 1999, 30(1): 1-9 (doi: 10.1046/j.1365-2052.1999.00377.x).
41. Sulima Iu.Iu., Kalendar' R.N., Sivolap Iu.M. The mapping of the barley genome by RAPD analysis using double haploid strains. *Cytol. Genet.*, 2000, 34(4): 41-49.
42. Owuor E.D., Fahima T., Beharav A., Korol A., Nevo E. RAPD divergence caused by microsite edaphic selection in wild barley. *Genetica*, 1999, 105(2):177-192.
43. Kalendar R., Schulman A.H. Transposon-based tagging: IRAP, REMAP, and iPBS. *Methods Mol. Biol.*, 2014, 1115: 233-255 (doi: 10.1007/978-1-62703-767-9_12).
44. Tomás D., Rodrigues J., Varela A., Veloso M.M., Viegas W., Silva M. Use of repetitive sequences for molecular and cytogenetic characterization of *Avena* species from Portugal. *Int. J. Mol. Sci.*, 2016, 17(2): E203 (doi: 10.3390/ijms17020203).
45. Shirasu K., Schulman A.H., Lahaye T., Schulze-Lefert P. A contiguous 66-kb barley DNA sequence provides evidence for reversible genome expansion. *Genome Res.*, 2000, 10(7): 908-915 (doi: 10.1101/gr.10.7.908).
46. Glazko V.I., Gladyr E.A., Feofilov A.V., Bardukov N.V., Glazko T.T. ISSR-PCR markers and mobile genetic elements in genomes of agricultural mammal species. *Agricultural Biology,* 2013, 2: 71-76 (doi: 10.15389/agrobiology.2013.2.71eng).
47. The Bovine Genome Sequencing and Analysis Consortium, Elsik C.G., Tellam R.L., Worley K.C. The genome sequence of taurine cattle: a window to ruminant biology and evolution. *Science,* 2009, 324(5926) 522-528 (doi: 10.1126/science.1169588).
48. Garcia-Etxebarria K., Jugo B.M. Evolutionary history of bovine endogenous retroviruses in the *Bovidae* family. *BMC Evolutionary Biology*, 2013, 13: 256 (doi: 10.1186/1471-2148-13-256).
49. Garcia-Etxebarria K., Sistiaga-Poveda M., Jugo B.M. Endogenous retroviruses in domestic animals. *Current Genomics,* 2014, 15(4): 256-265 (doi: 10.2174/1389202915666140520003503).
50. Zhang Y., Maksakova I.A., Gagnier L., van de Lagemaat L.N., Mager D.L. Genome-wide assessments reveal extremely high levels of polymorphism of two active families of mouse endogenous retroviral elements. *PLoS Genet.*, 2008, 4(2): e1000007 (doi: 10.1371/journal.pgen.1000007).
51. Nelleker C., Keane T.M., Yalcin B., Wong K., Agam A., Belgard T.G., Flint J., Adams D.J., Frankel W.N., Ponting C.P. The genomic landscape shaped by selection on transposable elements across 18 mouse strains. *Genome Biol.*, 2012, 13(6): R45 (doi: 10.1186/gb-2012-13-6-r45).
52. Mei L., Ding X., Tsang S.Y., Pun F.W., Ng S.K., Yang J., Zhao C., Li D., Wan W., Yu C.H., Tan T.C., Poon W.S., Leung G.K., Ng H.K., Zhang L., Xue H. AluScan: a method for genome-wide scanning of sequence and structure variations in the human genome. *BMC Genomics*, 2011, 12: 564 (doi: 10.1186/1471-2164-12-564).
53. Yang J.F., Ding X.F., Chen L., Mat W.K., Xu M.Z., Chen J.F., Wang J.M., Xu L., Poon W.S., Kwong A., Leung G.K., Tan T.C., Yu C.H., Ke Y.B., Xu X.Y., Ke X.Y., Ma R.C., Chan J.C., Wan W.Q., Zhang L.W., Kumar Y., Tsang S.Y., Li S., Wang H.Y., Xue H. Copy number variation analysis based on AluScan sequences. *Journal of Clinical Bioinformatics*, 2014, 4(1): 15 (doi: 10.1186/s13336-014-0015-z).
54. Garcia-Etxebarria K., Jugo B.M. Genome-wide detection and characterization of endogenous retroviruses in *Bos taurus*. *J. Virol.*, 2010, 84(20): 10852-10862 (doi: 10.1128/JVI.00106-10).
55. Van der Kuyl A.C. Characterization of a full-length endogenous beta-retrovirus, EqERV-Beta1, in the genome of the horse (*Equus caballus*). *Viruses,* 2011, 3(6): 620-628 (doi: 10.3390/v3060620).
56. Garcia-Etxebarria K., Jugo B.M. Genomic environment and digital expression of bovine endogenous retroviruses. *Gene*, 2014, 548(1): 14-21 (doi: 10.1016/j.gene.2014.06.048).
57. Oliveira S.G., Bao W., Martins C., Jurka J. Horizontal transfers of Mariner transposons between mammals and insects. *Mob DNA*, 2012, 3(1): 14 (doi: 10.1186/1759-8753-3-14).
58. Walsha A.M., Kortschaka R.D., Gardnerb M.G., Bertozzi T., Adelson D.L. Widespread horizontal transfer of retrotransposons. *PNAS USA*, 2013, 110(3): 1012-1016 (doi: 10.1073/pnas.1205856110).
59. Chalopin D., Naville M., Plard F., Galiana D., Volff J.N. Comparative analysis of transposable elements highlights mobilome diversity and evolution in Vertebrates. *Genome Biol. Evol.*, 2015, 7(2): 567-580 (doi: 10.1093/gbe/evv005).
60. Koonin E.V. Viruses and mobile elements as drivers of evolutionary transitions. *Phil. Trans. R. Soc. B*, 2016, 371(1701): 20150442 (doi: 10.1098/rstb.2015.0442).
61. Ahmed M., Liang P. Transposable elements are a significant contributor to tandem repeats in the human genome. *Comp. Funct. Genom.*, 2012, 2012: Article ID 947089 (doi: 10.1155/2012/947089).
62. Behura S.K., Severson D.W. Association of microsatellite pairs with segmental duplications in

insect genomes. *BMC Genomics*, 2013, 14: 907 (doi: 10.1186/1471-2164-14-907).
63. Sharma A., Wolfgruber T.K., Presting G.G. Tandem repeats derived from centromeric retrotransposons. *BMC Genomics*, 2013, 14: 142 (doi: 10.1186/1471-2164-14-142).
64. Bardukov N.V., Feofilov A.V., Glazko T.T., Glazko V.I. ISSR-PCR markers and mobile genetic elements in horse (*Equus caballus*) genome. *Agricultural Biology*, 2014, 4: 42-57 (doi: 10.15389/agrobiology.2014.4.42eng).
65. Glazko V., Kosovsky G., Glazko T. High density of transposable elements in sequenced sequences in cattle genomes, associated with AGC microsatellites. *Global Advanced Research Journal of Agricultural Science*, 2018, 7(2): 034-045.
66. Darvin Ch. *Izmeneniya domashnikh zhivotnykh i kul'turnykh rastenii. Tom 4* [Changes in domestic animals and cultivated plants. V. 4]. Moscow-Leningrad, 1951 (in Russ.).
67. Zeder M.A. Core questions in domestication research. *PNAS USA*, 2015, 112(11): 3191-3198 (doi: 10.1073/pnas.1501711112).
68. Ibeagha-Awemu E.M., Zhao X. Epigenetic marks: regulators of livestock phenotypes and conceivable sources of missing variation in livestock improvement programs. *Front. Genet.,* 2015, 6: 302 (doi: 10.3389/fgene.2015.00302).
69. Te Pas M.F.W., Madsen J., Calus M.P.L., Smits M.A. The importance of endophenotypes to evaluate the relationship between genotype and external phenotype. *Int. J. Mol. Sci.*, 2017, 18(2): 472 (doi: 10.3390/ijms18020472).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 673-686
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.018:573.22

doi: 10.15389/agrobiology.2018.4.673eng
doi: 10.15389/agrobiology.2018.4.673rus

INTERRELATION OF NERVOUS, IMMUNE, ENDOCRINE SYSTEMS AND NUTRITIONAL FACTORS IN THE REGULATION OF ANIMAL RESISTANCE AND PRODUCTIVITY

(review)

V.A. GALOCHKIN, K.S. OSTRENKO, V.P. GALOCHKINA, L.M. FEDOROVA

All-Russian Research Institute of Animal Physiology, Biochemistry and Nutrition — Branch of Ernst Federal Science Center for Animal Husbandry, Federal Agency of Scientific Organizations, pos. Institut, Borovsk, 249013 Russia, e-mail bifip@kaluga.ru (✉ corresponding author V.A. Galochkin), ostrenkoks@gmail.com
ORCID:
Galochkin V.A. orcid.org/0000-0002-5075-3647
Galochkina V.P. orcid.org/0000-0002-3121-7339
Ostrenko K.S. orcid.org/0000-0003-2235-1701
Fedorova L.M. orcid.org/0000-0002-1514-3050
The authors declare no conflict of interests
Received May 3, 2016

Abstract

Commercial livestock husbandry causes severe stress among animals resulting in up to 80 % emergence of secondary immunodeficiency. This review is an attempt to comprehensively analyze multiple interconnections between immune, neuroendocrine systems, together with nutrition, as essential factors for animal metabolism regulation, wellness, health, performance and productivity. Secretion of stress hormones and the degree of inhibition of the immune response depend on the type of animal's nervous system. Poor nutrition has a negative effect on the expression of immune response including humoral and cellular immunity synthesis of cytokine and plasma immunoreceptors (V.I. Fisinin et al., 2013; V.A. Galochkin et al., 2013; Y. Zhang et al., 2014; J.D. Ashwell et al., 2000; S. Cunningham-Rundles et al., 2005; V. Abhyankar et al., 2018; A. Haghikia et al., 2015; R.H. Oakley et al., 2013). Due to this relationship, immune, nervous and endocrine systems form virtual functionally integrated single super-system of immunobiological surveillance. Its purpose is to maintain body viability, efficiency and resistance to any physical, chemical, biological agents and psychosocial factors that can cause adverse effects or pathological conditions. The total body resistance reflects the combined effects of specific and nonspecific factors of innate and adaptive immune responses together with activity of a number of intracellular systems, including antioxidant-prooxidant system, monooxygenase, peroxisomal system (V.I. Lushchak, 2014; N. Sinha et al., 2015; V.A. Galochkin et al., 2015), which are together responsible not only for the neutralization of xenobiotics and endogenous toxins, but for the monitoring of homeostasis. Evolution of conceptual views on the immune system has developed into understanding of its function not only as a "shield and sword of the organism". In-deep look in immune function and mechanisms is unthinkable without examining immune system as a regulatory component of a single triad with the nervous and endocrine systems.

Keywords: productive animals, the immune system, nervous, endocrine regulation, nonspecific resistance, health, productivity

Animal productivity is closely connected with the functional activity of the immune system, which is a multicomponent and polyreactive system integrated with the other physiological body systems. If commercial animal husbandry results in severe stress, the proportion of livestock population with secondary immune deficiencies reaches 80%. The stresses and immune deficiencies precede numerous illnesses and directly induce pathological conditions of various degrees of severity reducing the volume and quality of animal husbandry products [1, 2].

The goal of this overview is to comprehensively analyze at interdisciplinary level the multiple interconnections essential for the formation of protective and regulatory functions of livestock in the context of livestock health and productivity.

In 2011, immunologists B.A. Beutler and J.A. Hoffmann were awarded a Nobel Prize in physiology and medicine for deciphering of revolutionary mecha-

nisms of immune system regulation. Their ideas were linked to the discovery and receptor protein trait of Toll-like receptors on cell surface activating the innate immune system, which had been previously believed to have been determined solely genetically [unlike adaptive immunity). The idea of a purely protective role of the immune system [protection against disease-producing and foreign factors) reigned until 1970-s, and still the function of maintaining genetic uniformity of cells in a living body [in the middle of the 20th century, Nobel Prize laureate F.M. Burnet introduced the term "the science of self") [3] is recognized as the most important function of the immune system. However, the understanding of this term in controlling homeostasis expands. Specifically, it has become almost axiomatic that a body forms a single and functionally undivided triad of regulatory systems: the nervous system, endocrine system and immune system [4-6]. The study of this triad requires interdisciplinary approach. Moreover, the task of identifying defensive mechanisms of a body cannot be solved without taking into account the following immunology phenomena: the general adaptation syndrome, the role of antioxidant and pro-oxidant systems, monooxygenase and peroxisomal systems, intracellular reparative processes, etc. The effects of multiple interconnections of the immune system with the nervous and endocrine systems and nutritive factors are rather numerous and cannot be ignored when developing new biologically active additives and feed additives.

The interaction between innate and acquired immunity. The innate immunity system is just the first line of defense against any foreign agents and begins at a molecular level. It is responsible for immediate nonspecific defense that does not include immunological memory about an antigen [7]. The adaptive immune system [also known as specific immune system) develops over days or weeks because it includes an antigen-specific response and creation of immunological memory due to which a subsequent encounter of the antigen is met with a quicker and stronger response. The initial mediators of adaptive immune response, the B-lymphocytes, produce antibodies neutralizing an antigen or mark the antigen for presentation to T-cells or subsequent destruction by macrophages [8]. As opposed to humoral immunity mediated by antibodies, the cell-mediated immunity is connected with T-cells, the lymphocytes produced by thymus gland. The subgroups of T-cells play different roles in the adaptive immune system: the cytotoxic T-killers attack and destroy alien cells, T-helpers amplify the immune response and help the function of the other lymphocytes, T-suppressors suppress and restrain the immune system [9].

The complement system, which includes about 20 protein factors activated in a cascade during immune response, irreversibly damages the structure and functions of an alien object. The phagocytosis dysfunction is often observed in case of lack of chemotactic polypeptides created during complement activation and ensuring phagocyte movement to the place of infection [10, 11]. The complement system modulates adaptive immune response and, as a component of both innate and adaptive immune system, may serve as an example of their interconnection.

The innate and acquired immunity function in unison via direct contact with the cells, and through the mediation of cytokine and chemokine communication molecules. i.e. small proteins contain cysteine that have chemotactic and attractant properties [12]. Consequently, an efficient immune system requires a coordinated effort of macrophages, neutrophiles, B- and T-lymphocytes with all other immune cell types. Furthermore, the immune response includes the growth of cell population of T-lymphocytes, reinforcing immunoglobulin synthesis with B-lymphocytes and acute-phase proteins with the formation of inflammatory mediators.

The cell-mediated response is required for lymphocyte clones to appear

and destroy objects with alien genetic information. Since diverse antigens can be found on the surface of alien cells, the immunoglobulins of different isotypes [A, M, G, E) are produced in activated plasmatic cells [13].

Stress and homeostasis. *Physiological stress*. In terms of physiological interpretation, stress can be defined as disturbance of internal equilibrium required for a living organism to respond in order to maintain psychophysiological integration. H.H.B. Selye, who outlined the triad necessary for all chromic stresses (involution of thymus-lymphatic system; hypertrophy of adrenal gland; stomach ulceration) [14], described maintaining stability of internal environment of a body as a generalized adaptation syndrome with a focus on adrenal cortex producing cortisol (corticosteroids). W. Cannon, the author of classic works on fight-or-flight response, who developed the definition of "stress" by introducing the term "homeostasis", focused on participation of sympathetic nervous system in stress response. At this time, the role of hypothalamic-pituitary-adrenal axis and sympathetic nervous system in the creation of a generalized response of a body to any stress [agent or event) is generally recognized.

Biochemical characteristics of stress. Oxidative metabolic stress. Intensive animal husbandry requires a widespread usage of preventive, therapeutic and stimulating means and technologies resulting in the emergence of additional chemical, biological and social factors causing stress to livestock population. During stress, a body generates an excessive amount of free radicals, which has a very negative impact on health of animals, their disease resistance and reduces the productivity and quality of products [15].

An in-depth analysis of state of all systems and formation of a generalized response to the incoming signals is possible only in the context of a supersystem of immunobiological control. It applies to the manifestations of nonspecific resistance. There exists a compelling opinion that immunobiological control should be understood as an interconnection of specific immune and non-specific biological factors and mechanisms of homeostasis directed against the agents and causes violating the structural and functional stability of the internal environment of a body and ensuring its preservation and recovery. These mechanisms are under genetic control and are regulated via direct correlations and response [16].

The activity of free-radical processes and lipid peroxidation required for normal operation of all intracellular components, regulation of lipid composition, fluidity and permeability of cytoplasmic membranes is biochemically homeostated [17] and is determined by tissue-specific balance of antioxidants and prooxidants. Oxidative stress occurs when the balance shifts in the direction of prooxidants [18] followed by disruption of defensive system functions and development of oxidative tissue damage. The free radicals are necessary for normal metabolism; however, their abundance has detrimental consequences [19]. The generality of free radical processes can be thought of as a unified mechanism of development of most (possibly, all) pathological process in any cell, tissue and organ regardless of pathology causes.

The almost strict dependence of suppression of free radical formation on activation of non-specific resistance systems on has always been observed [20]. The interconnection between loss of body resistance and increased activity of free radicals has also been observed. The metabolism controlled by the interaction among the nervous, immune and endocrine systems focuses specifically on their normalization during the initial stress phase [21]. Contemporary science has not yet managed to quantitatively assess the metabolic situation, the occurrence of which results in an uncontrolled overproduction of free radicals. A living organism has a multicomponent, in-depth antioxidant and antiradical system of defense [enzymatic and non-enzymatic) against oxidative stress [22], which is

a part of the non-specific resistance and immunobiological control.

Presently, the following catecholamines: adrenalin and noradrenaline are thought of as the main regulators of adaptive reactions [23]. They ensure fast, radical and relevant body response to stress due to their ability to initiate glycogenolysis and break down lipids, activate fatty acid oxidation, increase the concentration of glucose and unesterified fatty acids and triglycerols in blood, intensify the oxygen intake by tissues [24], change the luminal of vessels and bronchi, increase cardiac performance and performance of skeletal muscles [25], facilitate the agitation of the central nervous system. It is the catecholamines that are deemed to have the core function of establishing a link between the nervous, immune and endocrine systems via hypothalamus releasing factors, i.e. a large group of regulatory peptides common for these systems, and a cascade of hormones produced by the basal gland, as well as glands and cells synthesizing hormones in specific and non-specific organs [26].

The link between the hypothalamic-pituitary-adrenal axis and sympathetic part of the nervous system. Cerebral neurons in hypothalamus are the first to be stimulated in response to stress in order to synthesize and secrete corticotropin releasing hormone (CRH) and vasopressin (VP) [27]. The increased concentration of corticotropin releasing hormones and/or vasopressins in hypothalamic-pituitary portal blood initiates synthesis and secretion of neurohormones by the hypothalamus that activates the adrenal axis. The corticotropic cells of anterior pituitary gland stimulated by the corticotropin releasing hormones synthesize and secrete the adrenocorticotropic hormone (ACTH). In turn, the adrenocorticotropic hormone stimulates the production of glucocorticoids by the adrenal cortex. The glucocorticoids are responsible for negative hypothalamus and basal gland response via inhibiting the synthesis and/or secretion of corticotropin releasing factor, vasopressin и adrenocorticotropic hormone [28]. The implication is that vasopressin can stimulate the production of ACTH even at very low levels of CRH concentration. The glucocorticoid receptor can affect the hypothalamus-pituitary axis and can be inactivated by heat shock proteins hsp90, hsp70 and immunofilins, the low molecular weight proteins rich in cysteine that have the properties of cytokines. As a result of binding glucocorticoids with the receptors, the transcription of tissue-specific genes, which are related with the functioning of the immune system, is amplified or reduced, including synthesis of proinflammatory cytokines and anti-inflammatory cytokines, prostaglandins, cell adhesion molecules, etc. [29].

The sympathic nervous system is activated in response to numerous stress factors simultaneously with the hypothalamic-pituitary-adrenal axis or even earlier. Noradrenaline is secreted in case of stimulation of noradrenergic neurons in the brain and in postganglionic sympathetic neurons innervating the peripheral organs. Additionally, nerve impulse in the highest cortical centers from the brain transmits information for release of noradrenaline, serotonin and acetylcholine. The increased content of adrenaline in the brain acts as an alarm signal, which manifests itself in decreased neurovegetative activity, e.g. suppressed appetite and sleep disorders, and initiation of stress response via activation of the hypothalamic-pituitary-adrenal axis. Furthermore, noradrenaline secretion triggers the reactions of fear and aggression and boosts long-term memory of preserving hostile emotions reducing the functional activity of the immune system [30].

The reactions of the hypothalamic-pituitary-adrenal axis and sympathic nervous system are strictly and subtly interconnected. Both systems are activated in response to most stress factors and can synergistically produce response by helping each other. For instance, in the brain there exist reciprocal interconnections between noradrenaline and CRH, which activate each other. The release of

noradrenaline, serotonin and acetylcholine in the brain stimulated the secretion of CRH [31]. Similarly, noradrenaline induces the synthesis of ACTH and, correspondingly, of glucocorticoids. Likewise, CRH stimulates autonomic neurons in the brain stem for noradrenaline secretion [32]. The glucocorticoid receptors are also present in sympathetic neurons and enable glucocorticoids to regulate the synthesis, acceptance and noradrenaline content in brain tissue. The glucocorticoids can modulate the expression of β-adrenergic receptors via genomic and nongenomic mechanisms [33]. Together, catecholamine and glucocorticoids stimulate the cardiovascular system and catabolic effects, and inhibit numerous functions of the body, including the reproduction function and the immune system.

To summarize, the primary response to stresses of any aetiology is conducted by activating both the hypothalamic-pituitary-adrenal axis and the sympathic nervous system.

Stress, temperament and immune system. The hormonal panel analysis provides the most conclusive description of stress. The factors linked with the procedures associated with livestock breeding, e.g. weaning, castration, transportation, regrouping, tying, sudden change of ratios and quality of feeds, temperature conditions, negatively affect the immunity and productivity of animals. The behavioral response to challenges is differentiated and can be expressed as aggression, obedience, submission or adaptation. The response can be stronger or weaker depending on sensitivity of the animal [34]. The acute stress factors can multidirectionally affect the immune response, i.e. the effects can be immunosuppressive and immunomodulatory [35]. The chronic stresses usually result in suppression or dysfunction of innate and adaptive immune responses [36). Even under normal conditions the more excitable animals come close to stressed animals in terms of hormonal status, they have an increased basal concentration of glucocorticoids and catecholamines in blood, they are worse in terms of growth, their carcass quality is worse, and immune response in case of contact with pathogens is weaker. The increased concentration of glucocorticoids and catecholamines inhibits the immune system [37].

There are numerous works dedicated to the interconnections among the temperament, immune function and response of animals to stress factors, including tie-up and free stall housing. The cortisol concentration in young cattle stock is usually higher in case of free stall housing and goes down upon tie-up. Harsh treatment results in sharp decline of productivity and immune system of excitable (temperamental) animals and affects the calm animals to a lesser degree. The response to stresses of any aetiology is stimulated by different stress factors activating the hypothalamic-pituitary-adrenal axis and sympathetic nervous system [38].

The productivity and quality of the products are closely connected with behavioral response of animals and are reduced in case of negative emotions [39]. The stress hormone secretion, like immune response, depends on the temperament of animals. The calves with excitable (stress-non-resistant) temperament the basal concentration of cortisol and adrenaline is higher than that of calm animals [40]. Furthermore, in such animals the ACTH secretion in response to CRH is inhibited and in response to VP is boosted during depression. The nervous animals have a higher concentration of cortisol, and the calm animals the contents of neutrophils in blood positively correlates with cortisol concentration, which is not manifested in excitable animals [41]. The impact of temperament on production of stress hormones and the state of the immune system was identified in fattened male calves [41]. The easily excitable animals displayed a decline of the immune function along with reduction of the growth speed and carcass quality. In terms of lymphocyte proliferation and vaccine spe-

cific response (IgG products) the temperamental male calves were inferior to the calm ones. The excitable animals also showed a negative correlation between IgG and IgM concentration and lymphocyte proliferation (42). The cattle has higher population of B- and T-lymphocytes in blood, which identify antigens with membrane receptors and are responsible for immune response regulation, and the quantitative ratio of lymphocytes changes with age (43). The high concentration of catecholamines negatively affects the immune reactivity of cells treated with interleukines IL-1α and IL-1β. By affecting the secretion of cytokines with Th1 and Th2 T-helpers, the glucocorticoids and catecholamines can predominantly inhibit cell immunity controlled by Th1 cytokines, and not have the same active effect on Th2 cytokines controlled by humoral immunity [44].

To summarize, nervous animals display a higher degree of hypothalamic-pituitary-adrenal axis activation accompanied with suppressed immune response.

Nutritive factors and immune system. The nutrition, which is qualitatively and quantitatively deficient and can result in inadequate energy and nutrition substance ratio and deficit of a number of micronutrients (in some cases this can negatively affect the phagocytic function of innate immune system, synthesis of cytokines, antibodies and cell-mediated immunity), is the most frequent cause of immunodeficiencies and the risk factor of productive animals.

Macronutrients, food lipids. The disruptions of energy-protein feeds are the most frequent in animal husbandry, accompanied by the growth of vulnerability of livestock population to infections due to suppression of innate and adaptive immune systems [45]. Furthermore, the synthesis of certain cytokines and proteins of the complement systems is limited, the phagocytic function and cell-mediated immune reactions change [thymic atrophy occurs, the number of circulating T-cells declines along with the efficiency of immunological memory), the spleen and lymph node function is inhibited. The humoral immunity also suffers, the antibody affinity and response strength are reduced [16, 46].

Different food lipids are also connected to immune response modulation, first and foremost these are long-chain polyunsaturated fatty acids (LCPUSFA) ω-3 and ω-6, which are a part of the irreplaceable nutrient elements and should be received with food [47]. The eicosapentaenoic ($C_{20}H_{30}O_2$) and docosahexaenoic ($C_{21}H_{31}COOH$) ω-3 long-chain polyunsaturated fatty acids are involved in realization of the immune response and inflammatory response. They are included in the membranes of phospholipids of immune cells (including phagocytes and T-cells), induce the production of icosanoids, the 20-carbonic derivative of long-chain polyunsaturated fatty acids that play a key role in immune reactions, and other lipid mediators. In immune cell membranes the long-chain polyunsaturated fatty acids can metabolize enzymatically to icosanoids (prostaglandins, leukotrienes and thromboxanes) involved in the development of inflammatory response [16, 48].

The lipid mediators derived from the eicosapentaenoic and docosahexaenoic acids possess not only the anti-inflammatory properties, but are also capable of regulating the functions of T- and B-cells. Among icosanoids the derivatives of eicosapentaenoic acid are biologically less active than the derivatives of the arachidonic acid, therefore additions of eicosapentaenoic acid and other ω-3 long-chain polyunsaturated fatty acids are ineffective when treating inflammatory diseases. Presently, this area is actively studied because excessive consumption of ω-3 long-chain polyunsaturated fatty acids can suppress defensive mechanisms and increase vulnerability to infectious diseases [49]. The linoleic acid isomers [LA) with conjugated double bonds can modulate the immune function. The LA isomer cis-9, trans-11 is present in natural meat and milk of ruminant animals. It is also available as a food additive containing two LA isomer forms (cis-9, trans-11

and trans-10, cis-12). This additive increased the content of IgA and IgM in blood, while the number of proinflammatory cytokines declined and the number of anti-inflammatory cytokines increased [50].

Micronutrients: vitamins and minerals. The micronutrients play an essential role in the development and expression of immune response. Due to its immunosuppressive effect the deficit of specific micronutrients (vitamins, minerals) can have a negative impact on innate and adaptive immune system components increasing vulnerability to infectious and non-communicable diseases [51].

Vitamin A and its metabolites are involved in the reactions of the innate and adaptive immune systems. The skin and eye mucosa cells, as well as cells of the respiratory, gastrointestinal and genitourinary tract function as a barrier against infections (innate immune system). Vitamin A promotes the functioning of these mucosal cells and is necessary to maintain the activity of cells involved in the innate immune response (including natural killer cells, macrophages and neutrophils), and for the activity of T- and B-cells, the mediators of the adaptive immune system. Consequently, vitamin A is the essential factor of immune response. Vitamin A displays the key immune effects via the derivatives, specifically, via isomers of retinoic acid. The isomers of retinoic acid are steroid hormones, which are connected with retinoid receptors causing a cascade of molecular interactions initiating the expression of specific genes. The retinoic acid directly or indirectly regulates about 500 genes, some of which control cellular proliferation, which emphasizes the importance of vitamin A in the immune system [52].

The deficit of vitamin A results in immunodeficiency and increased risk of infectious diseases, especially at a young age. Vitamin A reduces the quantity and activity of killer cells. The subclinical deficiency of vitamin A increases the risk of infections, which, in turn, increase vitamin A deficiency in animals, including those caused by decreased feed intake, disorder of vitamin absorption and excretion, and reduced resorption. The impact of vitamin A on chemotaxis, phagocytosis and ability of immune cells to generate free radicals that destroy pathogens has been observed. By participating in the regulation of cytokine synthesis regulation vitamin A affects the development of inflammatory response of the innate immune system. The deficit of vitamin A affects humoral and cellular response of the adaptive immune system and has a particularly adverse effect on the growth and differentiation of B-cells that depend on retinol and its metabolites, and on manifestations of antibody response. The additions of vitamin A reduce the morbidity of diarrhea [53].

In terms of vitamin D, the function of the immune system modulator has been added to the impact on mineral homeostasis and bone metabolism. More than 200 genes are known, which are directly or indirectly regulated by dihydroxyvitamin D3. The receptor of vitamin D is expressed in immune cells of different types, including monocytes, macrophages, dendritic cells and activated T-cells. The macrophages also synthesize the hydroxyvitamin-D_3-1-hydroxylase enzyme, which locally converts vitamin D in its active form, which is involved both in the innate and adaptive immune responses. The antimicrobial peptides and cathelicidin are the key components of the innate immune system because they explicitly destroy pathogens, especially bacteria. The active form of vitamin D regulates cathelicidin synthesis and stimulates the other processes of the innate immune response, including proliferation of immune cells and biosynthesis of cytokines. By performing these functions vitamin D facilitates the increased efficiency of defense against infections. It suppresses the production of antibodies by B-cells and inhibits the proliferation of T-cells. Vitamin D can activate T-helpers and dendritic cells [54].

Vitamin C is a highly active natural antioxidant actively involved in innate and adaptive immune system response. Vitamin C functionally stimulates leucocytes, specifically neutrophils, lymphocytes and phagocytes (cell mobility, chemotaxis and phagocytosis). The neutrophils are the first line of cells activated by vitamin C [55]. At the same time it is believed to have an integrating role in the relations of immune cells. The neutrophils, mononuclear phagocytes and lymphocytes accumulate vitamin C in large quantities for protection of themselves and other cells against oxidative damage. The phagocytes produce a number of cytokines, including α-, β- and γ-interferons. The first two forms are thought of as classical and induced viruses, γ-interferon, being a typical cytokine, is synthesized by T- lymphocytes, natural killers and activated macrophages. Unlike α- and β-interferons, it actively stimulates the expression of components of the main complex of histocompatibility, antimicrobial and anti-tumor activity of macrophages and natural killers [56]. Furthermore, vitamin C is involved in regeneration of vitamin E from its oxidized form.

Vitamin E is an oil-soluble antioxidant; α-tocopherol form of vitamin E protects polyunsaturated fatty acid against peroxidation, which is the cause of damage of different cells of the immune system. The deficit of vitamin disrupts both the humoral and cell-mediated adaptive immune system, including the function of B- and T-cells. The additions of vitamin E above the recommended needs improve the immunity and reduce vulnerability to different infections, specifically at a young age [57). The functional activity of T-cells drops with age, which is confirmed by reduced proliferation and production by T-cells of cytokine IL-2 [58]. The experiments conducted on laboratory animals and clinical observations showed that vitamin E can compensate both age effects, improve the immune response on introduction of a vaccine against Hepatitis B and improve resilience against respiratory tract infections [59]. In other words, vitamin E can boost the immune system [60].

The deficiency of vitamin B_6 (pyridoxal) weakens the humoral and cell-mediated adaptive immune system. The specific effect of pyridoxal shortage manifests itself in suppression of proliferation, differentiation and maturation of lymphocytes, as well as biosynthesis of cytokines and antibodies. The adjusted content of pyridoxal in food ratios restores the affected immune functions [61].

The folic acid (Bc, B group vitamin) functions as a cofactor of enzymes transporting single-carbon fragments. The coenzymes containing folate act as acceptors and donators of single-carbon components in the endogenous synthesis reactions and RNA and DNA metabolism, as well as amino acids [62[. The deficiency of folate negatively affects the immune response, primarily the cell-mediated response. In case of folic acid deficiency the humoral arm of the immune system and antibody synthesis are suppressed [63].

Selenium is an essential element of the immune control system, it is a part of more than 30 enzymes (glutathione peroxidase, specifically) and vital biologically active compounds of animals. In case of selenium deficiency, animals, poultry and humans suffer numerous pathological changes similar to white-muscle disease, including T- and B- immunodeficiencies. Selenium affects the synthesis of IgG, IgM and IgA, lysozyme activity, β-lysines and general bactericidal activity, and activates the enzymatic antioxidant system [64]. By amplifying the activity of superoxide dismutase and glutathione peroxidase, selenium positively affects the non-specific resistance of the body, as well as cellular and humoral immune systems, which results in the growth of productivity and livability of animals and poultry [65]. Selenium additives stimulate immune response to foreign antigens, increase viral resistance, and expression of cytokines managing the immune response [66].

Zinc is a part of active centers of about 200 metalloenzymes and is through to be a critical element for the development and functioning of cells of the innate and adaptive immune system [67]. Zink does not accumulate in the body, it requires constant deliveries with feed. The Zn deficiency disrupts the complement system, cytotoxicity of natural killers, phagocytic activity of neutrophils and macrophages and ability of immune cells to generate free radicals that destroy pathogens. In case of strong Zn deficit, the immune system is suppressed and vulnerability to infectious agents increases [68].

Fe is involved in a number of immune functions, including differentiation and proliferation of T-lymphocytes, and in generation of reactive oxygen species neutralizing pathogens [69]. However, iron is also used by many infectious agents for reproduction and survival [70]. In case of acute inflammatory response, Fe concentration in blood serum decreases, and ferritin concentration increases. This is indicative of extraction of Fe, used by pathogens, as crucial response to infections. The increase of Fe concentration in blood, for instance, in case of hereditary haemochromatosis, can disrupt the immune system, biosynthesis of cytokines, complement activation and functions of T- and B-lymphocytes [71].

Copper plays an important role in the development and support of the immune system; however, the exact mechanisms of these interactions are still unclear. The copper deficit manifests itself as neutropenia, which can be the cause of significant increase of vulnerability to various infections [72]. At the same time it has been observed that chronic consumption of large copper doses negatively affects the immune system [73].

Probiotics [*immunobiotics*). More often than not these are lactobacilli and bifidobacteria, which are included in the ratios with fermentable products or as special additives. The probiotics are not digestible and access the colon, where they interact with receptors of intestinal epithelial and other cells associated with the immune system of the intestinal tract, including M- and dendritic cells [74]. The immune modulation effect is achieved only in case of constant intake of probiotics and change of intestinal flora. The probiotics have a positive effect on the innate and adaptive immune system [75], amplify epithelial intestinal barrier, including via inhibiting apoptosis and prolongation of life of intestinal epithelial cells, as well as stimulation of antibody production and proliferation of T-lymphocytes. The mechanism of impact of probiotics on the immune system of young livestock is rather complicated. As exemplified by *Lactobacillus jensenii* TL2937 strain, which in terms of its effects belongs to immunobiotics, this mechanism can broadly be described as follows [76]. A probiotic affects the signaling cascades of intestinal cells affecting the expression of cytokines and other proteins, and weakens the inflammatory response caused by the activation of a specific receptor of lymphocytes in lymphoid organs of intestinal walls. This process also involves the products of the major histocompatibility complex involved in antigen presentation to T- and B-lymphocytes. Consequently, the intestinal immunity of young livestock increases during the early postnatal period of ontogenesis. Since most of the cells of the immune system are localized in the intestinal mucosa and its mesogaster, the general immune status of animals is stimulated, which prevents the development of inflammatory intestinal disorders, diarrhea, allergies, gastrointestinal and other infections and improving health and growth of young livestock [77].

To summarize, the ability of the immune system to control the specifics of the internal environment of the body is implemented in conjunction with the nervous and endocrine systems. Together these systems, along with the antioxidant- pro-oxidant, monooxygenase and peroxisomal systems are combined in a suprasystem structure and ensure uniform immunobiological control of body re-

sistance. This structure maintains its resilience, control and metabolic regulation, as well as mobilization of non-specific resistance to the effect of any psychosocial, physical, chemical and biological agents and factors capable of causing adverse effect. The secretion of stress hormones and manifestation of immune response depend on the temperament of the animal because the hypothalamic-pituitary-adrenal axis and sympathetic nervous system are closely interconnected. The most frequent cause of immunodeficiency of productive animals is the deficiency of macronutrients and micronutrients. The interconnections between the immune, nervous and endocrine systems and nutritive factors are essential for the health and productivity of animals.

REFERENCES

1. Poryvaeva A.P., Krasnoperov A.S., Vereshchak N.A., Vaganova L.S. *Problemy veterinarnoi sanitarii, gigieny, ekologii*, 2017, 2: 83-87 (in Russ.).
2. Barashkin M.I. *Agrarnyi vestnik Urala*, 2015, 2: 113-119 (in Russ.).
3. Ribatti D. Peter Brian Medawar and the discovery of acquired immunological tolerance. *Immunol. Lett.*, 2015, 167(2): 63-66 (doi: 10.1016/j.imlet.2015.07.004).
4. Kalisch R., Müller M.B., Tüscher O. A conceptual framework for the neurobiological study of resilience. Behav. Brain Sci., 2015, 38(92): 1-79 (doi: 10.1017/S0140525X1400082X).
5. Hommers L., Raab A., Bohl A., Weber H., Scholz C.J., Erhardt A., Binder E., Arolt V., Gerlach A., Gloster A., Kalisch R., Kircher T., Lonsdorf T., Struhle A., Zwanzger P., Mattheisen M., Cichon S., Lesch K.P., Domschke K., Reif A., Lohse M.J., Deckert J. MicroRNA hsa-miR-4717-5p regulates RGS2 and may be a risk factor for anxiety-related traits. *Am. J. Med. Genet. Part B*, 2015, 168(4): 296-306. (doi: 10.1002/ajmg.b.32312).
6. Del Rey A., Besedovsky H.O. Immune-neuro-endocrine reflexes, circuits, and networks: physiologic and evolutionary implications. In: *Endocrine immunology*. W. Savino, F. Guaraldi (eds.). Front Horm Res. Basel, Karger, 2017, V. 48:1-18 (doi: 10.1159/000452902).
7. Panda S., Ding J.L. Natural antibodies bridge innate and adaptive immunity. *J. Immunol.*, 2015, 194(1): 13-20 (doi: 10.4049/jimmunol.1400844).
8. Cardamone C., Parente R., Feo G.D., Triggiani M. Mast cells as effector cells of innate immunity and regulators of adaptive immunity. *Immunol. Lett.*, 2016, 178: 10-14 (doi: 10.1016/j.imlet.2016.07.003).
9. Comrie W.A., Lenardo M.J. Molecular classification of primary immunodeficiencies of T lymphocytes. *Adv. Immunol.*, 2018, 138: 99-193 (doi: 10.1016/bs.ai.2018.02.003).
10. Dunkelberger J.R., Song W.C. Complement and its role in innate and adaptive immune responses. *Cell Res.*, 2010, 20(1): 34-50 (doi: 10.1038/cr.2009.139).
11. Surai P., Fisinin V.I. The modern anti-stress technologies in poultry: from antioxidants to vitagenes. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2012, 4: 3-13 (doi: 10.15389/agrobiology.2012.4.3eng).
12. Abhyankar V., Bland P., Fernandes G. The role of systems biologic approach in cell signaling and drug development responses — a mini review. *Med. Sci.*, 2018, 6(2): 1-9 (doi: 10.3390/medsci6020043).
13. Fisinin V.I., Surai P. *Ptitsevodstvo*, 2013, 5: 4-10 (in Russ.).
14. Goldstein D.S., McEwen B. Allostasis, homeostats, and the nature of stress. *Stress*, 2002, 5(1): 55-58 (doi: 10.1080/102538902900012345).
15. Galochkin V.A., Cherepanov G.G. *Problemy biologii produktivnykh zhivotnykh*, 2013, 1: 5-29 (in Russ.).
16. Cunningham-Rundles S., McNeeley D.F., Moon A. Mechanisms of nutrient modulation of the immune response. *J. Allergy Clin. Immunol.*, 2005, 115(6): 1119-1128 (doi: 10.1016/j.jaci.2005.04.036).
17. Galochkin V.A., Galochkina V.P., Ostrenko K.S. Development of theoretical bases and creation of antistress preparations of new generation for live-stock farming. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2009, 2: 43-55 (in Russ.).
18. Sinha N., Dabla P.K. Oxidative stress and antioxidants in hypertension — a current review. *Curr. Hypertens. Rev.*, 2015, 11(2): 132-142 (doi: 10.2174/1573402111666150529130922).
19. Lushchak V.I. Free radicals, reactive oxygen species, oxidative stress and its classification. *Chemico-Biological Interactions*, 2014, 224: 164-175 (doi: 10.1016/j.cbi.2014.10.016).
20. Jones D.P. Redefining oxidative stress. *Antioxidants & Redox Signaling*, 2006, 8: 1865-1879 (doi: 10.1089/ars.2006.8.1865).
21. Jones D.P. Radical-free biology of oxidative stress. *American Journal of Physiology—Cell Physiology*, 2008, 295(4): C849-C868 (doi: 10.1152/ajpcell.00283.2008).
22. Ostrenko K.S., Galochkina V.P., Galochkin V.A. Primenenie askorbata litiya dlya regulyatsii

lipidno-kholesterolovogo obmena i sistemy reduktsii glutationa u suporosnykh svinomatok. *Ukrainian Journal of Ecology*, 2018, 8(2): 59-66.
23. Belda X., Fuentes S., Daviu N., Nadal R., Armario A. Stress-induced sensitization: the hypothalamic-pituitary-adrenal axis and beyond. *Stress*, 2015, 18(3): 269-279 (doi: 10.3109/10253890.2015.1067678).
24. Zhao X.-J., Zhao Z., Yang D.-D., Cao L.-L., Zhang L., Ji J., Gu J., Huang J.-Y., Sun X.-L. Activation of ATP-sensitive potassium channel by iptakalim normalizes stress-induced HPA axis disorder and depressive behaviour by alleviating inflammation and oxidative stress in mouse hypothalamus. *Brain Res. Bull.*, 2017, 130: 146-155 (doi: 10.1016/j.brainresbull.2017.01.026).
25. Picard M., McManus M.J., Gray J.D., Nasca C., Moffat C., Kopinski P.K., Seifert E.L., McEwen B.S., Wallace D.C. Mitochondrial functions modulate neuroendocrine, metabolic, inflammatory, and transcriptional responses to acute psychological stress. *PNAS USA*, 2015, 112(48): 6614-6623 (doi: 10.1073/pnas.1515733112).
26. Lemos J.C., Wanat M.J., Smith J.S., Reyes B.A., Hollon N.G., Van Bockstaele E.J., Chavkin C., Phillips P.E. Severe stress switches CRF action in the nucleus accumbens from appetitive to aversive. *Nature*, 2012, 490: 402-406 (doi: 10.1038/nature11436).
27. Brindley R.L., Bauer M.B., Walker L.A., Quinlan M.A., Carneiro A.M.D., Sze J.Y., Blakely R.D., Currie K.P.M. Adrenal serotonin derives from accumulation by the antidepressant-sensitive serotonin transporter. *Pharmacol. Res.*, 2018, pii: S1043-6618(18)30429-8 (in press) (doi: 10.1016/j.phrs.2018.06.008).
28. Madalena K.M., Lerch J.K. The effect of glucocorticoid and glucocorticoid receptor interactions on brain, spinal cord, and glial cell plasticity. *Neural Plasticity*, 2017, 2017: Article ID 8640970 (doi: 10.1155/2017/8640970).
29. Ashwell J.D., Lu F.W., Vacchio M.S. Glucocorticoids in T cell development and function. *Annu. Rev. Immunol.*, 2000, 18(1): 309-345 (doi: 10.1146/annurev.immunol.18.1.309).
30. Lцwenberg M., Stahn C., Hommes D.W., Buttgereit F. Novel insights into mechanisms of glucocorticoid action and the development of new glucocorticoid receptor ligands. *Steroids*, 2008, 73(9-10): 1025-1029 (doi: 10.1016/j.steroids.2007.12.002).
31. Reeves J.W., Fisher A.J., Newman M.G., Granger D.A. Sympathetic and hypothalamic-pituitary-adrenal asymmetry in generalized anxiety disorder. *Psychophysiology*, 2016, 53(6): 951-977 (doi: 10.1111/psyp.12634).
32. Gil-Lozano M., Romanн-Pérez M., Outeirico-Iglesias V., Vigo E., González-Matías L.C., Brubaker P.L., Mallo F. Corticotropin-releasing hormone and the sympathoadrenal system are major mediators in the effects of peripherally administered exendin-4 on the hypothalamic-pituitary-adrenal axis of male rats. *Endocrinology*, 2014, 155(7): 2511-2523 (doi: 10.1210/en.2013-1718).
33. Oakley R.H., Cidlowski J.A. The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. *J. Allergy Clin. Immunol.*, 2013, 132(5): 1033-1044 (doi: 10.1016/j.jaci.2013.09.007).
34. Blalock J.E. The syntax of immune-neuroendocrine communication. *Immunology Today*, 1994, 15(11): 504-511 (doi: 10.1016/0167-5699(94)90205-4).
35. Edwards K.M., Morris N.B. Who's the boss: determining the control pathways of cardiovascular and cellular immune responses to acute stress. *Adv. Physiol. Educ.*, 2018, 42(2): 374-379 (doi: 10.1152/advan.00087.2017).
36. Zhang J.Y. Liu T.H., He Y., Pan H.Q., Zhang W.H., Yin X.P., Tian X.L., Li B.M., Wang X.D., Holmes A., Yuan T.F., Pan B.X. Chronic stress remodels synapses in an amygdala circuit-specific manner. *Biol. Psychiatry*, 2018, pii: S0006-3223(18)31633-0 (doi: 10.1016/j.biopsych.2018.06.019).
37. Curley K.O. Jr., Neuendorff D.A., Lewis A.W., Cleere J.J., Welsh T.H., Randel R.D. Functional characteristics of the bovine hypothalamic-pituitary-adrenal axis vary with temperament. *Hormones and Behavior*, 2008, 53(1): 20-27 (doi: 10.1016/j.yhbeh.2007.08.005).
38. Ramos R., Llabrés V., Monclús L., López-Béjar M., González-Solís J. Costs of breeding are rapidly buffered and do not affect migratory behavior in a long-lived bird species. *Ecology*, 2018, 0(0): Epub ahead of print (doi: 10.1002/ecy.2435).
39. Avitsur R., Padgett D.A., Sheridan J.F. Social interactions, stress, and immunity. *Neurologic Clinics*, 2006, 24(3): 483-491 (doi: 10.1016/j.ncl.2006.03.005).
40. Guzmán D.A., Lèche A., Contarde C.B., Nazar F.N., Marin R.H. Adrenocortical responses in Japanese quail classified by their permanence in proximity to either low or high density of conspecifics. *Poultry Sci.*, 2018, 0: pey269-pey269 (doi: 10.3382/ps/pey269).
41. Larauche M., Mulak A., Yuan P., Kanauchi O., Taché Y. Stress-induced visceral analgesia assessed non-invasively in rats is enhanced by prebiotic diet. *World J. Gastroenterol.*, 2012, 18(3): 225-236 (doi: 10.3748/wjg.v18.i3.225).
42. Savignac H.M., Kiely B., Dinan T.G., Cryan J.F. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. *Neurogastroenterol Motil.*, 2014, 26(11): 1615-1627 (doi: 10.1111/nmo.12427).
43. Savignac H.M., Tramullas M., Kiely B. Bifidobacteria modulate cognitive processes in an anxious mouse strain. *Behav. Brain Res.*, 2015, 287: 59-72 (doi: 10.1016/j.bbr.2015.02.044).

44. Zhang Y., Zhang Y., Gu W., He L., Sun B. Th1/Th2 cell's function in immune system. In: *T helper cell differentiation and their function. Advances in experimental medicine and biology*. B. Sun (ed.). Springer, Dordrecht, 2014, V. 841: 45-65 (doi: 10.1007/978-94-017-9487-9_3).
45. Jolly C.A., Fernandes G. Protein-energy malnutrition and infectious disease. In: *Nutrition and immunology: principles and practice*. M.E. Gershwin, J.B. German, C.L. Keen (eds.). Humana Press, Totowa, NJ, 2000: 195-202 (doi: 10.1007/978-1-59259-709-3_16).
46. Bourke C.D., Berkley J.A., Prendergast A.J._Immune dysfunction as a cause and consequence of malnutrition. *Trends Immunol.*, 2016, 37(6): 386-398 (doi: 10.1016/j.it.2016.04.003).
47. O'Shea M., Bassaganya-Riera J., Mohede I.C. Immunomodulatory properties of conjugated linoleic acid. *Am. J. Clin. Nutr.*, 2004, 79(6): 1199S-1206S (doi: 10.1093/ajcn/79.6.1199S).
48. Yaqoob P., Calder P.C. Fatty acids and immune function: new insights into mechanisms. *Brit. J. Nutr.*, 2007, 98(S1): S41-S45 (doi: 10.1017/S0007114507832995).
49. Miles E.A., Calder P.C. Fatty acids, lipid emulsions and the immune and inflammatory systems. In: *Intravenous lipid emulsions. World review of nutrition and dietetics*. P.C. Calder, D.L. Waitzberg, B. Koletzko (eds). Basel, Karger, 2015, V. 112: 17-30 (doi: 10.1159/000365426).
50. Haghikia A., Jörg S., Duscha A., Berg J., Manzel A., Waschbisch A., Hammer A., Lee D.H., May C., Wilck N., Balogh A., Ostermann A.I., Schebb N.H., Akkad D.A., Grohme D.A., Kleinewietfeld M., Kempa S., Thöne J., Demir S., Müller D.N., Gold R., Linker R.A. Dietary fatty acids directly impact central nervous system autoimmunity via the small intestine. *Immunity*, 2015, 43(4): 817-829 (doi: 10.1016/j.immuni.2015.09.007).
51. Richter F.C., Obba S., Simon A.K. Local exchange of metabolites shapes immunity. *Immunology*, 2018, 0(0): Epub ahead of print (doi: 10.1111/imm.12978).
52. Lo H.M., Wang S.W., Chen C.L., Wu P.H., Wu W.B. Effects of all-trans retinoic acid, retinol, and β-carotene on murine macrophage activity. *Food Funct.*, 2014, 5(1): 140-148 (doi: 10.1039/C3FO60309A).
53. Spinas E., Saggini A., Kritas S.K., Cerulli G., Caraffa A., Antinolfi P., Pantalone A., Frydas A., Tei M., Speziali A., Saggini R., Pandolfi F., Conti P. Can vitamin A mediate immunity and inflammation? *J. Biol. Reg. Homeos. Ag.*, 2015, 29(1): 1-6.
54. Sutton A.L., MacDonald P.N. Vitamin D: more than a "bone-a-fide" hormone. *Mol. Endocrinol.*, 2003, 17(5): 777-791 (doi: 10.1210/me.2002-0363).
55. Sorice A., Guerriero E., Capone F., Colonna G., Castello G., Costantini S. Ascorbic acid: its role in immune system and chronic inflammation diseases. *Mini-Reviews in Medicinal Chemistry*, 2014, 14(5): 444-452 (doi: 10.2174/1389557514666140428112602).
56. Berzina N., Markovs J., Dizhbite T., Apsite M., Vasilyeva S., Basova N., Smirnova G., Isajevs S. Oxidative stress and innate immunity status in chickens exposed to high dose of ascorbic acid. *Cell Biochem. Funct.*, 2013, 31(7): 551-559 (doi: 10.1002/cbf.2934).
57. Krueger L.A., Beitz D.C., Onda K., Osman M., O'Neil M.R., Lei S., Wattoo F.H., Stuart R.L., Tyler H.D., Nonnecke B. Effects of D-α-tocopherol and dietary energy on growth and health of preruminant dairy calves. *J. Dairy Sci.*, 2014, 97(6): 3715-3727 (doi: 10.3168/jds.2013-7315).
58. Sitrin J., Ring A., Garcia K.C., Benoist C., Mathis D. Regulatory T cells control NK cells in an insulitic lesion by depriving them of IL-2. *J. Exp. Med.*, 2013, 210(6): 1153-1165 (doi: 10.1084/jem.20122248).
59. El-Kannishy G., Arafa M., Abdelaal I., Elarman M., El-Mahdy R. Persistent oxidative stress in patients with chronic active hepatitis-C infection after antiviral therapy failure. *Saudi J. Gastroenterol.*, 2012, 18(6): 375-379 (doi: 10.4103/1319-3767.103429).
60. Reddy K.K., Ravinder T., Kanjilal S. Synthesis and evaluation of antioxidant and antifungal activities of novel ricinoleate-based lipoconjugates of phenolic acids. *Food Chem.*, 2012, 134(4): 2201-2207 (doi: 10.1016/j.foodchem.2012.04.046).
61. Cheng C.H., Chang S.J., Lee B.J., Lin K.L., Huang Y.C. Vitamin B_6 supplementation increases immune responses in critically ill patients. *Eur. J. Clin. Nutr.*, 2006, 60(10): 1207-1213 (doi: 10.1038/sj.ejcn.1602439).
62. Ströhle A., Bohn T. Folate and prevention of neural tube defects: new insights from a Bayesian model. *Int. J. Vitam. Nutr. Res.*, 2015, 85(3-4): 109-111 (doi: 10.1024/0300-9831/a000229).
63. Ströhle A., Wolters M., Hahn A. Safety of folic acid. *Med. Monatsschr. Pharm.*, 2015, 38(8): 297-306.
64. Galochkin V.A., Galochkina V.P. *Problemy biologii produktivnykh zhivotnykh*, 2008, 4: 3-20 (in Russ.).
65. Galochkin V.A., Cherepanov G.G. *Problemy biologii produktivnykh zhivotnykh*, 2013, 1: 5-29 (in Russ.).
66. Galochkin V.A., Agafonova A.V., Galochkina V.P., Cherepanov G.G. *Problemy biologii produktivnykh zhivotnykh*, 2015, 1: 5-24 (in Russ.).
67. Prasad A.S. Zinc in human health: effect of zinc on immune cells. *Mol. Med.*, 2008, 14(5-6): 353-357 (doi: 10.2119/2008-00033.Prasad).
68. Ibs K.H., Rink L. Zinc-altered immune function. *J. Nutr.*, 2003, 133(5): 1452S-1456S (doi: 10.1093/jn/133.5.1452S).
69. van Eijk L.T., Heemskerk S., van der Pluijm R.W., van Wijk S.M., Peters W.H., van der

Hoeven J.G., Kox M., Swinkels D.W., Pickkers P. The effect of iron loading and iron chelation on the innate immune response and subclinical organ injury during human endotoxemia: a randomized trial. *Haematologica*, 2014, 99(3): 579-587 (doi: 10.3324/haematol.2013.088047).
70. Khoshfetrat M.R., Mohammadi F., Mortazavi S., Rashidi A., Neyestani T., Kalantari N., Esmaillzadeh A. The effect of iron-vitamin C co-supplementation on biomarkers of oxidative stress in iron-deficient female youth. *Biol. Trace Elem. Res.*, 2013, 153(1-3): 171-177 (doi: 10.1007/s12011-013-9695-7).
71. Parveen N., Ahmad S., Shadab G.G. Iron induced genotoxicity: attenuation by vitamin C and its optimization. *Interdiscip. Toxicol.*, 2014, 7(3): 154-158 (doi: 10.2478/intox-2014-0021).
72. Beard J.L. Iron biology in immune function, muscle metabolism and neuronal functioning. *J. Nutr.*, 2001, 131(2): 568S-579S (doi: 10.1093/jn/131.2.568S).
73. Percival S.S. Copper and immunity. *Am. J. Clin. Nutr.*, 1998, 67(5): 1064S-1068S (doi: 10.1093/ajcn/67.5.1064S).
74. Bonham M., O'Connor J.M., Hannigan B.M., Strain J.J. The immune system as a physiological indicator of marginal copper status? *Br. J. Nutr.*, 2002, 87(5): 393-403 (doi: 10.1079/BJNBJN20020558).
75. de Vrese M., Schrezenmeir J. Probiotics, prebiotics, and synbiotics. In: *Food biotechnology. Advances in biochemical engineering/biotechnology*. V. 111. U. Stahl, U.E. Donalies, E. Nevoigt (eds.). Springer, Berlin, Heidelberg, 2008: 1-66 (doi: 10.1007/10_2008_097).
76. Ruemmele F.M., Bier D., Marteau P., Rechkemmer G., Bourdet-Sicard R., Walker W.A., Goulet O. Clinical evidence for immunomodulatory effects of probiotic bacteria. *J. Pediatr. Gastr. Nutr.*, 2009, 48(2): 126-141 (doi: 10.1097/MPG.0b013e31817d80ca).
77. Dowling D.J., Levy O. Ontogeny of early life immunity. *Trends Immunol.*, 2014, 35(7): 299-310 (doi: 10.1016/j.it.2014.04.007).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 687-697
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.084:636.087.7

doi: 10.15389/agrobiology.2018.4.687eng
doi: 10.15389/agrobiology.2018.4.687rus

USE OF PHYTOBIOTICTS IN FARM ANIMAL FEEDING
(review)

O.A. BAGNO[1], O.N. PROKHOROV[1], S.A. SHEVCHENKO[2], A.I. SHEVCHENKO[2, 3], T.V. DYADICHKINA[1]

[1]*Kemerovo State Agricultural Institute*, 5, ul. Markovtseva, Kemerovo, 650056 Russia, e-mail oaglazunova@mail.ru, oldao@mail.ru, dyadi-tanya@yandex.ru;)
[2]*Gorno-Altaisk State University,* 1, ul. Lenkina, Gorno-Altaisk, 649000 Russia, e-mail se-gal@list.ru (✉ corresponding author);
[3]*Gorno-Altaisk Research Institute of Agriculture,* Federal Agency for Scientific Organizations. 2, ul. Katunskaya, s. Maiama, Altai Republic, Russia, e-mail shaisol60@mail.ru
ORCID:
Bagno O.A. orcid.org/0000-0003-4047-2355
Prokhorov O.N. orcid.org/0000-0003-1916-661X
Shevchenko S.A. orcid.org/0000-0003-4361-3168
Shevchenko A.I. orcid.org/0000-0002-7753-1761
Dyadichkina T.V. orcid.org/0000-0003-1432-1166
The authors declare no conflict of interests
Acknowledgements:
This article is prepared under the Agreement with the Ministry of Education and Science of the Russian Federation № 14.610.21.0016 of 03.10.2017 "Development and implementation of a new series of highly effective medicinal-plant-born phytobiotics for highly productive and environmentally friendly animal farming", the unique identifier RFMEF161017X0016
Received April 2, 2018

Abstract

Realization of genetic potential of animal productivity in modern commercial livestock breeding necessitates the use of various biologically active dietary additives to ensure animal performance and homeostasis (R.R. Akhmedkhanova et al., 2010). The first such additives were feed antibiotics used since 1950s all over the world (R.I. Castillo-Lopez et al., 2017). However, it turned out that the excessive and uncontrolled use of antibiotics adversely affects the body of animals and birds. Microorganisms, when mutating, acquire resistance to antibiotics, thereby reducing the positive effect of the drugs. In addition, their cumulative and toxic effects occur. Over time, this led to a ban on the use of all types of feed antibiotics in the European Union (S.M. Alieva et al., 2017). After revealing the negative effects of feed antibiotics, a new tendency emerged in the world. That was a trend towards complete or partial replacement of these drugs with probiotics, the living microorganisms which are symbionts of the normal gut microflora. Probiotics are proven effective in growing young farm animals of different species in the early postnatal period and now proboitics are increasingly being used. In recent years, many scientists and practitioners of animal feeding have paid much attention to phytobiotics, the plant-born bioactive substances (W. Windisch et al., 2006). The fact that animals, including carnivores, when restricted in free walking, must eat green or at least dried plants was an empirical knowledge that humanity acquired simultaneously with the beginning of animal domestication. Targeted study on the impact of dietary bioactive plant-born compounds of different origin on animal and poutry performance, and the development of standardized phytobiotic preparations for livestock, including complex phytobiotics, are in the focus during last two or three decades (N.M. Kazachkova, 2017). Exterior and interior parameters are estimated in animals fed with dietary green and dried plants, their mixtures, coniferous and herbal flour of various composition, plant extracts, in particular essential oils, and phytobiotic complexes enriched with microelements and probiotics (N.A. Tabakov et al., 2008). The main benefit of phytogenic additives in livestock breeding is due to an improved feed digestibility, antimicrobial efficacy, the replacement of feed antibiotics, and growth stimulation. Their anti-inflammatory effect, better feed conversion and higher feed intake by animals are also noted (L.S. Ignatovich, 2017). Immune modulating plant-born fodder supplements have a significant effect on animal and poultry health and performance characteristics (B. Kiczorowska et al., 2017). High profitability of organic food production and animal and people wellness as a world trend are also the factors determining the relevance of the development of highly effective phytobiotics and their use in livestock and poultry farming.

Keywords: phytobiotics, probiotic, feed antibiotics, animal feeding, cattle, pigs, poultry, productivity, homeostasis.

The application of feed additives in contemporary livestock and poultry farming is required to prevent negative impact of certain feed and management factors [1]. The latter, in particular, cause depression of the immune system, due to which the livestock and poultry become more susceptible to different illnesses. Furthermore, the activity of numerous physiological bodily systems is distorted, which inevitably results in deterioration of the quality of products [2].

During the recent years, increased emphasis has been made on technologies based on comprehensive registration of crucial biotechnological factors and adoptions from wildlife. For example, phytobiotics (the biologically active substances that possess antimicrobial properties) [3] can offer an alternative to synthetic antimicrobial growth promoters. In contemporary studies, phytobiotics (phytogenic food additives or herbal products) are defined as natural plant-based additives that have varying impact on the body (antimicrobial, antiviral, immunomodulating, fungicidal or anti-inflammatory) and are used as animal feeds with the purpose of increasing the productivity of livestock and the quality of food products of animal origin [4, 5].

The goal of this survey is to study the contemporary usage of phytobiotics in livestock feed based on analysis of scientific publications in the leading Russian and international magazines, thesis databases, monographs and patents.

The history of a wide-scale application of phytobiotics in livestock and poultry feeds is inextricably connected with the prohibition of all forms of antibiotic growth promoters in the EU. The excessive and uncontrolled usage of antibiotic growth promoters negatively affects the bodies of livestock and poultry. Mutating, the microorganisms become immune to antibiotics thus excluding the positive effect of the drug [6\. Furthermore, antibiotics can build up in the body resulting in increased toxic effect [7].

In 2004-2005, a new feed concept was developed in the European Union, which precludes the usage of antimicrobial growth promoters and envisioning the usage of phytobiotics. The phytobiotics are divided into the following groups based on biological origin, chemical composition and other attributes: herbs (flowering, herbaceous and short-lived plants), spices (herbs with intensive odor or taste, usually added in food), ether oils (volatile lipophilic compounds obtained by cold pressing, steam or alcohol distillation) and resins (oleoresin, extracts obtained with the help of non-aqueous solvents). These substances can be used as antimicrobial agents and should become available for usage in livestock feeds [8, 9]. In a number of papers the authors underline the significant differences in the number of biologically active substances depending on geographical area or plant harvesting time [10, 11]. For instance, ether oils obtained after summer harvest have the highest antimicrobial activity immediately after flowering [12-14]. Presently, the production of phytobiotics in EU countries has reached significant amounts. Phytobiotics Futterzusatzstoffe GmbH is one of the largest global producers of phytobiotics, which was founded in Germany in 2000. The main product of the company is Sangrovit®, which is a natural plant-derived feed additive designed to increase livestock productivity [15].

The antimicrobial [10, 13, 16-18] and immunostimulating [19-22] properties of plant-derived ether oils are at the center of focus. The antimicrobial effect of 96 various ether oils has been analyzed, as well as 23 of their active agents. It was demonstrated that cinnamaldehyde, thymol, carvacrol and eugenol display the strongest antimicrobial activity against the strains of *Escherichia coli*, *Salmonella enterica* and *Listeria monocytogenes* [23]. The positive effect of *Origanum syriacum* ether oil on productivity and histology of the intestinal tract of poultry [24] has been determined, which is added to the diet of broilers in case of thermal stress.

The antimicrobial activity of ether oils is not defined by the only mechanism and is aimed at several goals in microbial cells [25, 26]. The ether oils can destabilize and change the permeability of bacterial membranes [27-30]. These changes result in the release of ions from the cell to the environment [31], and in the change of proton gradient and depletion of intracellular reserves of adenosine triphosphate [32, 33].

Unlike probiotics, the systemic impact of phytobiotics on the bodies of livestock and poultry is attributable not only to the antimicrobial effect, but also to their positive impact on digestive processes. The phytobiotics stimulate the production of endogenous enzymes improving the digestibility and nutrient intake of feed. Many of them act as natural flavoring agents stimulating feed consumption, which has a positive effect on livestock productivity [34].

The phytobiotics have specific impact on the microbial composition of the intestinal tract maintaining the optimal state of microbial flora [35]. The usage of phytobiotics stimulates digestive secretion and has positive impact on morphofunctional characteristics of gastrointestinal mucosa. The better and more efficient absorption in the small intestine results in reduction of valuable nutrient loss. The risk of undesirable microbial population developing in the colon is reduced [36]. The positive impact of gel phytogenic feed additive on growth performance, nutrient intake and morphological properties of the intestinal tract in post-weaning pigs has been reported [37].

M. Mohiti-Asli at al. [38] compared the effect of two phytogenic mixtures on growth and immune response of broilers. Adding oregano ether oil to the ratios had a positive impact on productivity and immune function of broiler chickens. A possibility of using plant-derived polyphenols to combat oxidative stress and inflammatory processes of agricultural animals is under consideration [39].

The phytobiotics are natural growth promoters (NGPs) and can become a promising replacement for antibiotic growth promoters in contemporary livestock farming [40]. The different NGPs combined in a balanced mix effectively combat the intrusion of pathogenic organisms and consequences of unfavorable keeping conditions [41].

The simplest method of using phytobiotics is to add plants to livestock feeds in their native or dry form. For instance, using fresh nettle and nettle meal as feed for poultry can cover up to 20% protein needs, up to 60-70% vitamin needs and up to 100% of needs in minor nutrient elements, and up to 30% combined feed can be saved. The taste of eggs and poultry meat and their biological value also increase significantly [42]. The usage of grass meal made of coroniferous saw-wort attributed to the increase of egg-laying capacity of white Hungarian goose, the weight of eggs, conception rate, hatchability and hatching out [43]. The inclusion of oak bark in broiler chicken ratios increased feed intake and had no negative impact on the bodies of poultry, and usage of oak bark extract along with enzyme preparation stimulated digestion [44]. The silver fir coniferous meals are recommended for all livestock and poultry in winter and spring season as an alternative to synthetic additives. Feeding fir meals to cows during lactation stimulated the increase of lactation productivity, improvement of vitamin content of milk and reproductive function and normalization of metabolism [45]. During factory testing of Pikhtovit (Solagift LLC, Russia), which was developed using silver fir needle extract, on cross-bred poultry, the broilers of the test group demonstrated better hematological figures compared with the control group, and body weight increased by 4% [46].

The data have been presented about usage of topinambur as a valuable feed and medicinal crop. Topinambur is a natural immunomodulator, which acts as a concentrator of inulin polysaccharide in combination with pectic substances,

vitamins, irreplaceable amino acids, macro and micro elements [47]. The effect of aqueous extracts of *Origanum vulgare* L. and *Rosmarinus officinalis* L. on the immune system, microbial population of the intestinal tract and productivity of broiler chickens has been studied. The extracts of these herbs increased the immune resistance of broilers, brought the microbial population of the intestinal tract into balance, which is required for digestive processes and protection against enteropathogenic microorganisms, and improved the productive qualities of poultry [48].

The introduction of multiple-component feed additives in the ratios of laying hens consisting of stinging nettle meal (0.3-1.0%), mountain pine fir needles (0.2-1.0%), laminaria (0.5 %), ginger plant (0.5-1.0%) and common yarrow (0.5-1.5%) turned out efficient for enrichment of rations with nutrient and biologically active substances [49].

When growing broilers, the addition of *Cinnamomum cassia* L. powder in the amount of 0.5 % per ration weight [50] can be used as phytobiotic alternative of antibiotics. An additive from *Punica granatum* L. improved the immune system and microbial ecosystem of the intestinal tract of broilers along with the reduction of emission of gas with litter [51]. *Boswellia serrata* Roxb. ex Colebr. resin is also considered safe and efficient biological additive for broilers positively affecting poultry productivity [52].

The application of *Thymus serpyllum* L. as phytogenic feed additive in pig farming facilitates the intoxication levels, stress-inducing impact on the immune system, stabilizes the endoecological situation and balance of gastrointestinal microbial population in the intestinal tract, increases feed attractiveness and intake, and absorption efficiency of essential nutrients, and in general stimulates the improvement of nutritional status of livestock, their optimal development and realization of their genetic potential [53].

Echinacea purpurea (L.) Moench is characterized by high biological plasticity, adaptivity, ecological sustainability, productive longevity, feed and medicinal properties, and stable fruit bearing [54]. During tests conducted on broiler chickens using *Echinacea purpurea* (L.) Moench, a 19.4 % growth intensity increase was identified as compared with the control group. The biggest effect was achieved by combining *Echinacea purpurea* (L.) Moench with Lactobifadolum. The usage of these agents as a whole increased live body weight gain by 20.9% during the entire period of chicken incubation, reduced feed costs by 17.3% and ensured 100% preservation of livestock population [55]. When aqueous alcoholic extracts of aboveground parts, roots and whole *Echinacea purpurea* (L.) Moench were added in the drinking water, the daily weight gain of broiler chickens increased by 3.3-12.5%, and feed costs decreased by 5.2-9.2% as compared with the control group [56]. An indication is made regarding a possibility of using *Cichorium intybus* L. as a feed additive to increase poultry productivity [57]. The positive effect was noted of Florabis phytobiotic agent (IPC Abis LLC, Russia), which is based on a complex of triterpenic acids of Siberian fir with cobalt ions, at a dose of 0.002 $ml \cdot units^{-1} \cdot day^{-1}$ on biochemical and immunological indicators of blood of broiler chickens of ISA F 15 cross-breed, digestibility and nutrient intake of combined feed [58].

As a rule, complex preparations have a more pronounced effect than each component individually. In this regard, the data about research of complex plant-derived concentrates with a probiotic based on *Bacillus subtilis* [59] bacterium are of interest. The sea buckthorn leaves fermented with a probiotic agent are used as a phytobiotic, as well as a mixture of *Echinacea purpurea* (L.) Moench herb with berries of *Silybum marianum*. The preparation in the form of a ProStor (CV-AgroTrade, Russia) biologically active additive (BAA) additionally

contains an association of *Bacillus licheniformis* bacteria, lactobacilluses, prebiotics mannan-oligosaccharides of cell walls of *Saccharomyces cerevisiae* yeast and beetroot pulp pectins. Feeding the complex plant-driven concentrate (sea buckthorn leaves) with a probiotic on the basis of *Bacillus subtilis* to lactating highly productive cows had a positive effect on milk productivity of livestock with reduced feed cost per product unit [59]. The live body weight of bulls receiving ProStor BAA at the age of 15-17 months was 13.5 kilos (3.1%) higher than that of the bulls in the control group by the end of the feed. The cost of energetic feed unit was lower by 7% in the test group as compared with the control group [59].

The addition of L-arginine Pro (Russia) contains a bioactive fir needle extract of Scots pine enriched with L-arginine. The feeding of ratios containing this additive in the amount of 1 g/100 g of combined feed ensures stable and intensive growth of rearing birds, facilitates the increase of egg-laying capacity of laying hens by 46.8%, egg weight by 4.7 g, increase of morphological indicators and egg quality [60].

The usage of bioactive substances of Volgograd Institute of Cattle Farming and Livestock Product Processing RAS (Russia) Laktofit (a composition of bioactive substances of topinambur, beetroot, carrot, pumpkin, milk thistle, chickpea with a concentration of lactulose and malic acid) and Lactoflex (a composition of bioactive substances of dandelion, mint, licorice, calendula, pumpkin seeds, milk thistle, chickpea with a concentration of lactulose and malic acid) enables body weight increase of laying hens, improvement of morphological and biochemical blood composition, increase of weight and linear indicators of reproductive organs of test birds and hatching out of healthy rearing birds [61].

Intebio natural substitution of antibiotic growth promoters (previously manufactured as Mix Oil, Biotrof" LLC, Russia) is a mixture of natural ether oils that has antimicrobial activity, antioxidant effect and anti-inflammatory effect. The introduction of Mix Oil phytobiotic agent in the ratios of brood sows had a positive impact on their preparation for farrowing. During the suckling period, the additive accelerated the growth of piglets and increased their livability. In the test group the daily live weight gain of piglets increased by 16.5% and feed costs per 1 kilogram of weight gain were 0.72 feed units lower as compared with the control group [62].

The usage of Provitol phytoprobiotic (Biotrof LLC, Russia), which includes living bacteria and compositions of ether oils extracted from plants with antioxidant properties in the ratios of freshly calved cows stimulated the increase in rough feed edibility and significant increase of milk productivity during days in milk (63). When determining the efficiency of Provitol phytoprobiotic in combined feed for laying hens containing novel corn, the age-specific decrease of productivity by 2.9% is determined in the control group, which was facilitated by poultry transition to novel corn. At the same time, in this context the addition of Provitol in combined feed for test group hens increased the egg-laying by 2.8% as compared with the previous month, and by 7.6% during the second productivity phase as compared with the control group [64].

The lab research and production tests have proven the synergistic effect of ether oils and organic acids that are included in Liptosa Premix Expert (Lipidos Toledo, S.A., Spain) preparation when used to inhibit clostridium, salmonella and *Escherichia coli*. The ether oils of thymol, carvacrol, eugenol are used as strong antiviral, anti-infectious, bactericidal and immunostimulating drugs. The sensibility of clostridium, salmonella and *Escherichia coli* in anaerobic conditions to thymol, carvacrol and oregano oil [17, 32, 33, 65] has been proven.

The production testing of Meth Plus phytobiotic (Levet-Agro LLC, the

Republic of Belarus) developed as alternative synthetic methionine was conducted in Hy-Line cross-breed chickens at the breeding stage. The poultry receiving Meth Plus in their ratios exceeded the standards in terms of body weight gain, and herd uniformity exceeded 80% (66). When conducting tests on Pietrain pigs, 1 kilogram of DL-methionine was replaced with a similar quantity of Meth Plus phytobiotic, as a result of which the livestock of one group demonstrated the highest daily live weight gain and the best feed conversion ratio and slaughter parameters as compared with the test livestock [66].

The activity of Biost-rong® 510, (Delacon Biotechnik GmbH, Austria) is conditioned on flavoring agents, anisic acid and glucuronic acid, saponins, thymol, borneol, carvacrol that stimulate biocatalytic and enzymatic process in the intestinal tract of poultry. It has been determined that Biostrong® 510 enables replacing antibiotic growth promoters, which ensures high digestibility and usage of main nutrients of combined feeds and livability [67].

Digestarom® 1317 (Micro-Plus Konzentrate GmbH, Germany) phytobiotic additive is a combination of spices, plant extracts and their ether oils that have a combined effect on livestock and poultry appetite. It has been determined that usage of this additive as a combined feed for goslings raised for meat facilitates the increase of livability, poultry live weight, increased meat qualities in the context of better digestion of feed components and reduction of costs per unit of products. The most effective additive feeding dosage is determined as follows: 20 g/100 kg of combined feed [68, 69].

Sangrovit® WS (Phytobiotics Fut-terzusatzstoffe GmbH, Germany) phytobiotic stimulates the consumption of feed and facilitates better functioning of digestive system of poultry. The primary active ingredient of the phytobiotic is contained in the *Macleya cordata* (Willd.) extract: 2.0-4.0% in dry leaves and stalks. The live weight of broilers receiving Sangrovit® WS from day 17 until day 21 of breeding at a rate of 100 g/t of water increased by 1.5%, and those receiving this agent from days 17 to 21 and from days 27 to 30 of breeding increased by 2.0% as compared to the control group. The livability of chickens from test groups during 35 days of raising was 1-4% higher as compared to the control group [70].

Liv 52 Vet (The Himalaya Drug Company, India) phytobiotic contains powders of different medicinal herbs (caper, blue dandelion, black nightshade, *Terminalia arjuna*, *Cassia occidentalis*, common yarrow, *Tamarix gallica*) and extracts of a mix of botanical raw materials (*Eclipta alba*, *Phyllanthus niruri*, spreading hogweed, *Tinospora cordifolia*, garden radish, medicinal emblica, plumbago, *Embelia ribes*, *Terminalia chebula*, *Fumaria officinalis*). During surveys conducted when feeding livestock with this phytobiotic agent the immune system activation of parent stock geese was observed: the phagocytic activity increased by 3.34-7.34%, the phagocytic number and index were respectively higher by 22.51-37.13 and 14.23-18.37% compared to the control group [71]. The usage of Liv 52 Vet additive in a dose of 200 and 250 g/t combined feed had immunostimulating effect on the bodies of broiler goslings, facilitated the stimulation of phagocyte functions and increased resistance to disease, which in turn resulted in increased livability of poultry [72].

An option is considered to use plant-derived agents to produce functional eggs. For instance, natural carotenoid pigments for poultry are obtained from medick, carrot, pumpkin, calendula petals. Oro Glo® 20 (Kemin Europa N.V., Belgium), which contains an extract of calendula petals, is used in a dose of 200-1000 g/t of combined feed to amplify the intensity of color of chicken egg yolk and accumulation of carotenoids in them [73]. It is recommended to use agents with carotene-containing (manufactured by Biocol Agro, Russia) Yellow

Biofon (natural pigment obtained from an extract of marigold petals) and Red Biofon (obtained from an extract of red pepper fruit) in the doses, respectively, of 600 and 500 g/t, and to achieve a brighter yellow yolk it is recommended to add doses of 300 and 600 g/t of feed [74] to increase nutrient properties of quail eggs in the context of insufficient amount of carotinoids in the feed.

The usage of phytobiotics wholly corresponds to the ideology of clean agricultural production and the tasks of improving the quality of life of people. The insignificant scope of phytobiotic usage in the Russian animal husbandry is due to the underdeveloped market of domestic products of this group, high price of imported phytobiotic feed additives, and lack of prohibition to use antibiotic growth promoters in Russia.

Therefore, comprehensive study of properties of plants containing phytobiotic components, the use of cutting-edge technologies to obtain and standardize these components, their experimental and production testing enable a wide usage of plant-derived extracts as biologically active last generation additives based on natural raw materials. In the context of intensive animal husbandry technologies, the phytobiotics recover reduced immune and antioxidant status of livestock, and ensure increase of all types of productivity due to consumption, digestibility, nutrient intake of feeds and normalization of intestinal microbial population and homeostasis in general.

REFERENCES

1. Akhmedkhanova R.R., Gamidov N.R. *Problemy razvitiya APK regiona*, 2010, 1(1): 73-77 (in Russ.).
2. Bushov A., Kurmanaeva V. *Ptitsevodstvo*, 2012, 1: 31-33 (in Russ.).
3. Gheisar M.M., Kim I.H. Phytobiotics in poultry and swine nutrition — a review. *Ital. J. Anim. Sci.*, 2018, 17(1): 92-99 (doi: 10.1080/1828051X.2017.1350120).
4. Świątkiewicz S., Arczewska-Włosek A., Józefiak D. Application of microalgae biomass in poultry nutrition. *World's Poult. Sci. J.*, 2015, 71: 663-672 (doi: 10.1017/S0043933915002457).
5. Windisch W., Schedle K., Plitzner C., Kroismayr A. Use of phytogenic products as feed additives for swine and poultry. *J. Anim. Sci.*, 2008, 86(Suppl. 14): 140-148 (doi: 10.2527/jas.2007-0459).
6. Wegener H.C. Antibiotics in animal feed and their role in resistance development. *Curr. Opin. Microbiol.*, 2003, 6(5): 439-445 (doi: 10.1016/j.mib.2003.09.009).
7. Hao H., Cheng G., Iqbal Z., Ai X., Hussain H.I., Huang L., Dai M., Wang Y., Liu Z., Yuan Z. Benefits and risks of antimicrobial use in food-producing animals. *Front. Microbiol.*, 2014, 5: 288 (doi: 10.3389/fmicb.2014.00288).
8. Windisch W., Kroismayr A. The effect of phytobiotics on performance and gut function in monogastrics. *Biomin World Nutrition Forum. 2007.* Rezhim dostupa: https://en.engormix.com/feed-machinery/articles/phytobiotics-on-performance-gut-function-in-monogastrics-t33528.htm. Data obrashcheniya: 07.08.2018.
9. Castillo-Lypez R.I., Gutiérrez-Grijalva E.P., Leyva-López N., López-Martínez L.X., Heredia J.B. Natural alternatives to growth-promoting antibiotics (GPA) in animal production. *J. Anim. Plant Sci.*, 2017, 27(2): 349-359.
10. Juliano C., Mattana A., Usai M. Composition and in vitro antimicrobial activity of the essential oil of *Thymus herba-barona* Loisel growing wild in Sardinia. *J. Essent. Oil Res.*, 2000, 12(4): 516-522 (doi: 10.1080/10412905.2000.9699578).
11. Faleiro M.L., Miguel M.G., Ladeiro F., Venancio F., Tavares R., Brito J.C., Figueiredo A.C., Barroso J.G., Pedro L.G. Antimicrobial activity of essential oils isolated from Portuguese endemic species of *Thymus. Lett. Appl. Microbiol.*, 2002, 36(1): 35-40 (doi: 10.1046/j.1472-765X.2003.01259.x).
12. McGimpsey J.A., Douglas M.H., Van Klink J.L., Beauregard D.A., Perry N.B. Seasonal variation in essential oil yield and composition from naturalized *Thymus vulgaris* L. in New Zealand. *Flavour Frag. J.*, 1994, 9(6): 347-352 (doi: 10.1002/ffj.2730090613).
13. Marino M., Bersani C., Comi G. Antimicrobial activity of the essential oils of *Thymus vulgaris* L. measured using a bioimpedometric method. *J. Food Protect.*, 1999, 62(9): 1017-1023 (doi: 10.4315/0362-028X-62.9.1017).
14. Delaquis P.J., Stanich K., Girard B., Mazza G. Antimicrobial activity of individual and mixed

fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int. J. Food Microbiol.*, 2002, 74(1-2): 101-109 (doi: 10.1016/S0168-1605(01)00734-6).
15. Jeroch H., Kozlowski K., Jeroch J., Lipinski K., Zdunczyk Z., Jankowski J. Efficacy of the phytogenic (*Papaveraceae*) additive Sangrovit® in growing monogastric animals. *Züchtungskunde*, 2009, 81(4): 279-293.
16. Si W., Gong J., Tsao R., Zhou T., Yu H., Poppe C., Johnson R., Du Z. Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. *J. Appl. Microbiol.*, 2006, 100: 296-305 (doi: 10.1111/j.1365-2672.2005.02789.x).
17. Burt S. Essential oils: their antibacterial properties and potential applications in foods — a review. *Int. J. Food Microbiol.*, 2004, 94(3): 223-253 (doi: 10.1016/j.ijfoodmicro.2004.03.022).
18. Dorman H.J.D., Deans S.G. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol.*, 2000, 88(2): 308-316 (doi: 10.1046/j.1365-2672.2000.00969.x).
19. Bakkali F., Averbeck S., Averbeck D., Idaomar M. Biological effects of essential oils — a review. *Food Chem. Toxicol.*, 2008, 46(2): 446-475 (doi: 10.1016/j.fct.2007.09.106).
20. Ait-Ouazzou A., Cherrat L., Espina L., Lorán S., Rota C., Pagán R. The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innov. Food Sci. Emerg.*, 2011, 12(3): 320-329 (doi: 10.1016/j.ifset.2011.04.004).
21. Adaszynska-Skwirzynska M., Szczerbinska D. Use of essential oils in broiler chicken production — a review. *Ann. Anim. Sci.*, 2017, 17(2): 317-335 (doi: 10.1515/aoas-2016-0046).
22. Kiczorowska B., Samolińska W., Al-Yasiry A.R.M., Kiczorowski P., Winiarska-Mieczan A. The natural feed additives as immunostimulants in monogastric animal nutrition — a review. *Ann. Anim. Sci.*, 2017, 17(3): 605-625 (doi: 10.1515/aoas-2016-0076).
23. Friedman M., Henika P.R., Mandrell R.E. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enteric. J. Food Protect.*, 2002, 65(10): 1545-1560 (doi: 10.4315/0362-028X-65.10.1545).
24. Konca Y., Cimen B., Yalcin H., Kaliber M., Beyzi S. Effect of hempseed (*Cannabis sativa* sp.) inclusion to the diet on performance, carcass and antioxidative activity in Japanese quail (*Coturnix coturnix japonica*). *Korean J. Food Sci. An.*, 2014, 34(2): 141-150 (doi: 10.5851/kosfa.2014.34.2.141).
25. Skandamis P., Koutsoumanis K., Fasseas K., Nychas G.-J.E. Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157: H7. *Ital. J. Food Sci.*, 2001, 13(1): 65-75.
26. Carson C.F., Mee B.J., Riley T.V. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage and salt tolerance assays and electron microscopy. *Antimicrob. Agents Ch.*, 2002, 46(6): 1914-1920 (doi: 10.1128/AAC.46.6.1914-1920.2002).
27. Sikkema J., de Bont J.A., Poolman B. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Mol. Biol. R.*, 1995, 59: 201-222.
28. Ultee A., Kets E.P.W., Alberda M., Hoekstra F.A., Smid E.J. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. *Arch. Microbiol.*, 2000, 174(4): 233-238 (doi: 10.1007/s002030000199).
29. Ultee A., Bennink M.H.J., Moezelaar R. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus. Appl. Environ. Microb.*, 2002, 68(4): 1561-1568 (doi: 10.1128/AEM.68.4.1561-1568.2002).
30. Sikkema J., De Bont J.A.M., Poolman B. Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.*, 1994, 269(11): 8022-8028.
31. Cox S.D., Mann C.M., Markham J.L., Bell H.C., Gustafson J.E., Warmington J.R., Wyllie S.G. The mode of antimicrobial action of essential oil of *Melaleuca alternifola* (tea tree oil). *J. Appl. Microbiol.*, 2000, 88(1): 170-175 (doi: 10.1046/j.1365-2672.2000.00943.x).
32. Ultee A., Kets W.E.P., Smid E.J. Mechanism of action of carvacrol on the food-borne pathogen *Bacillus cereus. Appl. Environ. Microb.*, 1999, 65(10): 4606-4610.
33. Davidson P.M. Chemical preservatives and natural antimicrobial compounds. In: *Food microbiology fundamentals and frontiers.* M.P. Doyle, L.R. Beuchat, T.J. Montville (eds.). ASM Press, NY, 1997: 520-556 (doi: 10.1128/9781555815912.ch33).
34. Nikolaev S.I., Melikhov V.V., Frolova M.V. *Vestnik Rossiiskoi sel'skokhozyaistvennoi nauki*, 2009, 2: 68 (in Russ.).
35. Sukhanova S.F., Azaubaeva G.S. *Veterinarnyi vestnik Kurganskoi GSKHA*, 2015, 1: 55-59 (in Russ.).
36. Juliani H.R., Koroch A.R., Simon J.E. Chemical diversity of essential oils of *Ocimum* species and their associated antioxidant and Antimicrobial Activity. In: *Essential oils and aromas: green extractions and applications.* F. Chemat, V.K. Varshney, K. Allaf (eds.). Dehradun, India, 2009.
37. Upadhaya S.D., Kim S.J., Kim I.H. Effects of gel-based phytogenic feed supplement on growth performance, nutrient digestibility, blood characteristics and intestinal morphology in weanling pigs. *J. Appl. Anim. Res.*, 2016, 44(1): 384-389 (doi: 10.1080/09712119.2015.1091334).
38. Mohiti-Asli M., Ghanaatparast-Rashti M. Comparison of the effect of two phytogenic compounds on growth performance and immune response of broilers. *J. Appl. Anim. Res.*, 2017, 45(1): 603-608.
39. Shapiro S., Guggenheim B. Inhibition of oral bacteria by phenolic compounds Part 1: QSAR

analysis using molecular connectivity. *Quant. Struct.-Act. Relat.*, 1998, 17(4): 327-337 (doi: 10.1002/(SICI)1521-3838(199808)17:04<327::AID-QSAR327>3.0.CO;2-O).
40. Delaquis P.J., Stanich K., Girard B., Mazza G. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int. J. Food Microbiol.*, 2002, 74(1-2): 101-109 (doi: 10.1016/S0168-1605(01)00734-6).
41. Molchanov A.A., Zhukova I.A., Antipin S.L. *Nauchnyi vestnik L'vovskogo natsional'nogo universiteta veterinarnoi meditsiny i biotekhnologii im. S.Z. Gzhitskogo*, 2016, 18(1-3): 76-81 (in Russ.).
42. Egorov I.A. *Ptitsevodstvo*, 2014, 6: 22-24 (in Russ.).
43. Khusnutdinov B., Gumarova G. *Ptitsevodstvo*, 2009, 10: 26-27 (in Russ.).
44. Kazachkova N.M. *Materialy Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Innovatsionnye tekhnologii v obrazovanii i nauke»* [Proc. Int. Conf. "Innovative technologies in education and science"]. Cheboksary, 2017: 14-16 (in Russ.).
45. Terent'ev V.I., Anikienko T.I. *Vestnik KrasGAU*, 2011, 5: 163-166 (in Russ.).
46. Tabakov N.A., Kozina E.A., Ki-yu-an N.A., Ryabinina L.A. *Kormlenie sel'skokhozyaistvennykh zhivotnykh i kormoproizvodstvo*, 2008, 6: 50-55 (in Russ.).
47. Yaroshevich M.I., Vecher N.E. *Trudy BGU*, 2010, 4(2): 1-12 (in Russ.).
48. Franciosini M.P., Casagrande-Proietti P., Forte C., Beghelli D., Acuti G., Zanichelli D., dal Bosco A., Castellini C., Trabalza-Marinucci M. Effects of oregano (*Origanum vulgare* L.) and rosemary (*Rosmarinus officinalis* L.) aqueous extracts on broiler performance, immune function and intestinal microbial population. *J. Appl. Anim. Res.*, 2016, 44(1): 474-479 (doi: 10.1080/09712119.2015.1091322).
49. Ignatovich L.S. *Dal'nevostochnyi agrarnyi vestnik*, 2017, 2(42): 75-81 (doi: 10.24411/1999-6837-2017-00012) (in Russ.).
50. Singh J., Sethi A.P.S., Sikka S.S., Chatli M.K., Kumar Pawan. Effect of cinnamon (*Cinnamomum cassia*) powder as a phytobiotic growth promoter in commercial broiler chickens. *Anim. Nutr. Feed Techn.*, 2014, 14(3): 471-479 (doi: 10.5958/0974-181X.2014.01349.3).
51. Ahmed T.S., Yang C.-J. Effects of dietary *Punica granatum* L. by-products on performance, immunity, intestinal and fecal microbiology, and odorous gas emissions from excreta in broilers. *J. Poul. Sci.*, 2017, 54: 157-166 (doi: 10.2141/jpsa.0160116).
52. Al-Yasiry A.R.M., Kiczorowska B., Samolińska W., Kowalczuk-Vasilev E., Kowalczyk-Pecka D. The effect of *Boswellia serrata* resin diet supplementation on production, hematological, biochemical and immunological parameters in broiler chickens. *Animal*, 2017, 11(11): 1890-1898 (doi: 10.1017/S1751731117000817).
53. Udintsev S.N., Zhilyakova T.P., Mel'nikov D.P. *Svinovodstvo*, 2010, 5: 18-21 (in Russ.).
54. Farnieva K.Kh. *Effektivnost' introduktsii i perspektivy ispol'zovaniya ekhinatsei purpurnoi (Echinacea purpurea (L.) Moench) v usloviyakh RSO-Alaniya. Avtoreferat kandidatskoi dissertatsii* [Introduction and use of *Echinacea purpurea* (L.) Moench in Alania. PhD Thesis]. Vladikavkaz, 2015 (in Russ.).
55. Khmyrov A., Fat'yanov A., Gorshkov G. *Zhivotnovodstvo Rossii*, 2012, 9: 16-17 (in Russ.).
56. Kushniruk T.N. *Intensivnost' rosta, sokhrannost', gematologicheskie i immunologicheskie pokazateli u ptits, potreblyavshikh dobavki ekhinatsei k kormu. Avtoreferat kandidatskoi dissertatsii* [Growth, preservation, hematological and immunological indicators in poultry fed with dietary *Echinacea*. PhD Thesis]. Belgorod, 2008 (in Russ.).
57. Saeed M., Abd El-Hack M.E., Alagawany M., Arain M.A., Arif M., Mirza M.A., Naveed M., Chao S., Sarwar M., Sayab M., Dhama K. Chicory (*Cichorium intybus*) Herb: chemical composition, pharmacology, nutritional and healthical applications. *Int. J. Pharmacol.*, 2017, 13(4): 351-360 (doi: 10.3923/ijp.2017.351.360).
58. Lantseva N.N., Martyshchenko A.E., Shvydkov A.N., Ryabukha L.A., Smirnov P.N., Kotlyarova O.V., Chebakov V.P. *Fundamental'nye issledovaniya*, 2015, 2: 1417-1423 (in Russ.).
59. Nekrasov R.V., Chabaev M.G., Ushakova N.A., Pravdin V.G., Kravtsova L.Z. *Izvestiya Orenburgskogo GAU*, 2012, 3: 225-228 (in Russ.).
60. Ryzhov V.A., Ryzhova E.S., Korotkii V.P., Zenkin A.S., Marisov S.S. *Nauchno-metodicheskii elektronnyi zhurnal Kontsept*, 2015, 13: 3236-3240 (in Russ.).
61. Komarova Z.B., Pilipenko D.N., Ivanov S.M. *Izvestiya Nizhnevolzhskogo agrouniversitetskogo kompleksa: nauka i vysshee professional'noe obrazovanie*, 2011, 3: 1-5 (in Russ.).
62. Laptev G.Yu., Bol'shakov V.N., Soldatova V.V. *Sel'skokhozyaistvennye vesti*, 2012, 1: 24 (in Russ.).
63. Duborezov V., Romanov V., Nekrasov R. *Zhivotnovodstvo Rossii*, 2013, Spetsvypusk: 38-40 (in Russ.).
64. Okolelova T., Laptev G., Bol'shakov V. Effektivnost' Provitola v kombikormakh dlya kur. *Ptitsevodstvo*, 2014, 1: 12-14.
65. Radaelli M., Parraga da Silva B., Weidlich L., Hoehne L., Flach A., da Costa L.A.M.A., Ethur E.M. Antimicrobial activities of six essential oils commonly used as condiments in Brazil against *Clostridium perfringens*. *Braz. J. Microbiol.*, 2016, 47(2): 424-430 (doi: 10.1016/j.bjm.2015.10.001).
66. Lopes I., Suika E., Lopes S., N'eto R., Rodriges A., Uspeshnyi A. *Kombikorma*, 2016, 1: 85-87 (in Russ.).

67. Egorov I., Egorova T., Rozanov B., Marechek E. *Ptitsevodstvo*, 2012, 1: 17-20 (in Russ.).
68. Khaziev D.D. *Vestnik BGAU*, 2013, 3: 79-81 (in Russ.).
69. Khaziev D.D. *Izvestiya Orenburgskogo GAU*, 2013, 5: 150-153 (in Russ.).
70. Zhirnova O.V., Gamko L.N., Shepelev S.I. *Zootekhniya*, 2016, 5: 26-27 (in Russ.).
71. Sukhanova S.F. *Vestnik APK Stavropol'ya*, 2017, 2: 109-119 (in Russ.).
72. Sukhanova S.F., Azaubaeva G.S. *Vestnik Altaiskogo GAU*, 2014, 12: 95-99 (in Russ.).
73. Tolstopyatov M.V., Salomatin V.V., Kalinina E.A. *Izvestiya Nizhnevolzhskogo agrouniversitetskogo kompleksa: nauka i vysshee professional'noe obrazovanie*, 2013, 4: 1-4 (in Russ.).
74. Afanas'ev G.D., Popova L.A., Komarchev A.S., Trepak Zh.G. *Ptitsa i ptitseprodukty*, 2014, 5: 62-64 (in Russ.).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 698-711
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 639.371.2.03

doi: 10.15389/agrobiology.2018.4.698eng
doi: 10.15389/agrobiology.2018.4.698rus

STURGEON (*Acipenseridae*) ARTIFICIAL REPRODUCTION PARADIGM CHANGEOVER UNDER CONDITIONS OF NATURAL STOCK DEFICIT OF STURGEON IN THE VOLGA-CASPIAN BASIN

(review)

N.V. SUDAKOVA[1], E.V. MIKODINA[2], L.M. VASILYEVA[1]

[1]*Astrakhan State University,* 20a, ul. Tatishcheva, Astrakhan, 414056 Russia, e-mail sudakorm@mail.ru, bios94@mail.ru; [2]*Russian Federal Research Institute of Fisheries & Oceanography,* Federal Agency for Fishery, 17, ul. Verkhnyaya Krasnoselskaya, Moscow, 107140 Russia, e-mail mikodina@vniro.ru (✉ corresponding author)
ORCID:
Sudakova N.V. orcid.org/0000-0002-7916-3720
Mikodina E.V. orcid.org/0000-0003-4874-1468
Vasilyeva L.M. orcid.org/0000-0002-4927-2088
The authors declare no conflict of interests
Received February 5, 2018

Abstract

As well known, natural populations of sturgeon (*Acipenseridae*), except sterlet (*Acipenser ruthenus*), reached critical values by the beginning of the 21st century (P. Bronzi et al., 1999; E.V. Makarov et al., 2000; L. Speer et al., 2000; G.I. Ruban et al., 2015). For the endangered species of sturgeon, artificial reproduction continues to be the main source of replenishment of their populations (M.S. Chebanov et al., 2007). This method of sturgeon breeding is the most developed in the Lower Volga of the Caspian basin (V.V. Mil'shtein, 1982; A.A. Kokoza, 2004; L.M. Vasilieva, 2011) in Russia. To increase the effectiveness of sturgeon aquaculture, existing technologies are being updated and new ones are being developed (M. Chebanov et al., 2011; S. Wuertz et al., 2018). One such is the formation of broodstock (production) herds at sturgeon hatcheries (V.V. Tyapugin, 2015; L.M. Vasilieva, 2015; A.A. Popova et al., 2002). Abroad, broodstocks of sturgeon fishes have been created in industrial farms mainly aimed at commercial activities. For the analytical study, we used our own data, literature sources and reports on sturgeon artificial reproduction of the North Caspian Basin Directorate for Fisheries and the Conservation of Aquatic Biological Resources (Sevkasprybvod). It should be noted that most of the Russian relevant papers on the issue have been published in regional periodicals with a restricted accessibility. In this review, we intend to fill the existing gap. Among the six sturgeon species habiting in the Volga-Caspian basin, beluga *Huso huso*, Russian sturgeon *Acipenser gueldenstaedtii*, the sturgeon *A. stellatus*, and the sterlet *A. ruthenus* (G.I. Ruban et al., 2011) are considered. The data on the use of sturgeon producers of different origin in artificial reproduction at six hatcheries of the Astrakhan region are analyzed for the period from 2012 to 2015. In the species composition of wild producers caught for fish breeding, Russian sturgeon predominates (85-90 %) with critically small proportions of beluga and stellate, the species which are almost on the verge of complete extinction in the natural habitat (A.A. Kokoza et al., 2014; G.I. Ruban et al., 2017). Among sturgeon producers, the number of individuals from natural populations decreased while those of broodstock increased. Production broodstocks are formed by two alternative methods, the domestication of wild adult fish and the cultivation of producers from birth (from fertilized eggs) to sexually mature stages. The domestication makes it possible to accelerate the formation of broodstocks, since it does not necessitate growing sturgeons during four periods of the life cycle (embryonic, larval, juvenile, and immature stage). The formation of brooocks by domestication method, which began in Russia in 1999, led to a decrease in the average age of reaching puberty in different species of sturgeon under captivity up to 2-9 years. The proportion of juveniles derived from sturgeon producers which were grown in artificial conditions in the Astrakhan region is dynamically increasing. Russian experts have developed scientific foundation and technologies for the formation of sturgeon broodstocks which are currently involved in artificial reproduction at federal sturgeon hatcheries in the Lower Volga. Thus, the change of the paradigm of sturgeon fish artificial reproduction is stated which in modern conditions should be based on the offspring of producers from broodstocks.

Keywords: Sturgeon, the Volga-Caspian basin, artificial reproduction, Sturgeon hatcheries, spring and winter races, sturgeon natural stocks shortage, brood stock, domestication, paradigm changeover

It is common knowledge that at the turn of the 20th century in most countries the *Acipenseridae* species had become extinct or almost extinct [15, 16]. Furthermore, in the water basins of Europe and North America the decline of their populations had occurred one and a half centuries before it happened in Russia [12]. Until now, in our country (as compared with the other countries) the total population of *Acipenseridae* has been the largest, but also close to critical [17, 18]. It has to be mentioned that the habitat of *Acipenseridae* in the Volga-Caspian basin of Russia changed so drastically overall that their natural reproduction became possible only for the species that do not have long migrations, specifically, for sterlet [16].

It was only during the last 30 years that the technologies of artificial reproduction developed in Russia (former USSR) have begun to be used extensively in the world in order to obtain young *Acipenseridae* fish as the source of restoring the natural populations and replenishing the needs of the market [19-21]. In many countries such reproduction is aimed at commercial breeding [22, 23]. The scientifically substantiated attempts to restore completely lost natural populations have been made [24-26]. The restoration of most species of European *Acipenseridae* in their natural habitat is possible due to vacant ecological niches and is performed in the context of aquaculture with the help of created broodstocks, for which in Europe they use own producers that are occasionally caught in their natural habitat or imported impregnated roe and young fish. This tendency is actively developed in Italy, Germany, France, Poland, the Czech Republic, Spain, Finland, Baltic States and Iran, Kazakhstan and Uzbekistan [27, 28]. Unlike those countries, in Russia *Acipenseridae* still exist in their natural habitat. There is also a network of federal sturgeon hatcheries established in the middle of the 20th century, where until the start of the 21st century the artificial reproduction of sturgeons was based on the usage of wild producers [16, 27]. However, catching wild producers gradually decreased, and currently domestic fish hatcheries are not supplied with the quantities of sturgeon males and females required for the reproduction of young fish.

The global problem of extinction of *Acipenseridae* in their natural habitat resulted in the signing of a Ramsar Declaration on Global Sturgeon Conservation [29]. One of the actions recommended in the declaration was broodstock development at fish hatcheries [28, 29]. Due to this, at the turn of the 21st century a new technological stage appeared in the biological engineering of artificial sturgeon reproduction. Its purpose is to establish broodstocks and use domesticated or hand-raised "from roe to roe" males and females [28].

It has to be mentioned that there does not exist such a system of artificial reproduction of sturgeons abroad similar to the Russian one; however, most articles on this subject were published in the regional periodical publications, which determines poor accessibility of the available data array in spite of their academic and practical interest. This survey aims to fill this gap by summarizing a large volume of information about the system of artificial reproduction of sturgeons in Russia.

It was our aim to analyze the activity of sturgeon hatcheries in the Lower Volga basin operating broodstocks in domestic aquaculture during the recent years, the results of which reflect the shift of paradigm of artificial sturgeon reproduction in Russia.

One of the factors that affected the size and condition of Caspian *Acipenseridae* was river control of the Volga and Kama rivers with a cascade of hydro power plants resulting in sharp decline of natural reproduction, because the migration routes of producers to spawning areas was significantly reduced. For instance, 11 hydro power plants were built during 1942-1987 on the rivers of

the Volga and Kama basin, of which eight were built on the Volga River: Ivankovskaya hydro power plant in 1942; Rybinskaya hydro power plant in 1950; Uglichskaya hydro power plant in 1955; Nizhegorodskaya (Gorkovskaya) hydro power plant in 1956; Zhigulevskaya (Kuybyshevskaya) hydro power plant in 1957; Volzhskaya (Stalingradskaya, Volgogradskaya) in 1961 hydro power plant, fish ladder; Saratovskaya hydro power plant in 1970, fish ladder; Cheboksarskaya hydro power plant in 1986; three hydro power plants on the Kama River: Kamskaya hydro power plant in 1956; Votkinskaya hydro power plant in 1966; Nizhnekamskaya hydro power plant in 1987 [30]. The dam of Volzhskaya hydro power plant (the lower stage of the Volga-Kama cascade) located 600 kilometers away from the Volga Delta in 1961 quite predictably and almost completely closed the spawning route for anadromous fish of the Caspian Sea, including sturgeons (with the exception of spawning areas of the Lower Volga) [31].

A new solution was added to the design of the Volzhskaya hydro power plant to minimize the damage of the migration route of sturgeons: a fish-passing facility — a hydraulic fish ladder [31] of a pit-lifting type which was commissioned in 1961. During the first years, when 200 to 700 thousand males and females migrating from the Caspian Sea for spawning assembled on the ebb side of the dam, about 20 thousand of them were moved through the fish ladder on the average, with the maximum number of 60 thousand in 1967 [32, 33]. However, this was just 10-15% of the number of migrating fish, which cannot be deemed efficient [32, 34, 35]. A mechanical fish ladder was built at Saratovskaya hydro power plant, which was reached only by 0.46-2.0% of all fish that overcame the Votkinskaya hydro power plant. Due to the fact that subsequently sturgeons stopped approaching the Votkinskaya hydro power plant, its fish ladder was put on hold in 1999, and later the same happened at Saratovskaya hydro power plant [31]. Consequently, this method of compensation to the fishing industry failed to accomplish its task of preserving natural reproduction of sturgeons in natural breeding areas of the mid-Volga and Upper Volga regions and the Kama River region. The reproduction of long-lived beluga and Russian sturgeon in the upper spawning areas took the biggest hit, and, to a lesser extent this affected the short cycle starred sturgeon and sterlet that do not make long migrations and all spawning areas of which are located below the dam of the Volga hydroelectric complex [33].

As far back as during the stage of designing the construction of Volga-Kama cascade of the hydro power plant the experts came to the conclusion that without a wide-scale implementation of artificial reproduction of sturgeons it would be impossible to preserve their natural populations [35, 36]. Consequently, fish breeding became the second method of compensating damage to natural populations of *Acipenseridae* in the Volga-Caspian basin [37, 38]. In the mid-20th century (1955-1981), 8 fish hatcheries were built in the Lower Volga beginning with the Volgograd hydroelectric complex for *Acipenseridae* rearing: one was built in the Volgograd region (Volzhskiy fish hatchery, which was later renamed to Volgogradskiy fish hatchery, town of Volzhskiy; commissioned in 1960), seven fish hatcheries in the Astrakhan region (Kizanskiy fish hatchery, Privolzhskiy region, village of Kizan, 1955; Lebyazhiy fish hatchery, Narimanovskiy district, town of Narimanov, 1979; Alexandrovskiy fish hatchery, Iskryaninskiy district, village Trudofront, 1974; Beryulskiy fish hatchery, village Algaza, 1961; Zhitninskiy fish hatchery, village of Zhitnoye, 1981; Iskryaninskiy fish hatchery, village Iskryanoye, 1962; Sergievskiy fish hatchery, village Sergievk, 1963). The Iskryaninskiy plant was converted several times and currently acts as a scientific and experimental facility of the Caspian Research and Development Fishery Institute (CaspNIRKH), the BIOS Center with a status of a sturgeon

rearing farm. The Alexandrovskiy fish hatchery became a branch of Severokaspiskiy basin administration for fishery and preservation of aquatic biological resources. Presently, 6 federal sturgeon fisheries have remained and continue operation in the Lower Volga and in the Astrakhan region [39], which are part of the Severokaspiskiy basin administration for fishery and preservation of aquatic biological resources, the first of which was commissioned before Volga became closed with a dam of the Votkinskaya hydro power plant (in 1958-1960). In the Lower Volga, Kizanskiy fish hatchery had been built first before Votkinskaya hydro power plant was commissioned but after commissioning of the Upper Volga hydro power plants (Iskryaninskaya, Rybinskaya, Uglichskaya, Nizhegorodskaya, Zhigulevskaya and Kamskaya hydro power plants). The latter blocked access to the spawning areas on the Kama River for beluga; however, in the mid-Volga Region they were still accessible until the Votkinskaya hydro power plant and Saratovskaya hydro power plant were commissioned.

The cumulative production capacity of fishing hatcheries exceeded 70 million standard young fish (3-5 g weigh) of three sturgeon species (beluga, Russian sturgeon and starred sturgeon), which were reared to viable stages and released to the water basins of the Volga-Caspian basin to replenish the natural resources [35]. This allowed restoring the natural reserves of sturgeons, and at the end of 1980-s their commercial yield amounted to 24-25 thousand tons, and production of caviar amounted to 2-2.5 thousand tons [40, 41]. Since 1956, more than 3 billion units of fishery-reared young sturgeons have been released in the Caspian Sea. At the turn of the 21st century, the percentage of reared fish in Caspian catch was 98% for beluga, 65 % for Russian sturgeon and 45% for starred sturgeon. Its has been determined that every million of standard young fish yielded 1030 tons of Russian sturgeon, up to 110 tons of starred sturgeon and 130 tons of beluga [41].

Because of the present adverse hydrological and environmental conditions and ongoing unceasing illegal fishing (illegal, unregistered and unregulated fishing; IUU-fishing) of migrating producers of sturgeons in the main water courses of the Lower Volga, the efficiency of their natural reproduction has declined [15, 16]. It turned out that natural reproduction is incapable of providing not only the legal population, but also of maintaining the biological diversity and generic heterogeneity of populations. In this context, the only option of preserving the sturgeons and their population is their rearing [38].

During the last years, the scope of sturgeon rearing in Russia has been declining, the existing fishing hatcheries in the Astrakhan region are operational only at 15-20%, and problems have been encountered with producers for industrial needs. The deficit of quality producers of natural generations increases every year. When in 1997-2004 the used fishing quotas for rearing in general for four sturgeon species amounted to 87%, in 2007-2010 this amounted to 32% [39]. In 2012-2015, this figure further declined and amounted to 46%, 21%, 7.2%, and to 25% of allocated quotas (Fig. 1).

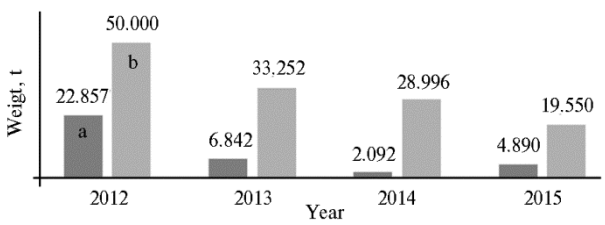


Fig. 1. Cumulative quota usage for catching of four sturgeon species for rearing purposes in the Volga-Caspian basin: a — catching, b — quota.

The comparative analysis of species composition of sturgeon producers caught in the lower Volga River for rearing indicates that the Russian sturgeon prevails in the catches (85-90%) (Table 1). Furthermore, only 0.877 tons of starred sturgeons were reared in 2012-2015 for the total allo-

cated quota of 8.4 tones (about 10% quota usage). Particularly critical is the situation with rearing beluga producers: no single beluga was caught in four years [39].

1. Fishing quota usage (tons) **for four sturgeon species for the purpose of rearing in the Volga-Caspian basin**

Species	Year							
	2012		2013		2014		2015	
	quota	catch	quota	catch	quota	catch	quota	catch
Beluga	3.800	0.000	1.272	0.000	2.083	0.000	1.910	0.000
Russian sturgeon	38.500	21.895	31.480	6.660	26.413	1.993	17.340	4.660
Starred sturgeon	7.400	0.665	0.400	0.082	0.400	0.000	0.200	0.130
Sterlet	0.300	0.297	0.100	0.100	0.100	0.099	0.100	0.100
Total	50.000	22.857	33.252	6.842	28.996	2.092	19.550	4.890

The declining numbers of sturgeon producers entering the Volga River for spawning resulted in prolongation of the catching period for rearing purposes. Whereas previously the fish rearing process involved primarily the producers of later run, in 2012-2015 it was dominated by spawning sturgeon migrants of the summer and autumn spawning run, i.e. the early runs, whose percentage turned out to be 12 higher and amounted to 678 individuals as compared with 55 individuals of later runs [41]. The early producers of sturgeons to be spawning in the next year require protracted autumn-winter reservation in fisheries, which predicates the need for technical modernization of fisheries, and, specifically, the construction of wintering fish basins for wintering of migrant fish. In this respect fish-rearing and physiological parameters of producers of two sturgeon species need to be studied (Operational and financial performance report of Severokaspiskiy basin administration for fishery and preservation of aquatic biological resources, Astrakhan, 2015).

The morphometric (weight, length), fish-rearing and biological parameters (amount of ovulating roe, oocyte diameter, percentage of conceptions) of early run sturgeon females entering the river for the winter and spawning in the next spring turned out to be higher than those of later runs that spawn in the same year they enter the river without wintering. Which means that early run females of beluga are better prepared for spawning than later run females [42; Operational and financial performance report of Severokaspiskiy basin administration for fishery and preservation of aquatic biological resources, Astrakhan, 2015]. At the same time, the average hemoglobin content of later run females was 69 g/l, and 48.2 g/l for early run females. Identical changes were observed concerning whey protein content in blood. The hematological parameters of early run females are indicative of expressed anaemia after the winter period spent in wintering fish basins due to reduced intensity of metabolic and oxidizing processes in the body during the winter period [43, 44]. In spite of the fact that fish-rearing and biological parameters of early run sturgeon females are better than those of later runs, the young fish produced by the latter is more viable, which was confirmed by its survival and growth rate. The concentration of hemoglobin and whey protein content in blood of the offspring of later runs were higher than those of the offspring of early run fish, which allows us to establish a connection between hematological parameters of females and young sturgeons [Operational and financial performance report of Severokaspiskiy basin administration for fishery and preservation of aquatic biological resources, Astrakhan, 2015].

Presently, the dominating factor limiting the scale of sturgeon rearing is the increasing deficit of producers of natural generations [45]. At this stage of biological engineering the only option of preserving the genetic material of natural populations of sturgeons is the guaranteed delivery to fishing hatcheries of producers from broodstocks created and maintained in fish farm conditions (46).

This implies a change of tactics of rearing for such species. The usage of wild sturgeon producers for rearing was abandoned of necessity and transition to the usage of males and females from broodstocks has occurred [38, 47].

In Russia, broodstocks of sturgeons began to be established relatively recently, and 20 years ago the necessity to create them was purely speculative. For most sturgeon species this process was considered extremely complicated and economically unjustified [35]. The deteriorating situation with the catching of sturgeon producers of natural generation dictated the need for rapid transition to the establishing of broodstocks in fish farm conditions to guarantee rearing processes. There are two key methods of establishing sturgeon broodstocks: rearing to reproductive age at fish farms according to the "roe to roe" principle and domestication of wild producers [48-50]. The first method of stock formation is a long lasting process requiring more than 10 years for producers to mature, because sturgeon species belong to the long-lived species that mature late. Due to this, the sturgeon hatcheries prefer domestication of mature wild individuals. For domestication purposes they use sturgeon females that first produce roe, and subsequently are transitioned to a rearing regime of maintenance and feeding. It is a known fact that during their lifetimes sturgeon females can spawn repeatedly [51], and in farm conditions they can yield fish-rearing roe 10-12 times [52-54]. This method allows reducing the time required for maturing of beluga, Russian sturgeon and starred sturgeon by two or three times after their successful adaptation for unusual maintenance conditions, and ensures a wide genetic diversity of offspring.

The sturgeon rearing farms of the Astrakhan region commenced creating production broodstocks in 1999. By now they have created and operate commercial broodstocks of beluga, Russian sturgeon, starred sturgeon and sterlet, and their size and biomass increase annually. In 2011, the total size of the stock in sturgeon broodstocks at federal fishing hatcheries amounted to 3746 individuals with total biomass equal to 62478 kilos, and in 2015 these figures increased to 4,428 individuals (by 18.2%) and 94100 kilos (more than by 5 %), respectively. In terms of species composition, Russian sturgeon (more than 85%) dominates fish hatcheries in domesticated production broodstocks, which exceed 80% of the total amount of females. The percentage of other species is an order less, for beluga 8 %, for starred sturgeon 4 %, for sterlet 3 %. In rearing female stocks the parameters created using the "roe to roe" method constitute 72%, including 58% for females; the fish of rearing groups that have not achieved reproductive age makes 28% [53, 55, 56].

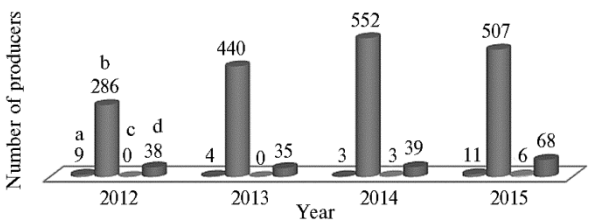


Fig. 2. The quantitative dynamics of domesticated producers of sturgeons used for breeding in fish farm conditions at six federal fish hatcheries located in the lower course of the Volga River: a — beluga, b — Russian sturgeon, c — starred sturgeon, d — sterlet.

In 2012-2015, the total number of sturgeon producers involved in fish rearing processes amounted to 2243 individuals from production broodstocks (where 2001 or 89.3% are domesticated females) and 242 individuals (or 10.7%) of the rearing female stocks completely raised and matured in fish farm conditions. During 4 years the number of domesticated sturgeon producers that matured in fish farm conditions during (Fig. 2) increased by 1.8 times, the largest share falls to Russian sturgeon (89.0%), the share for sterlet is 9.0%, for beluga 1.3%, for starred sturgeon 0.4% [35, 55].

All fish in production stocks at federal fish hatcheries of Severokaspiskiy

basin administration for fishery and preservation of aquatic biological resources in Astrakhan region have individual markers (chips), which enables creating rearing and genetic passports for each fish. The study of passport data of domesticated producers allowed determining that 1025 females matured in wintering fish basins, including 914 Russian sturgeons, 89 sterlets, 18 belugas, and 4 starred sturgeons. The interval between spawning varied from 2 to 9 years, for sterlet it was 1-2 years, for Russian sturgeon it was 3-4 years, for starred sturgeon it was 4-5 years and for beluga it was 5-6 years [50]. The age of the initial maturing of females in wintering fish basin was the largest (6-9 years) for three species, which took a long time to adapt to the farm conditions of growing and feeding [52]. Furthermore, the second maturing of these species happened faster (after 3-4 years), which is indicative of high genetic flexibility and successful adaptation of female fish to farm conditions [44, 51]. Eurybiontic sterlet turned out the most easily adapting species, while beluga turned out to be the hardest to adapt. These facts can be the crucial for the fact that created production stocks will be reliable for fish rearing [45, 47].

Since 2012, the individuals of rearing generation have begun to mature in farm stocks which were used to obtain reproductive products. Between 2012 and 2015, the total population of these sturgeon females involved in fish rearing amounted to 242 individuals and, with the exception of beluga which matures late, increased from 2012 to 2014 by 18 times (Table 2). The largest number of matured fish was sterlet females which in natural habitat mature at the ages of 4-5 [47]. The population of Russian sturgeon females involved in the fish rearing process displays a stable growth every year (see Table 2). The age of the first maturing of females of Russian sturgeon, starred sturgeon and sterlet primarily corresponded to the age of maturing of natural generation fish, and beluga reached reproductive age in wintering fish basins 2-3 years sooner than in its natural habitat. The obtained findings are indicative of the fact that duly established and raised rearing sturgeon broodstocks provide the rearing process with producers of farm-reared generation.

2. The species composition and number of sturgeon females that matured in the production stock during different years and involved in farm rearing at six federal fishing hatcheries located in the lower course of the Volga River

Species	Number of females			
	2012	2013	2014	2015
Beluga	–	–	–	2 (16)
Russian sturgeon	6 (13)	11 (14)	19 (15)	28 (16)
Starred sturgeon	–	–	4 (12)	–
Sterlet	–	82 (3)	85 (4)	5 (5)
Total	6	93	108	35

N o t e. The age of fish species in years is shown in brackets. Strikethrough means that females have not matured.

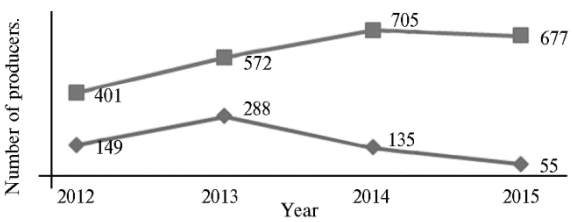


Fig. 3. Using sturgeon producers of different origin in farm rearing at six federal fishing hatcheries located in the lower course of the Volga River: ▲ — catching in a river, ■ — production stocks.

The comparative analysis of using sturgeon production of different origin indicates (Fig. 3) that during recent years, in the context of declining number of fish caught in the Volga-Caspian basin for rearing needs, the rearing process at sturgeon fisheries is maintained due to producers from the created production stocks. In 2012, the natural to fish-reared generation fish ratio was 1:2.5, in 2013 this ratio was 1:2, in 2014 1:5,

and in 2015 the number of producers from the created broodstocks was 12 times higher than those caught in the natural habitat. It is fair to say that in the nearest years fish rearing will be provided with producers from fishery stocks [55, 56].

Therefore, the unique relict sturgeon species inhabiting out planet for millions of years have adapted and survived many natural and man-made disasters. Their primary natural population, which is currently concentrated in the Volga-Caspian basin, is critically endangered and at the brink of extinction [1]. The analysis of these facts leads to the analogy with the theory of G.A. Zavarzin [57], according to which prokaryotes evolve with the habitat. Presumably, this theory can apply to eukaryotes, and specifically to *Acipenseridae*. The habitat conditions of Volga sturgeons changed drastically and fast due to anthropogenic influence. The early runs of sturgeons disappeared, the conditions for spawning in breeding areas above the Volgograd hydroelectric complex became unsuitable, and population of later runs became critically endangered. In order to preserve and restore these natural resources in a context of near-complete lack of natural reproduction [54], the scope of farm reproduction of sturgeon species is maintained using different methods [47], including improvement of biological engineering technologies and increase of efficiency of farm rearing [55].

While natural reserves of sturgeon species in the southern seas of Russia started to plummet [1] their farm rearing emerged and increased. This resulted in the closing of sturgeon farm hatcheries (36). The farm rearing of sturgeon species in the rivers of the Azov and Black Sea basins in Russia continues only on account of creation of rearing female stocks. Presently, they are available in the Askaysko-Donskiy fishery, Rogozhinskiy fish hatchery and Science Center of Aquaculture Vzmorye of Azov Fishery Research and Development Institute, which are a part of the Azovo-Donskoy Center of Aquaculture (the Don River), and Grivenckiy fish hatchery, Temryukskiom fish hatchery and Achuevskiy fish hatchery – the business units of the Azovo-Donskoy Center of Aquaculture (the Kuban River) [56]. In Astrakhan region six federal fish hatcheries of the Volga-Caspian basin eluded the threat of liquidation, as well as Volgograd sturgeon fish hatchery located inside the Volzhskaya hydro power plant (Volgograd region). Due to the fact that these fisheries [58] promptly commenced creating brood (production) stocks of sturgeon species and created them, it guarantees that natural reserves of sturgeon species in the Caspian Sea will not only preserve but will also be restored using contemporary aquaculture methods.

Consequently, it can be said that at this time a significant obstacle has been removed to implementing the principles of sturgeon breeding in the lower Volga established as the concept for preservation of sturgeon species as far back as in the middles of the 20th century prior to regulation of the Volga-Kama basin rivers by the cascade of hydro power plants. This concept was based on the usage of mature sturgeon producers in fish rearing caught in the Volga River during their spawning migration. However, during that time there was still no deficit of producers of natural generations, whereas now the availability of wild producers for fisheries is not sufficient for production capacities and does not exceed 5-10% [34, 35].

The experience of last years is indicative of the fact that processing methods of sturgeon rearing in Russia have been updated [58-60] and the model of sturgeon rearing has changed because fish rearing process at fisheries is supplied by progressively larger quantities of producers contained in production (rearing female) stocks [61]. For instance, sturgeon rearing female stocks at fisheries of the Astrakhan region were formed in two ways, by domestication of adult fish from natural populations and by breeding producers from birth to spawning in farm conditions. The population and biomass of such stocks grows

annually, as does involvement in breeding in the conditions of aquaculture [47]. The availability of such stocks guarantees supplies to the Russian fish hatcheries of fully-fledged males and females, which will help preserve the population of these relict fish species, at least in their natural habitat regions in the Volga-Caspian basin. The fish rearing of sturgeon species in the Volga-Caspian basin remains a unique domain of domestic aquaculture that has no analogues in the world in terms of the history of development, duration and scope [60, 61]. The new elements of intensifying sturgeon breeding [61-65] are such that when obtaining sturgeon offspring in farm conditions the tactic of fish rearing with wild producers, whose population is plummeting, is gradually replaced with the usage of females and males from brood (production) stocks [66-68] as this is performed with regard to most other species of fish aquaculture [69].

To summarize, Russia has developed scientific foundations and technologies of creating production stocks of sturgeon species involved in fish rearing at federal sturgeon fisheries of the Lower Volga. The fish rearing of sturgeon species in the Volga-Caspian basin among other things is aimed at maintaining the population of these species in natural water bodies, including that part of their natural Russian habitat, where their spawning sites still remain, and specifically, in the Lower Volga. The main paradigm of sturgeon rearing has changed 50 years after the federal sturgeon fisheries appeared in our country. The broodstocks are created at each of the six fisheries located in the Astrakhan region. Their formation is conducted via one of the following two methods, the domestication of the few wild producers and rearing of females and males at fisheries from roe obtained during fish rearing. The dynamics of this process indicates that in the nearest future the breeding will be completely supplied by producers from broodstocks.

REFERENCES

1. Makarov E.V., Zhiteneva L.D., Abrosimova N.A. *Zhivye iskopaemye blizki k vymiraniyu* [Living fossils are close to extinction]. Rostov-na-Donu, 2000 (in Russ.).
2. Ruban G.I., Akimova N.V., Goriounova V.B., Mikodina E.V., Nikolskaya M.P., Novosadova A.V., Rosenthal H.K., Sokolova S.A., Shagaeva V.G., Shatunovsky M.I. *Atlas of abnormalities in gametogenesis and early life stages of sturgeons.* World Sturgeon Conservation Society. Special Publ. Book of Demand, Nordenstedt, Germany, 2015.
3. Pourkazemi M. Caspian Sea sturgeon conservation and fisheries: past, present and future. *J. Appl. Ichthol.*, 2006, 22(s1): 12-16 (doi: 10.1111/j.1439-0426.2007.00923.x).
4. Kottelat M., Freyhof J. *Handbook of European Freshwater Fishes.* Publications Kottelat, Cornol and Freyhof, Berlin, 2007.
5. *FishBase. 2018. World wide web electronic publication.* R. Froese, D. Pauly (eds.). Available http://www.fishbase.org, version (02/2018). Accessed May 4, 2018.
6. Ruban G.I., Khodorevskaya R.P., Koshelev V.N. *Astrakhanskii vestnik ekologicheskogo obrazovaniya*, 2015, 1(31): 42-50 (in Russ.).
7. Ruban G.I., Kholodova M.V., Kalmykov V.A., Sorokin P.A. Morphological and molecular genetic study of the Persian sturgeon *Acipenser persicus* Borodin (*Acipenseridae*) taxonomic status. *Journal of Ichthyology*, 2008, 48(10): 891-903 (doi: 10.1134/S0032945208100068).
8. Ruban G.I., Kholodova M.V., Kalmykov V.A., Sorokin P.A. A review of the taxonomic status of the Persian sturgeon (*Acipenser persicus* Borodin). *J. Appl. Ichthyol.*, 2011, 27(2): 470-476 (doi: 10.1111/j.1439-0426.2011.01668.x).
9. Sergeev A. Evolutionary relations and population differentiation of *Acipenser gueldenstaedtii* Brandt, *Acipenser persicus* Borodin, and *Acipenser barii* Brandt [version 2; referees: 2 approved]. *F1000 Research*, 2016, 5: 2807 (doi: 10.12688/f1000research.10237.2).
10. Rastorguev S.M., Nedoluzhko A.V., Mazur A.M., Gruzdeva N.M., Volkov A.A., Barmintseva A.E., Mugue N.S., Prokhortchouk E.B. High-throughput SNP-genotyping analysis of the relationships among Ponto-Caspian sturgeon species. *Ecology and Evolution*, 2013, 3(8): 2612-2618 (doi: 10.1002/ece3.659).
11. Mikodina E.V. *Materialy Mezhdunarodnoi nauchnoi konferentsii, posvyashchennoi 100-letiyu GosNIORKH «Rybokhozyaistvennyi vodoemy Rossii. Fundamental'nye i prikladnye issledovaniya»* [Proc. Int. Conf. "Fisheries reservoirs of Russia. — fundamental and applied re-

search", dedicated to 100th Anniversary of GosNIORKH]. St. Petersburg, 2014: 71-80. Available http://mail.niorh.ru/download.pub/Conference/Fishery_ponds_2014.pdf. Accessed August 6, 2018 (in Russ.).
12. Zonn I.S. *Chernyi zhemchug Kaspiya (pochti vse o chernoi ikre)* [Black pearls of the Caspian Sea: almost all about black caviar]. Moscow, 2005 (in Russ.).
13. Speer L., Lauck L., Pikitch E., Boa S., Dropkin L., Spruill V. Roe to ruin: the decline of sturgeon in the caspian sea and the road to recovery. Wildlife Conservation Society, Natural Resource Defense Council, SeaWeb, 200. Available http://www.sturgeonaquafarms.com/News/roe_to_ruin.pdf. Accessed August 6, 2018.
14. Ruban G.I., Khodorevskaya R.P. Caspian Sea sturgeon fishery: a historic overview. *J. Appl. Ichthyol.*, 2011, 27(2): 199-208 (doi: 10.1111/j.1439-0426.2011.01725.x).
15. Ruban G.I., Khodorevskaya R.P., Shatunovskii M.I. *Voprosy rybolovstva*, 2015, 3: 269-277 (in Russ.).
16. Ruban G.I., Khodorevskaya R.P., Koshelev V.N. *Astrakhanskii vestnik ekologicheskogo obrazovaniya*, 2015, 1: 42-50 (in Russ.).
17. Arlati G., Bronzi P. Sturgeon farming in Italy. *Abstr. Int. Symp. on Sturgeon.* Moscow, 1993: 66-67.
18. Bronzi P., Rosenthal H., Arlati G., Williot P.A Brief overview on the status and prospects of sturgeon farming in Western and Central Europe. *J. Appl. Ichthyol.*, 1999, 15(4-5): 224-227 (doi: 10.1111/j.1439-0426.1999.tb00239.x).
19. Williot P., Sabeau L., Gessner J., Arlati G., Bronzi P., Gulyas T., Berni P. Sturgeon farming in Western Europe: recent developments and perspectives. *Aquat. Living Resour.*, 2001, 14(6): 367-374 (doi: 10.1016/S0990-7440(01)01136-6).
20. Kolman R. Kapusta A., Szczepkovski M., Duda A., Bogacka-Kapusta E. *Jesiotr bałtycki Acipenser oxyrhynchus oxyrhynchus Mitchill.* Olsztyn, Poland, 2008.
21. Domezain A. Main steps and proposals for a recovery plan of sturgeon in the Guadalquivir River (Spain). In: *Biology, conservation and sustainable development of sturgeons. Fish & Fisheries Series, V. 29.* R. Carmona, A. Domezain, M. García-Gallego, J.A. Hernando, F. Rodríguez, M. Ruiz-Rejyn (eds.). Springer, Dordrecht, 2009: 423-452 (doi: 10.1007/978-1-4020-8437-9_26).
22. Bronzi P., Rosenthal H., Gessner J. Global sturgeon aquaculture production: an overview. *J. Appl. Ichthyol.*, 2011, 27(3): 169-175 (doi: 10.1111/j.1439-0426.2011.01757.x).
23. *Aktualny stan i ochrona naturalnych populacji ryb jesiotrowatych Acipenseridae.* R. Kolman (ed.). Instytut Rybactwa Śrydłądowego, Olsztyn, 2014.
24. Kolman R. Kapusta A., Szczepkovski M., Bogacka-Kapusta E. *Jesiotr ostronosy — bałtycki (Acipenser oxyrhynchus oxyrhynchus Mitchill). Program restitucji bałtyckiej populacji jesiotra ostronosego w Polsce.* Olsztyn, Poland, 2014.
25. Friedrich T. *Sturgeons in Austrian rivers: historic distribution, current status and potential for their restoration.* World Sturgeon Conservation Society. Special Publ. Book of Demand, Nordenstedt, Germany, 2013.
26. Khodorevskaya R.P., Ruban G.I., Pavlov D.S. *Povedenie, migratsii, raspredelenie i zapasy osetrovykh ryb Volgo-Kaspiiskogo basseina* [Sturgeon behavior, migration, distribution and stocks in the Volga-Caspian basin]. Moscow, 2007 (in Russ.).
27. Dettlaff T.A., Ginzburg A.S., Schmalgausen O.I. *Sturgeon fishes. Developmental biology and aquaculture.* Springer-Verlag Berlin Heidelberg, 1993 (doi: 10.1007/978-3-642-77057-9).
28. Chipinov V.G. *Vestnik Astrakhanskogo gosudarstvennogo tekhnicheskogo universiteta. Seriya: Rybnoe khozyaistvo,* 2010, 1: 114-119 (in Russ.).
29. Ramsar Declaration on Global Sturgeon Conservation. *J. Appl. Ichthyol.*, 2006, 22: 5-12 (doi: 10.1111/j.1439-0426.2007.00922.x).
30. Nusenbaum L.M. *Trudy soveshchaniya ikhtiologicheskoi komissii*, 1961, vyp. 10: 235-238 (in Russ.).
31. Malevanchik B.S., Nikonorov I.V. *Rybopropusknye i rybozashchitnye sooruzheniya* [Fish-passing and fish-protecting constructions]. Moscow, 1984 (in Russ.).
32. Pavlov D.S., Skorobogatov M.A. *Migratsii ryb v zaregulirovannykh rekakh* [Migrations of fish in regulated rivers]. Moscow, 2014 (in Russ.).
33. Kalmykov V.A., Ruban G.I., Pavlov D.S. Migrations and resources of starlet *Acipenser ruthenus* (Acipenseridae) from the lower reaches of the Volga River. *Journal of Ichthyology*, 2010, 50(1): 44-51 (doi: 10.1134/S0032945210010066).
34. Mil'shtein V.V. *Osetrovodstvo* [Sturgeon farming]. Moscow, 1982 (in Russ.).
35. Kokoza A.A. *Iskusstvennoe vosproizvodstvo osetrovykh ryb* [Artificial reproduction of sturgeons]. Astrakhan', 2004 (in Russ.).
36. Vasileva L.M., Mikodina E.V. History of development and formation of the artificial reproduction of sturgeons in Russia. *Abstr. Conf. on Global Aquaculture: Securing our Future. Aqua-2012.* Prague, Czech Republic, 2012: 1141.
37. Vasil'eva L.M. *Ryba i moreprodukty*, 2009, 3: 21-25 (in Russ.).
38. Vasil'eva L.M., Naumov V.V., Sudakova N.V. *Estestvennye nauki*, 2015, 4: 90-95 (in Russ.).
39. Vasil'eva T.V., Naumov V.V. *Materialy dokladov konferentsii po voprosam razvitiya akvakul'tury v Rossiiskoi Federatsii.* Moscow, 2014 (in Russ.).

40. Vasil'eva L.M., Smirnova L.M., Yusupova A.Z. *Yug Rossii: ekologiya, razvitie*, 2012, 1: 40-44 (in Russ.).
41. Tyapugin V.V. *Materialy III Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Akvakul'tura osetrovykh ryb: dostizheniya i perspektivy razvitiya»* [Proc. III Int. Conf. "Sturgeon aquaculture: achievements and prospects"]. Astrakhan', 2004 (in Russ.).
42. Vasil'eva L.M. *Biologicheskie i tekhnologicheskie osobennosti tovarnoi akvakul'tury osetrovykh v usloviyakh Nizhnego Povolzh'ya.* [Biological features and technological of commercial aquaculture of sturgeon in the Lower Volga region]. Astrakhan', 2000 (in Russ.).
43. Podushka S.B. *Nauchno-tekhnicheskii byulleten' laboratorii ikhtiologii INENKO*, 2007, 12: 5-15 (in Russ.).
44. Vasil'eva L.M. *Materialy Mezhdunarodnogo nauchno-prakticheskogo seminara po industrial'noi akvakul'ture «Innovatsionnye tekhnologii rybovodstva v retsirkulyatsionnykh sistemakh»* [Proc. Int. Workshop on industrial aquaculture "Innovative technologies of fish farming in recycling systems"]. Belarus', Minsk, 2015: 7 (in Russ.).
45. Tyapugin V.V. *Osobennosti formirovaniya produktsionnykh stad beluga v sadkakh v usloviyakh Nizhnei Volgi. Avtoreferat kandidatskoi dissertatsii* [Formation of commercial herds in cages for beluga in the conditions of the Lower Volga. PhD Thesis]. Astrakhan', 2016. Available http://docplayer.ru/55564552-Tyapugin-vasiliy-vladimirovich-osobennsti-formirovaniya-produkcionnyh-stad-belugi-v-sadkah-v-usloviyah-nizhney-volgi.html. Accessed August 6, 2018 (in Russ.).
46. Popova A.A., Shevchenko V.N., Piskunova L.V., Chernova L.V. *Materialy nauchnoi konferentsii «Problema vosproizvodstva, kormleniya i bor'by s boleznyami ryb pri vyrashchivanii v iskusstvennykh usloviyakh»* [Proc. Conf. "Reproduction, feeding and combating diseases in fish grown under artificial conditions]. Petrozavodsk, 2002 (in Russ.).
47. Shipulin S.V. *Vestnik AGTU*, 2005, 3(26): 36-42 (in Russ.).
48. Shevchenko V.N., Popova A.A., Piskunova L V. *Materialy III Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Akvakul'tura osetrovykh ryb: dostizheniya i perspektivy»* [Proc. III Int. COnf. "Sturgeon aquaculture: advances and expectations]. Astrakhan', 2004: 139-142 (in Russ.).
49. Popova A.A., Shevchenko V.N., Piskunova L.V. *Materialy mezhdunarodnoi konferentsii «Sovremennoe sostoyanie i puti sovershenstvovaniya nauchnykh issledovanii v Kaspiiskom basseine»* [Proc. Int. Conf. "Scientific research of the Caspian basin: state of art and ways to improvement"]. Astrakhan', 2006: 214-222 (in Russ.).
50. Tyapugin V.V. *Materialy IV Mezhdunarodnoi nauchno-prakticheskoi konferentsii «Akvakul'tura osetrovykh ryb: dostizheniya i perspektivy razvitiya»* [Proc. Conf. "Sturgeon aquaculture: achievements and prospects]. Moscow, 2006: 130-131 (in Russ.).
51. *Promyslovye ryby Rossii. Tom 1* /Pod redaktsiei O.F. Gritsenko, A.N. Kotlyara, B.N. Kotenevayu [Commercial fisj of Russia. V. 1. O.F. Gritsenko, A.N. Kotlyar, B.N. Kotenev (eds.)]. Moscow, 2006 (in Russ.).
52. L'vov L.F. *Tezisy nauchnykh dokladov Vsesoyuznogo soveshchaniya «Formirovanie zapasov osetrovykh v usloviyakh kompleksnogo ispol'zovaniya vodnykh resursov»* [Workshop on sturgeon stock formation under complex use of water resources (Abstracts)]. Astrakhan', 1986: 201-203 (in Russ.).
53. Novikov A.V. *Materialy dokladov 2-i mezhdunarodnoi nauchnoi konferentsii «Vosproizvodstvo estestvennykh populyatsii tsennykh vidov ryb»* [Proc. 2nd Int. Conf. "Reproduction of natural populations of valuable fish species"]. St. Petersburg, 2013: 294-297 (in Russ.).
54. Kokoza A.A., Grigor'ev V.A., Zagrebina O.N. *Iskusstvennoe vosproizvodstvo kaspiiskikh osetrovykh s elementami ego intensifikatsii* [Artificial reproduction of Caspian sturgeon and its intensification]. Astrakhan', 2014 (in Russ.).
55. Burtsev I.A. *Biologicheskie osnovy i vzaimosvyaz' tovarnoi i pastbishchnoi akvakul'tury osetrovykh ryb* [Biological aspects and interrelation of commpdity and pasturable aquaculture of sturgeon]. Moscow, 2015 (in Russ.).
56. Vasil'eva L.M. *Tekhnologii pishchevoi i pererabatyvayushchei promyshlennosti APK — produkty zdorovogo pitaniya*, 2015, 2: 30-36 (in Russ.).
57. Zavarzin G.A. V knige: *Evolyutsiya i biotsenoticheskie sistemy* [In: Evolution and biocenotic systems]. Moscow, 1987: 144-158 (in Russ.).
58. Ruban G.I., Shatunovskii M.I. *Voprosy rybolovstva*, 2017, 18(1): 7-21 (in Russ.).
59. Sergieva Z.M., Burlachenko I.V., Nikolaev A.I., Yakhontova I.V. *Trudy VNIRO*, 2015, 153: 3-25 (in Russ.).
60. Chebanov M., Galich E. *Sturgeon hatchery manual. FAO, Fisheries and Aquaculture Technical Paper.* Ankara, Turkey, 2011.
61. Chebanov M.S., Savelyeva E.A. New strategies for brood stock management of sturgeon in the Sea of Azov basin in response to changes in patterns of spawning migration. *J. Appl. Ichthyol.,* 1999, 15(4-5): 183-190 (doi: 10.1111/j.1439-0426.1999.tb00230.x).
62. Chebanov M.S., Billard R. The culture of sturgeons in Russia: production of juveniles for stocking and meat for human consumption. *Aquat. Living Resour.*, 2001, 14(6): 375-381 (doi: 10.1016/S0990-7440(01)01122-6).

63. Williot P., Chebanov M.S. Controlled reproduction of farmed Siberian sturgeon *Acipenser baerii* Brandt. In: *The Siberian sturgeon (Acipenser baerii Brandt, 1869). V. 2. Farming*. P. Williot, G. Nonnotte, M.S. Chebanov (eds.). Springer International Publishing, 2018: 13-47.
64. *The Siberian sturgeon (Acipenser baerii Brandt, 1869). V. 1. Biology* /P. Williot, G. Nonnotte, D. Vizziano-Cantonnet, M.S. Chebanov (eds.). Springer International Publishing, 2018 (doi: 10.1007/978-3-319-61664-3).
65. Wuertz S., Guralp H., Psenicka M., Chebanov M. Sex determination in sturgeon. In: *Sex control in aquaculture. Chapter 37*. H. Wang, F. Piferrer, S.Chen (eds.). Wiley-Blackwell, 2018.
66. *Biology and conservation of the European sturgeon Acipenser sturio L., 1758. The reunion of the European and Atlantic sturgeons*. P. Willot, E. Rochard, N. Desse-Berset, F. Kirschbaum, J. Gessner (eds.). Springer-Verlag Berlin Heidelberg, 2011: 668 (doi: 10.1007/978-3-642-20611-*5*).
67. Willot P., Rouault T., Pelard M., Mercier D., Jacobs L. Artificial reproduction and larval rearing of captive endangered Atlantic sturgeon Acipenser sturio. *Endanger. Species Res.*, 2009, 6: 251-257 (doi: 10.3354/esr00174).
68. Billard R., Cosson J., Noveiri S.B., Pourkazemi M. Cryopreservation and short-term storage of sturgeon sperm, a review. *Aquaculture*, 2004, 236(1-4): 1-9 (doi: 10.1016/j.aquaculture.2003.10.029).
69. Mylonas K., Fostier A., Zanuy S. Broodstock management and hormonal manipulations of fish reproduction. *Gen. Comp. Endocr.*, 2010, 165(3): 516-534 (doi: 10.1016/j.ygcen.2009.03.007).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 712-722
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Genetics of breeding values

UDC 636.4:636.082:575.1

doi: 10.15389/agrobiology.2018.4.712eng
doi: 10.15389/agrobiology.2018.4.712rus

STUDY OF GENETIC AND ENVIRONMENTAL FACTORS, CHARACTERIZING THE FEED EFFICIENCY IN DUROC PIGS

A.A. BELOUS, A.A. SERMYAGIN, O.V. KOSTYUNINA, E.A. TREBUNSKIKH, N.A. ZINOVIEVA

Ernst Federal Science Center for Animal Husbandry, Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail belousa663@gmail.com, alex_sermyagin85@mail.ru, kostolan@yandex.ru, terramio7@mail.ru, n_zinovieva@mail.ru (✉ corresponding author)
ORCID:
Belous A.A. orcid.org/0000-0001-7533-4281
Sermyagin A.A. orcid.org/0000-0002-1799-6014
Kostyunina O.V. orcid.org/0000-0001-8206-3221
Trebunskikh E.A. orcid.org/0000-0002-5208-3376
Zinovieva N.A. orcid.org/0000-0003-4017-6863
The authors declare no conflict of interests
Acknowledgements:
The equipment of the Sharing Center for Farm Animal Bioresources and Bioengineering (FSC for Animal Husbandry) was used.
Supported financially by Ministry of Education and Science of the Russian Federation (the project unique identifier RFMEFI60417X0182)
Received April 28, 2018

Abstract

Feed efficiency is the most important economically relevant factor in swine breeding. The values of daily feed intake (DFI) and feed conversion rate (the ratio of feed intake to the body weight gain for a certain period, FCR). A group of factors that can affect the feed efficiency is the feeding behavior. In this regard, it is relevant to study the genetic and environmental variability of a number of factors that affect the growth, feed efficiency, and ethological features of feed intake in Duroc pigs based on the automatic feeding station data records. The aim of our study was to select at test population the factors which can be associated with feed efficiency, including following traits: body weight (BW, kg), average daily gain (ADG, g), daily feed intake (DFI, g/day), time spent at the feeding station (TPD, min), the number of visits to the feeding station per day (NVD, times), feed intake per visit (FPV, g), feed rate (FR, g/min), and the time at the feeding station per visit (TPV, min). Three different approaches were applied to calculate the feed conversion rate: (1) the ratio of feed intake to the body weight gain for the whole feeding period (FCR_1); (2) the ratio of feed intake to the body weight for 10-day feeding periods (FCR_2); (3) the calculation based on daily data records taking into account the daily fluctuations of BW and DFI (FCR_3).The values of average daily gain (ADG_1, ADG_2, ADG_3) were calculated according to FCR_1, FCR_2, and FCR_3, respectively. The initial dataset of individual records included 99867 observations of each trait for 71 boars. After the evaluation of data for normal distribution and presence of at least 60 % of records, 60 boars were selected for the further analysis. The final dataset included 4138 daily values for every boar. The decomposition of phenotypic variability was performed using the analysis of variance without interaction. The analysis of variance parameters of genetic and environmental types and the evaluation of relationships between variables were based on REML method with a multi-variable model. Boars accessed the feeding station at the age of 74.2±1.0 days (Cv = 10.6 %), the age at the body weight of 100 kg was 149.9±1.0 days (Cv = 5.0 %). Average values of FCR differed depending on the calculation approach and ranged from 2.52 kg/kg to 3.08 kg/kg. The higher variability was observed for FCR_2 and FCR_3 — 23.2 % and 19.2%, respectively. The variability of feeding behavior (TPD, NVD, FPV, FR, and TPV) was 13.7 %, 27.4 %, 21.6 %, 17.7 %, and 21.8 %, respectively. The genetic ratio of parent boar was maximal for the following factors: FCR_2 (11.7 %), FCR_3 (15.4 %), TPD (28.2 %), and NVD (30.8 %). The heritability coefficient of FCR_3 was low (0.019), while the related variables of feeding behavior and body weight revealed more reliable results: h^2 = 0.134-0.368 and h^2 = 0.744. The higher level of genetic correlations were observed between FCR_3 and TPD (0.585), FR (-0.368), FPV (–0.274), and NVD (0.368). ADG_2 and FCR_2 were characterized by negative correlation. Our results can be used in the developing the breeding programs based on genetic and genomic evaluation of pigs for a number of traits.

Keywords: pig, Duroc breed, feed conversion, feeding behavior, body weight, average daily

Feed efficiency is the major aspect of the economic attractiveness of pork production. To assess this feature, indicators of daily feed intake (DFI) and feed conversion rate (FCR) as the ratio of feed consumed to body weight gain over a certain period of time are used. A positive correlation (r from 0.32 to 0.70) was established between DFI and average daily gain (ADG) [1]. Varying DFI may account for up to 59% of the ADG variability [2]. The variability of feed efficiency indicators is due to both external and internal factors. The external factors include the composition and energy nutrition of the feeds, management and climatic conditions. Internal factors are associated with various physiological processes (appetite regulation, intestinal absorption, nutrient availability, thermal regulation, muscle activity, etc.), as well as the status of anabolic and catabolic metabolism [3].

However, from the point of view of population genetic studies, indicators of feed efficiency are of interest in the context of factors of the pig feeding behavior. If you have information on the patterns of inheritance and variability of ethological characteristics, you can regulate the functional needs of animals using technological methods, based on the breed-specific features. The development of computerized feeding systems (feed stations, feedlots) enabled an automatic, high-precision daily individual accounting of not just feed consumption rate and weight gain, but also signs of feeding behavior [4]. This information opens up new horizons in investigating its characteristics in pigs in conjunction with feed efficiency indicators.

It is known that the efficiency of feed use and feeding behavior vary among different breeds of pigs [1, 5, 6]. If feed conversion by breed is relatively stable (there has been a steady decline over the past 15 years, due to extensive selection by this indicator and diet improvements), the values of feed behavior in different studies demonstrate a great inter- and intrabreed variability [7-9]. It has been established that indicators of feed conversion and feeding behavior are characterized by moderate and high degree of heritability [10-12], however, the use of the latter in breeding requires information about their relationships with signs of feed use. Although the conducted studies showed the presence of correlations between the above indicators, in most cases, the identified patterns were of breed and population-specific nature [1, 13, 14]. The studies previously performed on Russian populations of pigs were mainly aimed at identifying the relationship between feed conversion and other economically useful traits not taking into account their variability [15, 16]. Studies conducted on other types of farm animals show the prospects of including feed conversion in the characteristics of the meat and fattening qualities of the livestock [17, 18].

In the present study, for the first time in Russia, results were obtained that characterize the features of the feeding behavior of Durok young hogs in relation to the indicators of feed use at automatic feed stations.

Our goal was to investigate the impact of genetic and paratypical factors on the efficiency of feed use and the feeding behavior of pigs of the Russian reproduction.

Techniques. Studies were conducted from July 2017 to March 2018 (SGC, LLC selection and genetic center, Verkhnyaya Khava, Voronezh region). The accounts were performed using GENSTAR test feeding automatic stations (Cooperl, France). Animals received complete feed stuff PK-56-1 made according to GOST 21055-96 (Full-ration mixed feeding stuffs for bacon feeding of pigs) according to recipes SK-6, SK-7 and SK-52 (Verkhnekhavsky Elevator OJSC, Russia). The content of the main nutrient components in the diet varied

over the follow-up within the limits allowed by the technical requirements of the standard, and was 12.61-13.59 MJ/kg of the metabolic energy, 14.16-16.77% of the mass fraction of crude protein, 3.83-4.49% of fiber, 0.81-1.14% of lysine, 0.51-0.73% of methionine and cystine, and 0.15-0.21% of tryptophan.

The population included 71 young Duroc hogs of the Russian-based reproduction. All animals were assigned with an individual identifier (electronic chip). Growing of animals at the stations was carried out in groups of 10-15 animals (in batches) at the age of 70 days (live weight of 29 to 33 kg). The duration of the control growing varied and ended at the age of 138 to 174 days. During the entire period of the control growing, we estimated the body weight (BW, kg), average daily feed intake (DFI, g/day) and indicators of feeding behavior, including the total time of visits to a feed station per day (TPD, min/day), number of daily feeder visits (NVD, units), average feed intake per visit (FPV, g), feeding rate (FR, g/min; FR = DFI/TPD), average duration of a visit (TPV, min; TPV = TPD/NVD). The abbreviations of terms correspond to generally accepted acronims in feed conversion, growth, and feed behavior [5].

Three approaches were used to evaluate the feed conversion rate (FCR, kg/kg). The first one involved the calculation of the indicator based on the initial and final live weight for the control growin and the amount of feed consumed:

$$\overline{\mathrm{FCR}_1} = \frac{\sum_{i=1}^{1} \mathrm{CR}_{i=n}}{(\mathrm{W}_n - \mathrm{W}_1)}, \quad (1)$$

where $\overline{\mathrm{FCR}_1}$ is the feed conversion for the entire growing period, $\sum_{i=1}^{1} \mathrm{CR}_{i=n}$ is the amount of feed consumed over n observations, starting with $i = 1$; (W_n – W_1) is the gain in live weight during the growing period, W_n is the live weight at the end of the period, W_1 is the live weight at the beginning of the period.

The second approach was based on an estimate of the average values of body weight, its average daily gains and feed conversion obtained during 10-day periods:

$$\overline{\mathrm{FCR}_2} = \sum\left(\frac{\sum_{i=0}^{10} \mathrm{CR}_{i=0\,(i+1\ldots i+10)}}{(\mathrm{W}_{i+10} - \mathrm{W}_{i+1})}\right)/\mathrm{t}\ , \quad (2)$$

where $\overline{\mathrm{FCR}_2}$ is an indicator of feed conversion by 10-day periods per animal; $\sum_{i=0}^{10} \mathrm{CR}_{i=0\,(i+1\ldots i+10)}$ is the total feed intake by an animal over a 10-day period; W_{i+10} is the live weight of an animal on Day 10 of each 10-day period; W_{i+1} is the live weight of an animal on Day 1 of each 10-day period, i is a 10-day period step with t > 1, t is the number of 10-day periods.

In the third case, the parameters of the daily assessment of the pig productivity were used along with adjustments of the feed conversion rate by negative values (correction for an average value of the positive variances), taking into account daily fluctuations in live weight and feed intake:

$$\overline{\mathrm{FCR}_3} = \sum_{i=1}^{i}\left(\frac{\mathrm{CR}_i}{\mathrm{W}_{i+1} - \mathrm{W}_i}\right)/i, \quad (3)$$

where $\overline{\mathrm{FCR}_3}$ is the daily feed conversion rate per animal, CR_i is the total daily feed intake, W_{i+1} is the live weight of the animal at the time of observation, W_i is the live weight of the animal at the previous observation, i is the observation number.

The calculation of average daily gains (ADG_1, ADG_2, ADG_3) was performed for FCR_1, FCR_2, FCR_3. To assess the growth rate of young hogs, the age of attaining a live weight of 100 kg (AGE_{100}, days) was determined.

The primary information on the control growing of young hogs was collected into electronic files, based on which a database was formed with elements of the logical control of input information, such as dates, repetitions, a lack of

one of the estimated parameters. Initially, an array of 99867 entries was monitored for compliance with the law of normal distribution of the analyzed indicators (individual measurements). The detected outliers in the daily data for each animal, exceeding the threshold of a ±10.0% deviation from the previous value, were excluded from further processing. The analysis included only individual animals, the number of test values (based on the sum of the recorded values) in which was not lower than 60.0%. The final sample size consisted of 60 young hogs (descendants of 13 sires and 37 dams), with an average quality indicator for data collecting and recording at 91.2% (limits of 63.3 to 99.5%). The database included 4,138 daily average values for each parameter studied. When assessing the feed conversion rate, an analysis of the indicator variability magnitude was performed involving a sample size of seven 10-day periods from the beginning to the end of growing, which included 413 observations.

To assess the effects of genetic and environmental nature, an equation was chosen that is characterized by the least variance value of unaccounted factors (error variance) using the fixed-effects analysis of variance with no factor interaction. Then, the least square method (LSM) using STATISTICA 10 (StatSoft, Inc., USA) was used to calculate the average values of the estimates:

$$y = \mu + \text{Feeding Station}_i + \text{Batch}_k + \text{Age}_{l(1)} + \text{Sire}_j + e_{iklj}, \quad (4)$$

where y are indicators of feed conversion rate (DFI, TPD, NVD, FPV, FR, TPV, FCR, BW, ADG); μ is an average population constant; Feeding Station_i is an effect of the feeding station ($i = 1 \ldots 3$); Batch_k is an effect of the lot number of the control growing ($k = 1 \ldots 4$); Age_l is an effect of the initial age of the control growing ($l = 1 \ldots 18$) or in a similar model $\text{Age}_{l\ (1)}$ is an effect of the age of the animal for the entire period of the control growing ($l_{(1)} = 1 \ldots 102$), Sire_j is a genetic effect of the a hog breeder (sires of the offspring being estimated) ($j = 1 \ldots 13$), e_{iklj} is a random error (unallocated variance).

The values of genetic and paratypical variantces of individual animals for obtaining selection and genetic parameters were calculated using a similar model through information on hogs' parents to construct an additive relationship matrix according to the restricted maximum likelihood approach (REML, $n = 110$ animals, including 60 with indicators of their own productivity):

$$y = \mu + \text{Test-day}_e + \text{Feeding Station}_i + \text{Batch}_k + \text{Age}_l b_1 + \text{Animal}_j + e_{eikli}, \quad (5)$$

where Test-day_e is a fixed effect of observation during the entire period of the experiment ($e = 1 \ldots 226$); $\text{Age}_l b_1$ is the initial age of control growing, b_1 is a linear regression coefficient, Animal_j is a randomized effect of an animal, having a normal distribution with a mean at 0, and a variance at $A\sigma a^2$, where A is an additive relationship matrix ($j = 1 \ldots 110$), e_{eiklj} is a random error (unallocated variance).

Estimates of values by traits were calculated based on the least square method using STATISTICA 10 (StatSoft, Inc., USA). The reliability of the effect of organized groups of factors, included in the model, was determined based on the MANOVA method. When applying descriptive statistics (indicators of variance), as well as decomposition of phenotypic variability, generally accepted approaches [19] were used, BLUPF90 software family was used for analyzing variances and calculating correlations by a model for a number of interrelated features [20].

Results. The average age of the start of the test fattening-off using feed stations was 74.2±1.0 days ($Cv = 10.6\%$). The young hogs adapted well, which enabled obtaining high average daily gains in body weight (950±19 g) (Fig. 1). The patterns of its increase over 10-day periods were uniform. The age of attaining a live weight of 100 kg averaged 149.9±1.0 days ($Cv = 5.0\%$). Phenotypic

variability remained within the biological limits, reaching a maximum of variance in the second and third ten-days of fattening, at 15.9 and 16.1%, respectively, and a minimum in the first (13.7%) and last (12.0%) periods.

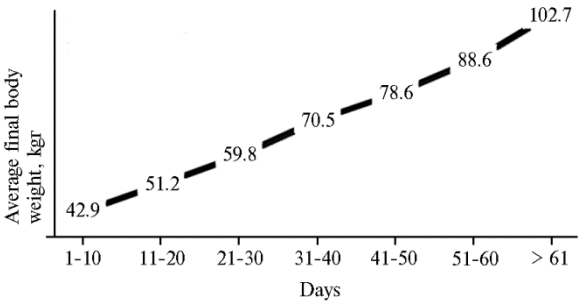


Fig. 1. Patterns of live weight gains in Duroc pigs (*Sus scrofa*) by periods of growing at feed stations (SGC, LLC selection and genetic center, Verkhnyaya Khava, Voronezh region, July 2017 to March 2018).

Among the parameters of feeding behavior, the lowest variability was recorded for the time spent at the feeding station (TPD, 85.3 min/day, C_v = 13.7%) (Table 1). The intermediate position was noted for the feed feeding rate (FR, 27.6 g/min), with *Cv* at 17.7%. For the average visit duration (TPV, 19.6 min) and the amount of feed consumed per visit (FPV, 499.1 g), the phenotypic variation was within 21.6 to 21.8%. The highest variability (*Cv* = 27.4%) was typical for the number of daily feed station visits (NVD, 5.2 units).

We used three different approaches to calculate feed conversion rates (FCR). Fluctuations in the live weight of pigs, caused by the impact of technological, paratypical factors, and by the state of animal health, can be significant. In the first version of the calculation (FCR_1), both high daily average gains and weight losses are disguised. The second method of calculation (FCR_2) is focused on determining the feed conversion rate by ten-day periods, which allows smoothing out fluctuations in the average daily changes in the live weight and more reliably estimate the very parameter. The third approach (FCR_3) suggests involving in the calculations the daily data on feed consumption and weight gains in pigs, taking into account positive values of the variability magnitude for feed conversion.

Parameters of the feed conversion rates, calculated using three above methods, were characterized by the different variability (Table 1). Indeed, in the FCR_2 and FCR_3 methods, the value of *Cv* was 23.2 and 19.2%, respectively, with means of 3.08 and 2.52 kg/kg. For FCR_1, a low degree of the value variation was found (12.3%), with its close mean at 2.55 kg/kg.

1. Parameters of feed use efficiency and feed behavior in a population of Duroc pigs (*Sus scrofa*) (SGC, LLC selection and genetic center, Verkhnyaya Khava, Voronezh region, July 2017 to March 2018)

Parameter	*M*±SEM	σ	*Cv*, %
AGE_{100}, days	149.9±1.0	7.5	5.0
ADG_1, g	950±19	150	15.8
ADG_2, g	892±21	162	18.2
ADG_3, g	986±24	187	19.0
DFI, g/day	2309.1±36.5	282.7	12.2
FCR_1, kd/kg	2.55±0.04	0.31	12.3
FCR_2, kd/kg	3.08±0.09	0.72	23.2
FCR_3, kd/kg	2.52±0.06	0.48	19.2
TPD, min/day	85.3±1.5	11.7	13.7
NVD, u.	5.2±0.2	1.4	27.4
FPV, g	499.1±13.9	107.6	21.6
FR, g/min	27.6±0.6	4.9	17.7
TPV, min	19.6±0.6	4.3	21.8

N o t e. The abbreviations correspond to generally accepted cuttings for indicators [5].

The impact of the genetic factor of the sire of the studied offspring for feed conversion parameters was within 4.5-15.4% of the total variance taken into

account by the model (Table 2). For FCR_2 and FCR_3, the largest proportion of the sire effect was noted, with 11.7% and 15.4%, respectively, moreover, the model equation for FCR_3 had a reliable distribution of all components of variability by impact (F= 2.32; $p < 0.05$; $R^2 = 73.3\%$). At the same time, no significant determination was established for FCR_2 ($F = 1.47$; $R^2 = 63.5\%$). Of interest, for FCR_1, highly significant results were obtained ($F = 4.57$; $p < 0.001$), which explain up to 84.4% of the total phenotypic variability in the model. In other words, the linear dependence of the predicted (expected) results for feed conversion in the first method of the calculation compared to the observed ones is limited by strictly selected components of the dispersion at the minimum variance error.

2. Separation of the components of phenotypic variability (%) for a set of traits of feed conversion in fractions of genetic and environmental factors for a population of Duroc pigs (*Sus scrofa*) (SGC, LLC selection and genetic center, Verkhnyaya Khava, Voronezh region, July 2017 to March 2018)

Parameter	Component of variability						
	sire	feeding station	batch	age	e	R^2	F
Growth indicators							
BW	8.2	0.5	3.4	13.2	18.3	81.7	3.77***
ADG_1	7.6	4.9	0.9	15.4	15.8	84.2	4.49***
ADG_2	9.0	4.9	0.5	13.9	25.5	74.5	2.46**
ADG_3	6.5	3.5	0.6	8.7	23.0	77.0	2.83**
Feed efficiency indicators							
DFI	10.2	4.7	4.5	21.3	16.9	83.1	4.15*
FCR_1	4.5	0.2	2.1	12.7	15.6	84.4	4.57***
FCR_2	11.7	7.1	0.0	29.1	36.5	63.5	1.47
FCR_3	15.4	4.8	1.6	28.9	26.7	73.3	2.32*
Feed behavior indicators я							
TPD	28.2	0.0	2.3	18.2	29.9	70.0	1.98***
NVD	30.8	4.4	0.7	6.2	26.7	73.3	2.32*
FPV	9.4	10.7	2.2	5.9	15.8	84.2	4.51***
FR	9.8	2.6	4.9	12.3	26.9	73.1	2.29*
TPV	9.4	5.6	0.2	13.8	36.8	63.2	1.45

N o t e. The abbreviations correspond to generally accepted cuttings for indicators [5]; e is the residual (unallocated) variance of the model, R^2 is the coefficient of determination, F is the Fisher test.
*, **, *** The contribution of the impact of the variability component on the parameter is statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

3. Population and genetic constants for parameters of feed use efficiency and feed behavior in a population of Duroc pigs (*Sus scrofa*) (SGC, LLC selection and genetic center, Verkhnyaya Khava, Voronezh region, July 2017 to March 2018)

Parameter	BW	DFI	FCR_3	TPD	NVD	FPV	FR	TPV
BW	0.744c	0.565	-0.067	–0.233	0.037	0.000	0.588	–0.516
DFI	0.155	0.079c	0.099	0.200	0.587	0.187	0.292	–0.017
FCR_3	–0.223	0.219	0.019c	0.585	0.368	–0.274	–0.368	0.145
TPD	0.010	0.839	0.083	0.134c	0.584	0.057	–0.138	0.133
NVD	0.163	0.370	0.017	0.242	0.218c	–0.827	0.029	–0.629
FPV	0.012	0.037	0.086	–0.127	–0.393	0.258c	0.145	0.577
FR	0.280	0.534	0.041	–0.696	–0.018	0.367	0.458c	–0.226
TPV	–0.120	–0.402	0.045	0.484	–0.336	0.702	–0.678	0.368c

N o t e. The abbreviations correspond to generally accepted cuttings for indicators [5]; c — diagonally are located the heritability coefficients h^2 (below the diagonal, there are genetic correlations, above the diagonal, there are paratypic correlations).

However, paratypical factors, such as the feeding station and the batch number of the control growing, had a minimal effect (0.0-7.1%) in all FCR variants. The exception was the age of putting for a test fattening-off: the high dependence of this factor seems to be related to the initial live weight of animals in groups at feeding stations. For FCR_3 compared with FCR_2 and FCR_1, a significant impact of the male parent genotype was shown, which was higher in its contribution than the compared indicators by 10.9 percentage points (pp) and 3.7 pp, respectively. It is fair to say that the database being accumulated

will allow attracting seasonal fluctuations into an organized group of factors (the effect of the year, month and day of indicator monitoring).

The additive genetic group determines 6.5-9.0% effects of the average daily gains for ADG (1 ... 3) and the body weight of fattening hogs, with R^2 = 74.5 to 84.2% (F = 2.46-4.49; p <0.01...0.001).

The heritability of the feed conversion rate (FCR_3) is low (h^2 = 0.019), which, in our view, is due to the high proportion of daily (environmental) variation of the trait in the studied sample (Table 3). Moderate heritability coefficients were obtained, with FR 0.458, TPV 0.368, FPV 0.258, NVD 0.218. A low value was noted for TPD, such as 0.134. A high proportion of the genetic component was found for the indicator of body weight (h^2 = 0.744), which was generally typical for the traits of meat productivity in the special Duroc breed.

The correlations between feed behavior, feed conversion and live weight varied in their directions and were moderate in magnitude. In pigs, the live weight was mostly predetermined by the DFI values (r_g = 0.565) and FR (r_g = 0.588). An inverse genetic relationship is found for TPV (r_g = –0.516), that is, the choice of animals with a long stay at the feeding station per visit will not allow for effective selection by live weight. The choice based on FCR_3 will be more effective with the following parameters of animal feeding behavior taken into account: a shorter daily stay at the feeding station (TPD, r_g = 0.585), high feed intake per visit (FPV, r_g = –0.274) and a high feeding rate (FR, r_g = –0.368), a lower number of the feeding station visits per day (NVD, r_g = 0.368). In this regard, the development of a selection index, which takes into account a set of two factors, such as the feeding behavior and the feed conversion rate, is most preferable for the Duroc pig breeding program.

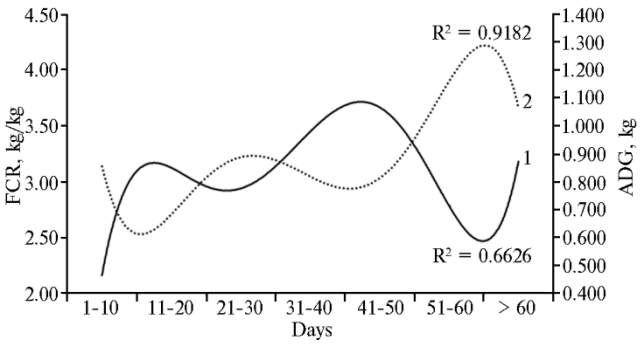


Fig. 2. Dependence of feed conversion rates (FCR_2, 1**) and average daily weight gains** (ADG_2, 2) **in a population of Duroc pigs (*Sus scrofa*) by control ten-days of growing** (SGC, LLC selection and genetic center, Verkhnyaya Khava, Voronezh region, July 2017 to March 2018).

An analysis of variations in the accounted indicators by 10-day cycles (Fig. 2) reveals a step-wise growth pattern: respectively, from 851±51 g for ADG_2 and 2.19±0.13 kg/kg for FCR_2 at the beginning of fattening-off (days 1 to 10) up to 1,072±41 g and 3.31±0.18 kg/kg at the end of the test (> day 61). The trend in the feed conversion rate (the fifth degree polynomial) was in a form of a sinusoidal curve, the change patterns of which was described with an accuracy by period R^2 = 66.3%. A similar distribution was obtained for the ADG_2 parameter trend (the fifth degree polynomial), which was inversely ("mirror-like") related to FCR_2 (r_p = –0.592). Consequently, high gains in live weight were provided with the best results if based on the feed conversion rate.

To smooth out the environmental effects (the feeding station and the batch number of the tested hogs), we obtained the least squares (LSM) estimates. The results (Fig. 3) are described using a third-order polynomial (according to the model equation, the forecast accuracy is R^2 = 73.5%). From the date of putting to the fattening-off and up to 100 days, a smooth increase in the feed conversion was observed due to the high rates of growth and development of animals. Further, during Days 100 to 140, the feed conversion value was stabilized and a so-called plateau

was formed with a minimum variation (2.52-2.54 kg/kg). From the age of 140 days, there was a sharp increase in the trend curve, indicating a decrease in the efficiency of fattening-off of the pigs due to the fat deposition. The growth tempo and relative gains slowed down, indicating the completion of the physiological and biological growth phase in animals.

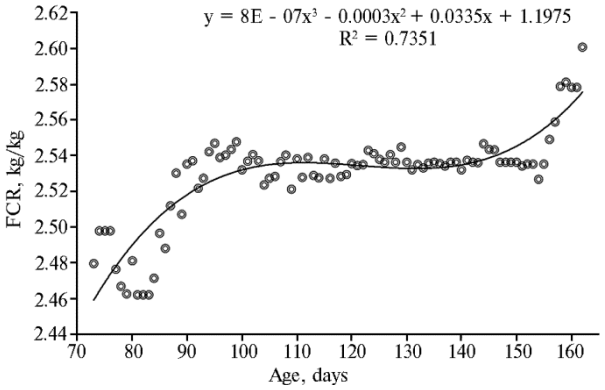


Fig. 3. Patterns of LSM (least square means) values of feed conversion rates (FCR_3) **depending on the age of the test fattening-off of Duroc pigs (*Sus scrofa*)** (SGC, LLC selection and genetic center, Verkhnyaya Khava, Voronezh region, July 2017 to March 2018).

Individual differences in the feed use efficiency and feeding behavior caused by genetic factors allow identification of valuable molecular biomarkers for predicting these traits, as well as their use in pig breeding [8, 9, 21]. In addition, observations related to feeding behavior can be used as a tool in automated monitoring systems to assess the health of animals and better detect diseases, in order to control management [8, 22, 23]. Our findings can be applied in the development of genomic selection programs, which are currently associated with genetic advances in livestock breeding [24, 25].

Therefore, in fattening pigs at automatic feeding stations, the impact of genetic factors determined 15.4% of the total variability by the feed conversion rate indicator, FCR_3, while for the parameters of feeding behavior, the additive variation was 28.2% (the total time spent at the feeding station per day, TPD) and 30.8% (the number of daily feeding station visits, NVD). The analysis of genetic correlations between traits demonstrates the promise of using the parameters of feeding behavior to increase the reliability of the feed conversion rate estimate. The pattern of changes in the feed conversion rate due to the age of animals tends to increase, despite the degree of fluctuations in average daily gains by fattening period. The use of a daily variation in the feed conversion rate (FCR_3) along with the conventional indicator estimate (FCR_1) will allow to more effectively choice of terminal hogs through engaging some selection parameters such as feeding behavior.

REFERENCES

1. Do D.N., Strathe A.B., Jensen J., Mark T., Kadarmideen H.N. Genetic parameters for different measures of feed efficiency and related traits in boars of three pig breeds. *J. Anim. Sci.*, 2013, 91(9): 4069-4079 (doi: 10.2527/jas.2012-6197).
2. Young R.J., Lawrence A.B. Feeding behaviour of pigs in groups monitored by a computerized feeding system. *Anim. Prod.*, 1994, 58(1): 145-152 (doi: 10.1017/S0003356100007182).
3. Maselyne J., Saeys W., Van Nuffel A. Review: Quantifying animal feeding behaviour with a focus on pigs. *Physiol. Behav.*, 2015, 138: 37-51 (doi: 10.1016/j.physbeh.2014.09.012).
4. Hyun Y., Ellis M. Effect of group size and feeder type on growth performance and feeding patterns in finishing pigs. *J. Anim. Sci.*, 2002, 80(3): 568-574 (doi: 10.2527/2002.803568x).
5. Baumung R., Gerhard G., William A., Söelkner J. Feed intake behaviour of different pig breeds during performance testing on station. *Arch. Tierz., Dummerstorf.*, 2006, 49(1): 77-88.
6. Lewis C.R.G., McGlone J.J. Modelling feeding behaviour, rate of feed passage and daily feeding cycles, as possible causes of fatigued pigs. *Animal*, 2008, 2(4): 600-605 (doi: 10.1017/S1751731108001766).
7. Rohrer G.A., Brown-Brandl T., Rempel L.A., Schneider J.F., Holl J. Genetic analysis of behavior traits in swine production. *Livest. Sci.*, 2013, 157(1): 28-37 (doi: 10.1016/j.livsci.2013.07.002).

8. Brown-Brandl T., Rohrer G., Eigenberg R. Analysis of feeding behavior of group housed growing—finishing pigs. *Comput. Electron. Agr.*, 2013, 96: 246-252 (doi: 10.1016/j.compag.2013.06.002).
9. Reyer H., Shirali M., Ponsuksili S., Murani E., Varley P.F., Jensen J., Wimmers K. Exploring the genetics of feed efficiency and feeding behaviour traits in a pig line highly selected for performance characteristics. *Mol. Genet. Genomics*, 2017, 292(5): 1001-1011 (doi: 10.1007/s00438-017-1325-1).
10. Herd R., Arthur P. Physiological basis for residual feed intake. *J. Anim. Sci.*, 2009, 87(Suppl. 14): E64-E71 (doi: 10.2527/jas.2008-1345).
11. Hall A.D., Hill W.G., Bampton P.R., Webb A.J. Genetic and phenotypic parameter estimates for feeding pattern and performance test traits in pigs. *Anim. Sc*i., 1999, 68: 43-48.
12. Chen C., Misztal I., Tsuruta S., Herring W., Holl J., Culbertson M. Influence of heritable social status on daily gain and feeding pattern in pigs. *J. Anim. Breed. Genet.*, 2010, 127(2): 107-112 (doi: 10.1111/j.1439-0388.2009.00828.x).
13. Morgan C.A., Emmans G.C., Tolkamp B.J., Kyriazakis I. Analysis of the feeding behavior of pigs using different models. *Physiol. Behav.*, 2000, 68(3): 395-403 (doi: 10.1016/S0031-9384(99)00195-X).
14. Rauw W.M., Soler J., Tibau J., Reixach J., Gomez Raya L. Feeding time and feeding rate and its relationship with feed intake, feed efficiency, growth rate, and rate of fat deposition in growing Duroc barrows. *J. Anim. Sci.*, 2006, 84(12): 3404-3409 (doi: 10.2527/jas.2006-209).
15. Tagirov Kh. Kh., Asaev E.R. *Izvestiya Orenburgskogo gosudarstvennogo agrarnogo universiteta*, 2007, 1: 118-120 (in Russ.).
16. Larina O.V., Aristov A.V., Kudinova N.A. *Vestnik Ryazanskogo gosudarstvennogo agrotekhnologicheskogo universiteta im. P.A. Kostycheva,* 2017, 2: 26-29 (in Russ.).
17. Gal'pern I.L., Dzholova M.N. *Genetika i razvedenie zhivotnykh*, 2015, 1: 30-34 (in Russ.).
18. Levakhin B.I., Azhmuldinov E.A., Titov M.G., Lasygina Yu.A., Ryabov N.I. *Vestnik Kurskoi gosudarstvennoi sel'skokhozyaistvennoi akademii*, 2015, 7: 145-146 (in Russ.).
19. Kuznetsov V.M. *Osnovy nauchnyh issledovanii v zhivotnovodstve* [Fundamentals of scientific research in animal husbandry]. Kirov, 2006 (in Russ.).
20. Misztal I., Tsuruta S., Strabel T., Auvray B., Druet T., Lee D.H. BLUPF90 and related programs (BGF90). *Proc. 7th World Congress on genetics applied to livestock production*. Montpellier, Communication No. 28-27, 2002, 28: 21-22.
21. Ding R., Yang M., Wang X., Quan J., Zhuang Z., Zhou S., Li S., Xu Z., Zheng E., Cai G., Liu D., Huang W., Yang J., Wu Z. Genetic architecture of feeding behavior and feed efficiency in a Duroc pig population. *Frontiers in Genetics*, 2018, 9: 220 (doi: 10.3389/fgene.2018.00220).
22. Weary D., Huzzey J., Von Keyserlingk M. Board-invited review: using behavior to predict and identify ill health in animals. *J. Anim. Sci.*, 2009, 87(2): 770-777 (doi: 10.2527/jas.2008-1297).
23. Cross A.J., Keel B.N., Brown-Brandl T.M., Cassady J.P., Rohrer G.A. Genome-wide association of changes in swine feeding behaviour due to heat stress. *Genet. Sel. Evol.*, 2018, 50: 11 (doi: 10.1186/s12711-018-0382-1).
24. Sermyagin A.A., Gladyr' E.A., Kharitonov S.N., Ermilov A.N., Strekozov N.I., Brem G., Zinov'eva N.A. Genome-wide association study for milk production and reproduction traits in Russian Holstein cattle population. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2016, 51(2): 182-193 (doi: 10.15389/agrobiology.2016.2.182eng).
25. Zinov'eva N.A., Sermyagin A.A., Kostyunina O.V. *Zhivotnovodstvo Rossii*, 2018, tematicheskii vypusk «Svinovodstvo»: 53-55 (in Russ.).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 723-734
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.4:636.082:575.1

doi: 10.15389/agrobiology.2018.4.723eng
doi: 10.15389/agrobiology.2018.4.723rus

EFFECTS OF GENOTYPES FOR *IGF2*, *CCKAR* AND *MC4R* ON THE PHENOTYPIC ESTIMATIONS AND BREEDING VALUES FOR PRODUCTIVE TRAITS IN PIGS

E.E. MELNIKOVA[1], N.V. BARDUKOV[1], M.S. FORNARA[1], O.V. KOSTYUNINA[1], A.A. SERMYAGIN[1], A.M. ZAITSEV[2], N.A. ZINOVIEVA[1]

[1]*Ernst Federal Science Center for Animal Husbandry,* Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail melnikovaee@vij.ru, bardukv-nikolajj@mail.ru, margaret-fornara@gmail.ru, kostolan@yandex.ru, alex_sermyagin85@mail.ru, n_zinovieva@mail.ru (✉ corresponding author); [2]*All-Russian Research Institute for Horse Breeding,* Federal Agency of Scientific Organizations, pos. Divovo, Rybnovskii Region, Ryazan Province, 391105 Russia, e-mail amzaitceff@mail.ru
ORCID:
Melnikova E.E. orcid.org/0000-0002-7498-1871
Bardukov N.V. orcid.org/0000-0002-5497-2409
Fornara M.S. orcid.org/0000-0002-8844-177X
Kostyunina O.V. orcid.org/0000-0001-8206-3221
Sermyagin A.A. orcid.org/0000-0002-1799-6014
Zaitsev A.M. orcid.org/0000-0003-4260-602X
Zinovieva N.A. orcid.org/0000-0003-4017-6863
The authors declare no conflict of interests
Acknowledgements:
The equipment of the Sharing Center for Farm Animal Bioresources and Bioengineering (FSC for Animal Husbandry) was used.
Supported financially by Ministry of Education and Science of the Russian Federation (the project unique identifier RFMEFI60417X0182)
Received March 21, 2018

Abstract

Development of programs for marker-assisted selection has to be based on genetic polymorphisms, whose effect on the production traits and breeding values of animals is reliable and significant. Prospects for the use of genomic selection in pigs are associated with the development of low-density (LD) DNA arrays, which include the SNPs (single nucleotide polymorphisms) selected by the results of genome-wide association studies (GWAS) with HD-panels. Genes of insulin-like growth factor 2 (*IGF2*), cholecystokinin receptor A (*CCKAR*) and melanocortin 4 receptor (*MC4R*) are of interest for inclusion in LD-panels. Numerous studies have shown a significant effect of these genes on feed conversion rate, growth rate, meat content, and fat deposition. The aim of this work was to evaluate the effect of complex genotypes for *IGF2*, *CCKAR* and *MC4R* on growth and carcass traits of the Landrace and Large White pigs raced in Russia. In total, 1262 animals, including Large White (n = 667) and Landrace pigs (n = 595) were studied. Pig phenotypes were determined for muscle depth (MD, mm), adjusted age at 100 kg (AGE100, day) and back fat thickness (BF, back fat) at three points: BF1 (at ribs 6-7, mm), BF2 (at rib 10, mm), BF3 (at 14 rib, mm). DNA was extracted from tissue samples (ear pluck) using a DNA-Extran-2 Kit (Sintol, Russia). Polymorphism of *IGF2* was determined by real-time PCR. Causative SNPs in *CCKAR* and *MC4R* genes were defined by multiplex PCR with FLASH detection. The allele frequencies of DNA-markers were pA = 27.2 % and pA = 86.3 % for *IGF2*, pA = 0.6 % and pA = 21.1 % for *CCKAR*, pA = 54.1 % and 60.0 % for *MC4R* in Landrace and Large White pigs, respectively. The heritability coefficients (h^2) were 0.204-0.242 for BF1, BF2, and BF3, 0.309 for MD, and 0.366 for AGE100. We developed an equation model for the pig's breeding traits and found the significant effects of fixed factors in the model (breed, sex, year of birth), including specific genotypes for the analyzed genes on the phenotypic variations (for *IGF2* and *MC4R* on BF1, BF2 and BF3, $P > 0.95$), and estimated breeding values (EBV) for growth and carcass traits (for each of the three markers the ratio of additive genetic variation ranged from 0.5 to 7.6 %, $P > 0.95$-0.999). We identified the economically desired alleles for *IGF2* (allele *A*) and *MC4R* (allele *A*) genes. Animals which carried the homozygous genotypes for the desired alleles (*AA* for both of *IGF2* and *MC4R* genes) were characterized by the significantly better scores for analyzed traits, estimated by least squares method, comparing to the individuals which were homozygous for the alternative allele *G*. The additive compensating effect of genotypes' combinations for *IGF2* and *MC4R* on the pig growth traits was established. The animals with the highest number of the *A* alleles for *IGF2* and *MC4R* had preferable characteristics for the back fat thickness comparing to animals with *GG* genotypes (for both DNA markers). The differences between groups of animals carrying in their genotypes from four to single copy of the

A alleles comparing to animals which do not have *A* alleles (*GG* genotypes for both markers) varied from 7.9 to 21.0 % for BF1, from 8.5 to 21.4 % for BF2, from 9.9 to 22.6 % for BF3, and from 2.8 to 3.2 % for MD. In this regard, the *IGF2* and *MC4R* genotypes can be used in breeding programs of Large White and Landrace pigs raced in Russia to select the pigs with desired growth and carcass characteristics.

Keywords: pigs, Large White breed, Landrace, *IGF2*, *CCKAR*, *MC4R* genes, polymorphisms, estimated breeding value (EBV), growth and carcass traits

The genomic selection is thought of as a promising strategy of genetic improvement of livestock, including pigs [1-3]. The integration of genome methods in pig breeding became possible due to the development of commercial SNP-arrays (single nucleotide polymorphisms) for highly productive genotyping with density varying from 10.2 thousand to more than 650 thousand SNPs [4]. If in livestock breeding the genomic selection programs are implemented on the basis of using SNP-arrays of medium density (MD) and high density (HD) [2, 5, 6], in pig breeding the application of MD- and HD-arrays is economically unjustified. The implementation of genomic selection of pigs is associated with the development of low density DNA-matrixes with inclusion of SNPs in them selected by the results of GWAS (genome-wide association studies) analysis using MD- and HD-arrays [7-10]. In order to increase the information content of LD-arrays, they additionally include SNPs localized in "primary" genes associated with QTL. Presently, more than ten genes are known that have a noticeable effect on economic traits of pigs [11]. Their inclusion in LD-arrays will facilitate the improved forecast accuracy of estimated breeding values (EBV). In order to select economically significant SNPs, their impact on economic traits in populations should first be evaluated, which implements the programs of marker and genomic selection. The insulin-like growth factor 2 (*IGF2*) genes, Cholecystokinin A receptor (*CCKAR*) and Melanocortin 4 receptor (*MC4R*) act as potential DNA markers for inclusion in LD-arrays.

The *IGF2* gene is localized to the distal end of SSC2 pig chromosome [12]. G3072A has been identified, which has a significant impact on the rate of growth, muscle gain and fat deposition of these animals [13-15]. Subsequently, the effect of this mutation was confirmed in numerous studies conducted in various pig populations of both foreign (16, 17) and domestic selection [18, 19]. The advantage of using *IGF2* as a DNA marker is attributable to its paternal nature, i.e. the effect of paternal genotype for *IGF2* manifests itself in progeny regardless of maternal genotype [13]. Another advantage of *IGF2* is related to the positive impact of potentially "desirable" allele A on reproductive qualities from the point from view of feeding qualities [18].

CCKAR is one of the principal receptors participating in hunger regulation [20, 21]. The *CCKAR* gene of pigs is mapped on SSC8 chromosome [22]. An economically significant SNP A179G (exon 1) [23] associated with the growing power and feed conversion rate of pigs is known: the animals with at least *G* allele are on average characterized by at least a 3% higher daily body weight gains and at least 5 % higher cost of feed per 1 kg of weight gain [23, 24].

MC4R is involved in regulation of hypothalamic-pituitary-adrenal axis (HPA) via vasopressin and corticotropic neurons [25]. Due to MC4R expression in the brain region regulating appetite its connection with feed consumption and energy balance can safely be presumed. The *MC4R* locus is mapped on SSC1 chromosome in the q22-27 region [26]. A G→A mutation is known, which results in Asp298Asn ammo-acid replacement, which establishes a connection to the increase of fatness, body weight gain and increased feed consumption [26]. The population-genetic studies showed significant differences in *MC4R* (p_A = 0.24-1.00) allele frequencies both among and inside varying pig breeds [27-29]. A

connection has been identified between allocations of *MC4R* alleles with the selection strategies deployed. For instance, lines of Large White pigs selected for high fleshing and low feed conversion rate, the *MC4R A* allele frequency was $p_A = 0.52$ against $p_A = 1.00$ in the line selected for low fleshing and high feed conversion rate [30]. The available data about the effect of *MC4R* polymorphism on productive indicators are not universal. However, a marked impact of this gene on daily live weight gain of purebred pigs [28-30] and crossbred pigs [31-33], feed consumption [27], as well as muscle weight gain and carcass fat content [33-35] has been identified. A significant effect of *MC4R* polymorphism has been identified, which is manifested with regard to feed consumption which, in turn, influenced back fat and growth rate (variations of 5-8%) [27]. The impact of this mutation on the aforementioned characters was noted in almost all analyzed commercial lines. The maximum mean differences for combined genotypes constituted 2 mm for back fat, 70 g/day for average daily live weight gain and 2 % for meat content. K. Salajpal at al. [36] determined the impact of *MC4R* genotype on back fat (*GG* < *AA*) and percentage content of meat in the carcass (*GG* > *AA*, $p < 0.05$) in marketable pigs.

The inclusion of several DNA markers in selection programs requires evaluation of their combined effect on manifestation of economic traits and establishing quantitative relations with the estimated breeding values (EBV).

In this study, for the first time we analyzed the associations of polymorphisms of several genes involved in regulating a number of metabolic and physiological processes with carcass and growth traits of pigs bred in Russia, and identified a reliable connection between certain *IGF2*, *CCKAR* and *MC4R* genotypes with variability of these attributes.

Our goal was to study the combined effect of *IGF2*, *CCKAR* and *MC4R* genotypes on carcass and growth traits of Large White and Landrace pigs.

Techniques. In 2017-2018, 1262 pigs (*Sus scrofa*) were selected to form a test sample which included Large White pigs ($n = 667$) and Landrace pigs ($n = 595$) of Russian selection (Selection and Hybrid Center LLC, Voronezh region). The pig phenotypes were identified using muscle depth (MD), mm, age of 100 kg live weight (AGE_{100}, days) and back fat (BF) in three points: BF1 (in the area of 6-7 rib, mm), BF2 (in the area of 10 rib, mm) and BF3 (in the area of 14 rib, mm).

The DNA was separated from boar tissue samples (ear notch) using a set of DNA-Extran-2 (Syntol R&D company, Russia) reagents. The DNA concentration and quality were evaluated with a Qubit 2.0 desktop fluorimeter (Invitrogen/Life Technologies, USA) and NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, USA). *IGF2*gene polymorphism (G→A in position 16144, Accession No. AY242112) was determined via PCR method in real time using a QuantStudio 5 device (Thermo Fisher Scientific, USA). The single nucleotide polymorphisms in *CCKAR* gene (A→G in position +179, Accession No. DQ496228.1) and *MC4R* gene (G→A in position +1426, Accession No. NM_214173.1) were defined by multiplex PCR method with FLASH-detection (fluorescent amplification-based specific hybridization) at the end point using high performance genotyping system Fluidigm EP1 (Fluidigm Corporation, USA).

The development of a model to evaluate the degree of impact of *IGF2*, *CCKAR*, *MC4R* animal genotypes for variability of test parameters included consideration of a number of equations, which, apart from the studied genetic factors, contained the factors reflecting paratypic effects, possibly affecting the phenotypic manifestations of carcass and growth traits. Using the multivariate analysis of variance (MANOVA) model equations with a varying number of fixed factors were tested. The equation characterized with the lowest error variance value was selected for

further analysis, which was used to calculate mean appraisal values using the least squares method (LSM) via STATISTICA 10 (StatSoft, Inc., USA) software:

$$y = \mu + B_i + Sex_j + Year_k + G_l + e,$$

where y is the calculated indicators of carcass and growth traits (BF1, BF2, BF3, MD, AGE_{100}); μ is mean population constant; B_i is "breed" factor (Landrace, Large White); $Year_k$ is the year of birth of an animal (years 2009-2017); G_l is the effect of the genotype for each of *IGF2*, *CCKAR* and *MC4R* markers; e is a random error (unallocated variance).

The estimated breeding values of livestock units when comparing with marker genotypes were calculated using a similar model using additive kinship matrix information according to the BLUP Animal Model (n = 1752, including 1262 animals with genotypes of corresponding genes) method. The EBV calculations and analysis of variation components were conducted using BLUPF90 (37) program family.

When studying the combined impact of genotype combination for two markers (*IGF2*/*MC4R*), all animals were divided into groups by desirable allele *A* frequency. Group 4*A* (100%) includes pigs with genotype *AA*/*AA* for *IGF2* and *MC4R*; 3*A* pigs (75%) are with *AA*/*AG*, *AG*/*AA*; 2*A* pigs (50%) are with *AA*/*GG*, *AG*/*AG*, *GG*/*AA*; 1*A* pigs (25%) with *AG*/*GG*, *GG*/*AG*; 0A (0 %) means *GG*/*GG*.

Fisher criterion was used to evaluate statistical significance of factors taken into account (*F*-criterion, the ratio between the considered factor variance and residual variance $F = \sigma_f^2/\sigma_e^2$) for the corresponding number of degrees of freedom (df). The accuracy of mean bias of traits for the compared genotype groups was determined using *t*-Student criterion for the corresponding number of degrees of freedom and levels of confidence factors $P > 0.95$, $P > 0.99$, $P > 0.999$.

Results. The studied pig sample had relatively high mean values of carcass and growth traits (Table 1). The moderate nature of heritability for studied traits was identified (h^2 = 0.204-0.366), which is in line with the results of works by other authors [38].

1. Characterization of carcass and growth traits in the analyzed sample of Large White and Landrace pigs (*Sus scrofa*) (Selection and Hybrid Center LLC, Voronezh region, 2017-2018)

Traits	*M*	σ	BF1	BF2	BF3	MD	AGE_{100}
BF1	14.74	3.44	0.242[c]	0.931	0.926	0.400	0.070
BF2	12.10	2.92	0.878	0.236[c]	0.969	0.165	0.008
BF3	11.74	2.75	0.807	0.843	0.204[c]	0.216	–0.136
MD	56.36	6.06	0.327	0.325	0.351	0.309[c]	–0.070
AGE_{100}	159.6	8.26	0.116	0.127	0.099	0.099	0.366[c]

N o t e. BF1 — back fat thickness in the region of rib 6-7, mm; BF2 — back fat thickness in the region of rib 10, mm; BF3 — back fat thickness in the region of rib 14, mm; MD — muscle depth, mm; AGE100 — age of 100 kg body weight, days; *M* — mean value of an indicator, σ — mean square deviation; c — diagonally located heritability coefficients h^2 (under the diagonal line are the phenotypic correlations, above the diagonal line are genetic correlations).

In the course of the analysis we identified high positive interrelations among phenotypic (r_1 from +0.807 to +0.878) and genetic (r_2 from +0.926 to +0.969) indicators of BF1-BF2, BF1-BF3, BF2-BF3 trait pairs, which is indicative of uniformity of fat deposit (fatty tissue) of pigs by analyzed control points when they achieve body weight of 100 kg and is considered a positive trait of raised livestock population. At the same time, the values of back fat thickness indicated an average and low (for genetic properties of units) degree of connection with muscle depth and age of live weight gain of 100 kg, i.e., from the genetic point of view they turned out almost completely independent. That being said it seems feasible to conduct genetic evaluation of livestock population for each of the mentioned traits individually, and to include them in integrated evaluation of car-

cass and fattening traits of pigs.

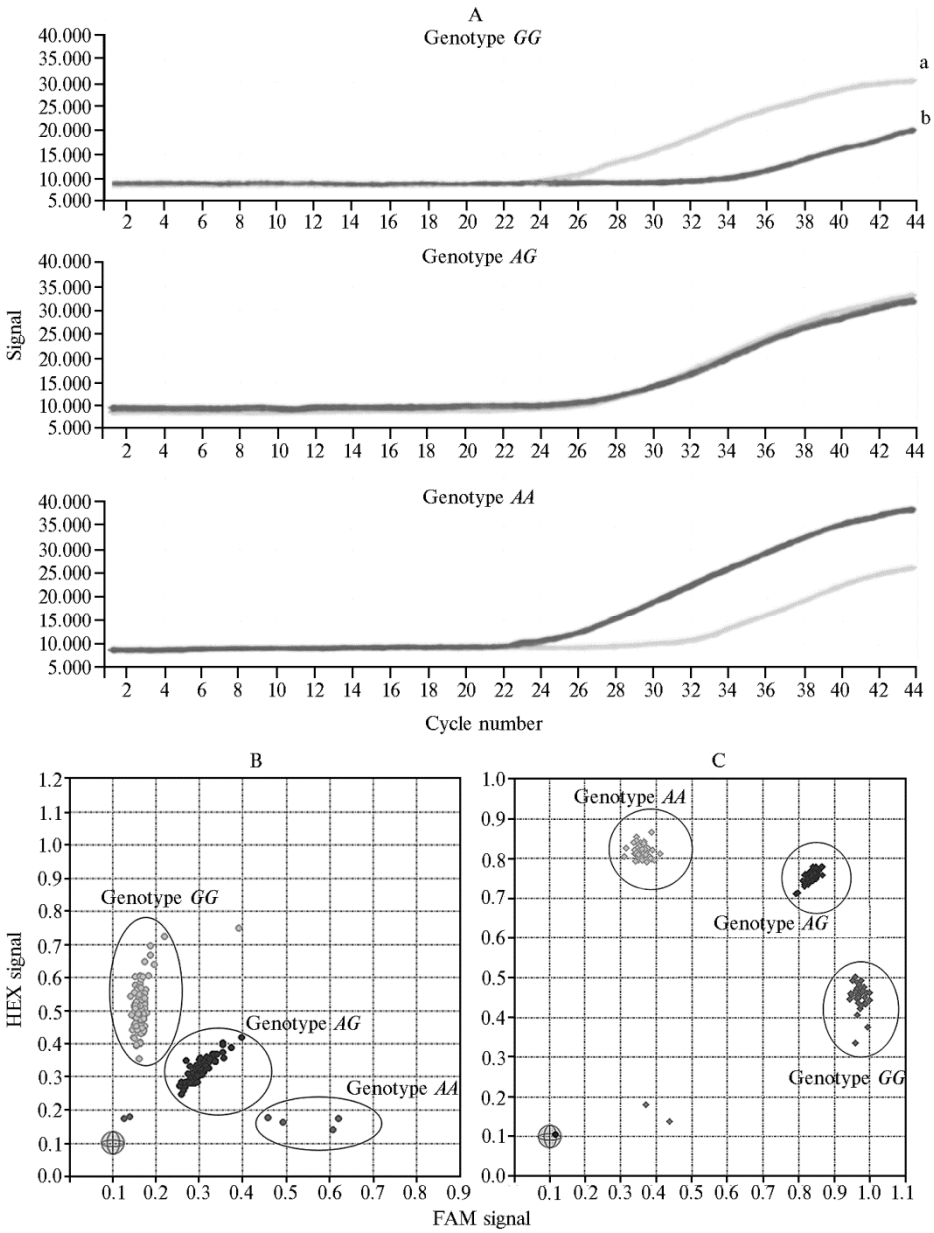


Fig. 1. Genotyping *IGF2*, *CCKAR* and *MC4R* DNA markers of Large White and Landrace pigs (*Sus scrofa*): A — genotyping *IGF2* DNA marker using the allele-specific real time PCR method (QuantStudio 5, Thermo Fisher Scientific, USA), B and C — genotyping *CCKAR* and *MC4R* DNA markers respectively using FLASH-PCR (fluorescent amplification-based specific hybridization) technology with end point detection (Fluidigm EP1, Fluidigm Corporation, USA); a — G allele, b — A allele (Selection and Hybrid Center LLC, Voronezh region, 2017-2018).

The genotyping of *IGF2*, *CCKAR* and *MC4R* using high performance PCR-analysis methods allowed us clearly identify genotypes of DNA markers in question (Fig. 1).

All DNA markers of analyzed pig breeds turned out polymorphous (Fig. 2). The analysis shows lack of essential differences in frequencies of occurrence of *MC4R* genotypes and alleles between Large White and Landrace pig breeds (p_A corresponds to 54.1 and 60.0%). At the same time, uneven distribution of

genotypes and alleles of other DNA markers are identified, i.e. there are p_A 27.2 and 86.3% for *IGF2*, and p_A 0.6 and 21.1% for *CCKAR* for Landrace and Large White pigs respectively.

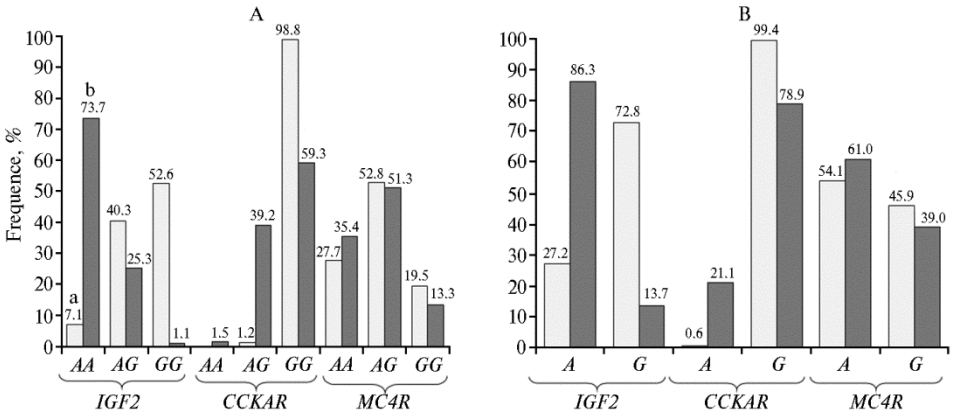


Fig. 2. Distribution of genotype frequencies (A) **and alleles** (B) **of *IGF2*, *CCKAR* and *MC4R* DNA markers in Large White** (a) **and Landrace** (b) **pigs (*Sus scrofa*)** (Selection and hybrid center LLC, Voronezh region, 2017-2018)**.**

2. The reliability values of model factor impact on variance of carcass and growth traits of Large White and Landrace pigs (*Sus scrofa*) based on multivariate analysis of variance without factor interaction (Selection and hybrid center LLC, Voronezh region, 2017-2018)

Factor	df	*F*-criterion				
		BF1	BF2	BF3	MD	AGE_{100}
Breed	2	2.03	3.18*	2.20	0.02	6.01*
Gender	1	12.10*	14.37*	16.87*	0.41	14.50*
Year of birth	8	21.16*	26.85*	17.54*	40.35*	19.35*
IGF2 genotype	2	12.06*	11.67*	13.83*	0.64	2.16
CCKAR genotype	2	0.02	0.24	0.15	0.28	0.06
MC4R genotype	2	21.27*	18.59*	15.66*	2.07	2.81

N o t e. df — number of degrees of freedom; *F*-criterion — statistical Fisher distribution criterion; BF1 — back fat thickness in the region of rib 6-7, mm; BF2 — back fat thickness in the region of rib 10, mm; BF3 — back fat thickness in the region of rib 14, mm; MD — muscle depth, mm; AGE100 — age of 100 kg body weight, days.
* Impact of the factor on variance of the indicator is statistically significant at P > 0.95.

3. The contingency of estimated breeding values (EBV) **for carcass and growth traits of Large White and Landrace pigs (*Sus scrofa*) depending on genotype for *IGF2*, *CCKAR* and *MC4R* genes** (Selection and Hybrid Center LLC, Voronezh region, 2017-2018)

Marker	Comparable genotype pair	EBV				
		BF1	BF2	BF3	MD	AGE_{100}
IGF2	*AA*	–0.086	–0.005	–0.024	–0.519	+0.043
	AG	+0.122	+0.137	+0.122	–0.303	–0.114
	GG	+0.491	+0.450	+0.399	–0.186	–0.042
	F-criterion	33.0^{***}	31.3^{***}	33.6^{***}	3.2^{*}	0.3
	R^2, %	4.99	4.75	5.07	0.51	0.05
CCKAR	*AA*	–0.245	–0.010	–0.038	–2.276	+0.553
	AG	–0.029	+0.059	+0.035	–0.732	+0.155
	GG	+0.172	+0.183	+0.157	–0.247	–0.090
	F- criterion	4.6^{**}	2.5	3.0*	11.4^{***}	0.9
	R^2, %	0.73	0.40	0.48	1.78	0.14
MC4R	*AA*	–0.244	–0.123	–0.132	–0.616	+0.327
	AG	+0.191	+0.207	+0.178	–0.345	–0.120
	GG	+0.599	+0.527	+0.480	+0.073	–0.481
	F- criterion	49.0^{***}	46.4^{***}	51.6^{***}	8.5^{***}	5.6^{**}
	R^2, %	7.24	6.88	7.59	1.33	0.88

N o t e. BF1 — back fat thickness in the region of rib 6-7, mm; BF2 — back fat thickness in the region of rib 10, mm; BF3 — back fat thickness in the region of rib 14, mm; MD — muscle depth, mm; AGE100 — age of 100 kg body weight, days.
*, **, *** Impact of the factor on variance of the indicator is statistically significant at P > 0.95, P> 0.99 and P > 0.999.

In the course of the multivariate analysis of variance (MANOVA) it was observed that for the analyzed group of animals the following factors had significant impact on the variance of productivity: (for BF2 and AGE_{100} traits $P > 0.95$), gender and year of birth (for all traits $P > 0.95$, with the exception of MD), and *IGF2* and *MC4R* market genotypes (for traits BF, $P > 0.95$) (Table 2). A genotype of pigs regarding *CCKAR* marker had no reliable impact on manifestation of productive traits in question.

Of particular interest for the usage of DNA markers in the selection is the study of their impact on estimated breeding values of units (Table 3). Determining the criterion of significance (*F*-criterion) for "genotype for DNA marker" trait enabled identifying reliable impact of each of the genetic traits in question on the change of estimated breeding values for all five traits for *MC4R*, for four out of five traits for *IGF2* (apart from AGE_{100}) and for three traits for *CCKAR* (apart from BF2 and AGE_{100}). The findings confirm the reliable breeding value superiority ($P > 0.95$-0.999) of pigs with *IGF2 AA* genotype over those with *AG* and *GG* genotype in terms of back fat thickness. Furthermore, *AA* pigs stood out by decreased EBV values for MD and AGE_{100}. Identical tendencies are identified for *CCKAR* and *MC4R* markers, for which desirable *AA* genotypes were defined for BF1, BF2 and BF3 genetic values and *GG* genotypes for MD and AGE_{100} traits. The highest value of interconnection linearity between the traits analyzed were observed for trait pairs "genotype for *MC4R* marker—back fat thickness in three points" ($R^2 = 6.88$-7.59 %) (see Table 3).

The identified regularities are confirmed upon comparison of the results received for both pig breeds (Table 4). Furthermore, for Large White pigs statistically significant variances have been determined between *MC4R* homozygous genotype for AGE_{100} trait ($P > 0.95$-0.999), which is indirectly indicative of a relation between this trait and carcass trait (back fat thickness in three measurement points, BF1, BF2 and BF3).

4. The weighted appraisal values for productive traits for *IGF2*, *CCKAR* and *MC4R* genotypes in pigs (*Sus scrofa*) of different breeds obtained by the least squares method (Selection and Hybrid Center LLC, Voronezh region, 2017-2018)

DNA marker	Genotype (*n*)	LSM estimates for productive traits				
		BF1	BF2	BF3	MD	AGE_{100}
		Landrace				
IGF2	*AA* (*n* = 42)	13.69±0.60[a]	10.63±0.48[a]	9.66±0.46[a]	55.18±0.99	160.12±1.40
	AG (*n* = 240)	14.46±0.38	11.67±0.30	10.62±0.29	53.74±0.62	159.84±0.88
	GG (*n* = 313)	15.22±0.39	12.34±0.31	11.20±0.30	53.12±0.65	161.19±0.91
CCKAR	*AA* (*n* = 0)					
	AG (*n* = 7)	14.38±1.32	11.60±1.06	10.29±1.01	54.37±2.17	160.15±3.07
	GG (*n* = 588)	14.68±0.36	11.83±0.28	10.76±0.27	53.63±0.58	160.39±0.83
MC4R	*AA* (*n* = 165)	13.88±0.41[b]	11.11±0.32[b]	10.26±0.31[b]	53.39±0.68	160.44±0.97
	AG (*n* = 314)	14.82±0.37	11.97±0.29	10.80±0.28	53.62±0.62	160.36±0.87
	GG (*n* = 116)	15.84±0.45	12.82±0.36	11.63±0.35	54.19±0.76	160.42±1.07
		Large White				
IGF2	*AA* (*n* = 481)	13.68±0.38[a]	11.25±0.33[a]	10.69±0.32[a]	52.52±0.65	153.71±0.94
	AG (*n* = 165)	14.45±0.44	11.68±0.38	11.22±0.36	52.47±0.75	154.54±1.08
	GG (*n* = 7)	16.64±1.22	13.47±1.07	13.39±1.02	57.20±2.11	158.63±3.03
CCKAR	*AA* (*n* = 10)	13.83±1.05	11.54±0.91	10.87±0.88	51.35±1.80	154.58±2.58
	AG (*n* = 256)	13.85±0.41	11.27±0.36	10.77±0.35	52.45±0.71	153.82±1.02
	GG (*n* = 387)	13.86±0.39	11.39±0.34	10.85±0.32	52.62±0.67	153.96±0.96
MC4R	*AA* (*n* = 231)	13.12±0.42[b]	10.88±0.37[b]	10.37±0.35[b]	51.94±0.73	155.24±1.04[b]
	AG (*n* = 335)	13.81±0.39	11.30±0.34	10.78±0.33	52.65±0.68	153.96±0.98
	GG (*n* = 87)	14.73±0.46	11.97±0.40	11.39±0.39	52.99±0.80	152.43±1.14

Note. BF1 — back fat thickness in the region of rib 6-7, mm; BF2 — back fat thickness in the region of rib 10, mm; BF3 — back fat thickness in the region of rib 14, mm; MD — muscle depth, mm; AGE100 — age of 100 kg body weight, days. [a] The differences with the *GG* (*IGF2*) are statistically significant at $P > 0.95$-0.999. [b] The differences with the *GG* (*MC4R*) are statistically significant at $P > 0.95$-0.999.

IGF2 and *MC4R* DNA markers can not only have a significant and similar impact on carcass and growth traits, but also be interconnected among each

other and have compensatory effect (the additive effect of genes). In order to test this hypothesis we analyzed evaluations of carcass and growth traits of animals depending on genotype combinations for two markers (*IGF2/MC4R*). The findings (Table 5) confirm the validity of the proposed hypothesis relative to the traits of fattening productivity of pigs (back fat thickness in three measurement points). Moreover, the pigs with the best traits are the ones with the highest frequency of occurrence of desirable *A* alleles according to *IGF2* and *MC4R*. The identified regularities confirm the availability of correlative interconnections of *IGF2* and *MC4R* genotypes with carcass and growth traits, which can be used in practical selection.

5. The weighted appraisal values for productive traits for genotype combinations for *IGF2/MC4R* DNA markers in large white and landrace pig groups (*Sus scrofa*) obtained by the least squares method (LSM) (Selection and Hybrid Center LLC, Voronezh region, 2017-2018)

Group	*n*	LSM estimates for productive traits				
		BF1	BF2	BF3	MD	AGE_{100}
4*A*	195	12.96 ± 0.41^{abc}	10.35 ± 0.34^{abc}	9.61 ± 0.33^{abc}	52.71 ± 0.71	160.81 ± 1.02
3*A*	400	13.65 ± 0.35^{ab}	10.85 ± 0.30^{ab}	10.17 ± 0.28^{ab}	53.54 ± 0.61	159.93 ± 0.87
2*A*	339	14.19 ± 0.38^{abeд}	11.25 ± 0.32^{abe}	10.60 ± 0.31^{abe}	53.01 ± 0.66	159.69 ± 0.94
1*A*	258	15.14 ± 0.40^{acde}	12.05 ± 0.34^{acde}	11.18 ± 0.32^{acde}	52.96 ± 0.70	160.21 ± 0.99
0*A*	64	16.43 ± 0.53^{bcde}	13.17 ± 0.45^{bcde}	12.41 ± 0.43^{bcde}	54.47 ± 0.92	160.52 ± 1.32

N o t e. BF1 — back fat thickness in the region of rib 6-7, mm; BF2 — back fat thickness in the region of rib 10, mm; BF3 — back fat thickness in the region of rib 14, mm; MD — muscle depth, mm; AGE100 — age of 100 kg body weight, days..

[a] The differences are statistically significant when comparing with the 0*A* group, P > 0.95-0.999.
[b] The differences are statistically significant when comparing with the 1*A* group, P > 0.95-0.999.
[c] The differences are statistically significant when comparing with the 2*A* group, P > 0.95-0.999.
[d] The differences are statistically significant when comparing with the 3*A* group, P > 0.95-0.999.
[e] The differences are statistically significant when comparing with the 4*A* group, P > 0.95-0.999.

Therefore, proven impact of certain *IGF2*, *CCKAR* and *MC4R* genotypes on variability of carcass and growth traits of Large White and Landrace pigs has been determined. The presence of a desirable genotype for these genes can be used as a factor in marker selection of pigs based on back fat thickness traits (in three measurement points) and muscle depth for *IGF2* (P > 0.95-0.999), and for back fat thickness traits (in three measurement points), muscle depth and age of live weight gain of 100 kg for *MC4R* (P > 0.99-0.999). Connectivity of *CCKAR* gene genotype with genetic estimates for BF1 (P > 0.99) and MD (P > 0.999) traits has been identified. Our findings identify the additive interconnection of genotypes for *IGF2* and *MC4R* markers with fattening pig traits in question and prove superiority of pigs with the highest frequency of a desirable allele above those homozygous for an alternative genotype.

To conclude, presence of a certain genotype for markers in question can conventionally characterize the genetic potential of a pig for its carcass and growth traits. When evaluating and allocating animals to primary selection groups it is expedient to take into consideration both the presence of desirable individual alleles for *IGF2* and *MC4R* markers and combinations of genes displaying proven correlated impact on variance of economically significant traits. When developing DNA biochips, the analyzed mutations associated with the quantitative traits of pig productivity, as reliably significant for breeding value of animal, should be taken into account in the array design. This will improve the efficiency of selection during pig breeding.

REFERENCES

1. Sermyagin A.A., Naryshkina E.N., Karpushkina T.V., Strekozov N.I., Zinov'eva N.A. *Molochnoe i myasnoe skotovodstvo*, 2015, 7: 2-5 (in Russ.).
2. Sermyagin A.A., Gladyr' E.A., Kharitonov S.N., Ermilov A.N., Strekozov N.I., Brem G., Zi-

nov'eva N.A. Genome-wide association study for milk production and reproduction traits in Russian Holstein cattle population. *Agricultural Biology* [*Sel'skokhozyaistvennaya Biologiya*], 2016, 51(2): 182-193 (doi: 10.15389/agrobiology.2016.2.182eng).
3. Zinov'eva N.A., Sermyagin A.A., Kostyunina O.V. *Zhivotnovodstvo Rossii*, 2018, 7: 53-55 (in Russ.).
4. Ramos A.M., Crooijmas R.P., Affara N.A., Amaral A.J., Archibald A.L., Beever J.E., Benedixen C., Churcher C., Clark R., Dehais P., Hansen M. S., Hedegaard J., Hu Z.L., Kerstens H.H., Law A.S., Megens H.-J., Milan D., Nonneman D.J., Rohrer G.A., Rothschild M.F., Smith T.P.L., Schnabel R.D., Van Tassell C.P., Taylor J.F., Wiedmann R.T., Schook L.B., Groenen M.A.M. Design of a high density SNP genotyping assay in the pig using SNPs identified and characterized by next generation sequencing technology. *PLoS ONE*, 2009, 4(8): 6524 (doi: 10.1371/journal.pone.0006524).
5. Meuwissen T.H.E., Hayes B.J., Goddard M.E. Prediction of total genetic value using genome-wide dense marker maps. *Genetics*, 2001, 157(4): 1819-1829.
6. VanRaden P.M., Null D.J., Sargolzaei M., Wiggans G.R., Tooker M.E., Cole J.B., Sonstegard T.S., Faust M.A., Doak G.A. Genomic imputation and evaluation using high-density Holstein genotypes. *J. Dairy Sci.*, 2013, 96(1): 668-678 (doi: 10.3168/jds.2012-5702).
7. Habier D., Fernando R.L., Dekkers JCM. Genomic selection using low-density marker panels. *Genetics*, 2009, 182(1): 343-353 (doi: 10.1534/genetics.108.100289).
8. Huang Y., Hickey J.M., Cleveland M.A., Maltecca C. Assessment of alternative genotyping strategies to maximize imputation accuracy at minimal cost. *Genet. Sel. Evol.*, 2012, 44(1): 25 (doi: 10.1186/1297-9686-44-25).
9. Cleveland M.A., Hickey J.M. Practical implementation of cost-effective genomic selection in commercial pig breeding using imputation. *J. Anim. Sci.*, 2013, 91(8): 3583-3592 (doi: 10.2527/jas.2013-6270).
10. Abell C.E., Dekkers J.C.M., Rothschild M.F., Mabry J.W., Stalder K.J. Total cost estimation for implementing genome-enabled selection in a multi-level swine production system. *Genet. Sel. Evol.*, 2014, 46(1): 32 (doi: 10.1186/1297-9686-46-32).
11. *Pig QTLdb*. Available https://www.animalgenome.org/cgi-bin/QTLdb/SS/index. Accessed May 10, 2018.
12. Van Laere A.-S. From QTL to QTN. *Identification of a quantitative trait nucleotide influencing muscle development and fat deposition in pig. Doctoral thesis.* Swedish University of Agricultural Sciences, Uppsala, 2005.
13. Van Laere A.S., Nguyen M., Braunschweig M., Nezer C., Collette C., Moreau L., Archibald A.L., Haley C.S., Buys N., Tally M., Andersson G., Georges M., Andersson L. A regulatory mutation in *IGF2* causes a major QTL effect on muscle growth in the pig. *Nature*, 2003, 425 (6960): 832-836 (doi: 10.1038/nature02064).
14. Chang K.C. Key signalling factors and pathways in the molecular determination of skeletal muscle phenotype. *Animal*, 2007, 1(5): 681-698 (doi: 10.1017/S1751731107702070).
15. Estellé J., Mercadé A., Noguera J.L., Pérez-Enciso M., Óvilo C., Sánchez A., Folch J.M. Effect of the porcine IGF2-intron3-G3072A substitution in an outbred Large White population and in an Iberian×Landrace cross. *J. Anim. Sci.*, 2005, 83(12): 2723-2728. (doi: 10.2527/2005.83122723x).
16. Heuven H.C.M., Bovenhuis H., Janss L.L.G., Van Arendonk J.A.M. Efficiency of population structures for mapping of Mendelian and imprinted quantitative trait loci (QTL) in outbred pigs using variance component methods. *Genet. Sel. Evol.,* 2005, **37:** 35-655 (doi: 10.1051/gse:2005019).
17. Oczkowicz M., Tyra M., Walinowicz K., Różycki M., Rejduch B. Known mutation (A3072G) in intron3 of the *IGF2* gene is associated with growth and carcass composition in Polish pig breeds. *J. Appl. Genet.*, 2009, 50(3): 257-259 (doi: 10.1007/BF03195681).
18. Kostyunina O.V., Svezhentseva N.A., Zinov'eva N.A., Dotsev A.V., Shakhina A.V., Sizareva E.I., Gladyr' E.A. Effect of ESR and IGF2 marker genotypes on the breeding values of the large white boars. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2011, 6: 54-59.
19. Kostyunina O.V., Kramarenko S.S., Svezhentseva N.A., Sizareva E.I., Zinov'eva N.A. The association of ***IGF2*** with productive traits of pigs of large white breed in the aspect of sexual differentiation. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2015, 50(6): 736-745 (doi: 10.15389/agrobiology.2015.6.736eng).
20. Crawley J.N., Fiske S.M., Durieux C., Derrien M., Roques B.P. Centrally administered cholecystokinin suppresses feeding through a peripheral-type receptor mechanism. *J. Pharmacol. Exp. Ther.*, 1991, 257: 1076-1080.
21. Asin K.E., Bednarz L. Differential effects of CCK-JMV-180 on food intake in rats and mice. *Pharmacol. Biochem. Be.*, 1992, 42(2): 291-295.
22. Clutter A.C., Sasaki S., Pomp D. Rapid communication: the cholecystokinin type-A receptor (CCKAR) gene maps to porcine chromosome 8. *J. Anim. Sci.*, 1998, 76(7): 1983-1984.
23. Houston R.D., Haley C.S., Archibald A.L., Cameron N.D., Plastow G.S., Rance K.A. A polymorphism in the 5' untranslated region of the porcine Cholecystokinin Type-A Receptor

(CCKAR) gene affects feed intake and growth. *Genetics*, 2006, 174(3): 1555-1563 (doi: 10.1534/genetics.106.059659).
24. Johnson R. Effect of DNA markers in Nebraska selection lines. *Nebraska Swine Reports*, 2010: 44-50. Available https://digitalcommons.unl.edu/cgi/viewcontent.cgi?article=1260&context=coopext_swine. Accessed May 10, 2018.
25. Mountjoy K.G., Mortrud M.T., Low M.J., Simerly R.B., Cone R.D. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol. Endocrinol.*, 1994, 8(10): 1298-1308.
26. Kim K.S., Larsen N.J., Rothschild M.F. Rapid communication: linkage and physical mapping of the porcine melanocortin-4 receptor (MC4R) gene. *J. Anim. Sci.*, 2000, 78(3): 791-792.
27. Kim K.S., Larsen N., Short T., Plastow G., Rothschild M.F. A missense variant of the melanocortin 4 receptor (MC4R) gene is associated with fatness, growth and feed intake traits. *Mamm. Genome*, 2000, 11(2): 131-135.
28. Hernández-Sánchez J., Visscher P., Plastow G., Haley C. Candidate gene analysis for quantitative traits using the transmission disequilibrium test: the example of the melanocortin 4-receptor in pigs. *Genetics*, 2003, 164(2): 637-644.
29. Stachowiak M., Szydlowski M., Obarzanek-Fojt M., Switonski M. An effect of a missense mutation in the porcine melanocortin-4 receptor (MC4R) gene on production traits in Polish pig breeds is doubtful. *Anim. Genet.*, 2005, 37(1): 55-57 (doi: 10.1111/j.1365-2052.2005.01373.x).
30. Houston R.D., Cameron N.D., Rance K.A. A melanocortin-4 receptor (MC4R) polymorphism is associated with performance traits in divergently selected large white pig populations. *Anim. Genet.*, 2004, 35(5): 386-390 (doi: 10.1111/j.1365-2052.2004.01182.x).
31. Chen J.F., Xiong Y.Z., Zuo B., Zheng R., Li F.E., Lei M.G., Li J.L., Deng C.Y., Jiang S.W. New evidences of effect of melanocortin-4 receptor and insulin-like growth factor 2 genes on fat deposition and carcass traits in different pig populations. *Asian-Australian Journal of Animal Science*, 2005, 18(11): 1542-1547 (doi: 10.5713/ajas.2005.1542).
32. Óvilo C., Fernández A., Rodríguez M.C., Nieto M, Silióa L. Association of *MC4R* gene variants with growth, fatness, carcass composition and meat and fat quality traits in heavy pigs. *Meat Science*, 2006, 73(1): 42-47 (doi: 10.1016/j.meatsci.2005.10.016).
33. Van den Maagdenberg K., Stinckens A., Claeys E., Seynaeve M., Clinquart A., Georges M., Buys N., De Smet S. The Asp298Asn missense mutation in the porcine melanocortin-4 receptor (MC4R) gene can be used to affect growth and carcass traits without an effect on meat quality. *Animal*, 2007, 1(8): 1089-1098 (doi: 10.1017/S1751731107000456).
34. Park H.B., Carlborg O., Marklund L., Andersson L. *Melanocortin-4 receptor* (*MC4R*) genotypes have no major effect on fatness in a Large White × Wild Boar intercross. *Anim. Genet.*, 2002, 33(2): 155-157 (doi: 10.1046/j.1365-2052.2002.00824.x).
35. Bruun C.S., Jшrgensen C.B., Nielsen V.H., Andersson L, Fredholm M. Evaluation of the porcine *melanocortin 4 receptor* (*MC4R*) gene as a positional candidate for a fatness QTL in a cross between Landrace and Hampshire. *Anim. Genet.*, 2006, 37(4): 359-362 (doi: 10.1111/j.1365-2052.2006.01488.x).
36. Jokubka R., Maak S., Kerziene S., Swalve H.H. Association of a melanocortin 4 receptor (MC4R) polymorphism with performance traits in Lithuanian White pigs. *J. Anim. Breed. Genet.*, 2006, 123(1): 12-22 (doi: 10.1111/j.1439-0388.2006.00559.x).
37. Salajpal K., Đikić M., Karolyi D., Šurina J., Matković M., Liker B. Effect of MC4R polymorphism on physiological stress response in pigs. Poljoprivreda, 2007, 13(1): 46-50. Available https://hrcak.srce.hr/16049. Accessed May 10, 2018.
38. Misztal I., Tsuruta S., Strabel T., Auvray B., Druet T., Lee D.H. BLUPF90 and related programs (BGF90). *Proc. 7th World Congress on genetics applied to livestock production*. Montpellier, France, 2002, 28(28-07): 21-22.
39. Dube B., Mulugeta S.D., Dzama K. Genetic relationship between growth and carcass traits in Large White pigs. *S. Afr. J. Anim. Sci.*, 2013, 43(4): 482-492.

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 35-742
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Reproductive biotechnologies

UDC 636.1:636.082:591.463.15:591.11:575

doi: 10.15389/agrobiology.2018.4.735eng
doi: 10.15389/agrobiology.2018.4.735rus

ASSOCIATED CONNECTION OF ERYTHROCITARY ANTIGENS WITH CHARACTERISTICS OF STALLION SEMEN AFTER CRYOCONSERVATION

A.V. TKACHEV[1, 2], O.L. TKACHEVA[3], V.I. ROSSOKHA[3]

[1]*National University of Pharmacy,* 53, vul. Pushkinskaya, Kharkov, Ukraine 61002, e-mail sasha_sashaola@mail.ru (✉ corresponding author);
[2]*Gorin Belgorod State Agricultural University*, 1, ul. Vavilova, pos. Maiskii, Belgorod Region, Belgorod Province, 308503 Russia, e-mail sasha_sashaola@mail.ru (✉ corresponding author);
[3]*Institute of Animal Science of National Academy of Agrarian Sciences of Ukraine*, 3, vul. 7-i Gvardeiskoi Armii, Kharkov, Ukraine 61120, e-mail tkacheva.olga2017@gmail.com, rossokha.v@ukr.net
ORCID:
Tkachev A.V. orcid.org/0000-0002-7721-5742 Rossokha V.I. orcid.org/0000-0001-8709-7535
Tkacheva O.L. orcid.org/0000-0002-5573-6117
The authors declare no conflict of interests
Received July 10, 2017

Abstract

Cryopreservation of animal sperm is the most important way to preserve endangered breeds and species. This is of particular relevance in the horse breeding of Russia and Ukraine given the critical decrease in the livestock of pedigree animals to 1.3 million and up to 300 thousand heads, respectively. For example, in Ukraine only 3 out of 12 officially registered breeds have the minimum required number of breeding stallions and mares. Therefore, the increase in the characteristics of stallion sperm and the improvement of the ability to predict the effectiveness of cryopreservation are of great scientific and practical importance. This article presents the results of the studies on the associated dependence of erythrocyte antigens A, C, D and K of the blood groups of horses of Ukrainian selection. The associated relationship of the efficiency of cryopreservation of stallion sperm has been revealed depending on the antigenic characteristics of erythrocytes according to A, C, D, and K blood group systems. It has been shown that the obtaining of the average parameters of stallion spermatozoa motility and survival after cryopreservation of 2.5 points and 4 hours, respectively, was accompanied by the presence of alleles of erythrocyte antigens *bcm/cgm*, *bcm/de*, *bcm/dg*, *bcm/dk*, *cegm/cgm*, *cegm/d*, *cegm/dg*, *cegm/dk*, *cgm/*cgm, *cgm/d*, *cgm/dg*, *de/d*, *de/dk*, *dk/d*, *dk/de*, *dk/dk* of the blood group D system. High semen characteristics after cryopreservation, i.e. the motility more than 4 points and survival spermatozoa more than 4 hours were obtained from the stallions with alleles of erythrocyte antigens *bcm/d*, *cgm/de*, *dg/dk* of the blood group D. The degree of the influence of erythrocyte antigens of the blood group D on the cryoresistance of the stallion semen is 32.5 % ($p < 0.001$), on the motility of the thawed semen — 18.2 % ($p < 0.01$), and on the survival rate of the thawed spermatozoa — 25.2 % ($p < 0.001$). The absence of the blood group A antigens in the stallions was accompanied by a significant increase in the biological characteristics of the semen after cryopreservation. In the absence of erythrocyte antigens of the blood group A, the activity of the sperm in stallions was higher by 0.51 points ($p < 0.05$) as compared to the control, by 1.36 points ($p < 0.01$) as compared to the carriers of *a/–*, by 0.17 points as compared to the stallions with *ad/–*. It has been shown that the presence of *a/–* of the blood group C contributed to a significant ($p < 0.05$) impairment of the activity (by 0.33 points), of the survival (by 0.78 hours) and of the semen durability (by 6.26 %) as compared to the stallions in which there were no alleles of this blood group system. If the antigens of the blood group K were not inherited by stallions, the activity of the thawed sperm was 0.35 points higher ($p < 0.05$) than in the carriers of *a/–* of the K system, and by 0.40 points ($p < 0.05$) higher than in the control stallions in which the antigenic profile of erythrocytes was not determined. It has been found out that the degree of the influence of the alleles of the blood group K on the activity of spermatozoa after thawing was 1.4 % ($p < 0.05$), on the semen durability — 2.0 % ($p < 0.05$), on the absolute value of durability — 1.5 % ($p < 0.05$), on the spermatozoa preservation — 1.2 % ($p < 0.05$). The generalized dispersion analysis of the obtained data has shown that the degree of the influence of the antigenic characteristics of erythrocytes on sperm cryoresistance in the stallions of Ukrainian selection was 38.7 % ($p < 0.001$) for blood group D, 1.7 % ($p < 0.05$) for blood group A, 16.6 % ($p < 0.01$) for blood group C, and 12.9 % ($p < 0.01$) for blood group K.

Keywords: erythrocyte antigens, alleles of systems of blood groups, cryopreservation of se-

Cryopreservation of animal sperm is the most important way to preserve endangered breeds and species. This is of particular relevance in the horse breeding of Russia and Ukraine given the critical decrease in the livestock of pedigree animals to 1.3 million and up to 300 thousand heads, respectively [1-3]. For example, in Ukraine only 3 out of 12 officially registered breeds have the minimum required number of breeding stallions and mares [4]. Therefore, the increase in the characteristics of stallion sperm and the improvement of the ability to predict the effectiveness of cryopreservation are of great scientific and practical importance.

The modern physiology focuses more on the impact of microorganisms on the primary characteristics of semen after cryopreservation, which is explained by the subsequent application of the preserved semen doses in the system of artificial insemination of horses [5-7]. Many other factors are analyzed, which can affect the main spermogram parameters of stallions: endocrine profile, breed, age, time of year, overall physiological condition, mycotoxins, overall chromosomal instability, etc. [8-12]. Work is underway to evaluate the efficiency of cryopreservation of epididymal semen of stallions [1].

At the same time, the associated link between the erythrocyte antigens with ejaculate parameters after cryopreservation is almost not studied at all [13-15] in spite of the fact that their association with fertile potential of horses was proven as far back as in 1940-s [16-18]; only a few researches point out the necessity of such work [19-22]. Whereas 20 blood group systems of humans have been officially recognized today out of 35 existing groups [23], horses have only 9 such systems, where four are of particular practical value, A, C, D, and K, the associated link of which with spermogram parameters after cryopreservation has not been analyzed. The ability to improve mare fertilization rate during mating is displayed based on impact of immunogenetic markers; however, the association of these markers with stallion spermogram parameters have not been studied [24]. The only research of biological properties of stallion native semen properties in combination with the antigenic erythrocyte profile showed that these properties are associated, but their association with the cryopreservation performance was not taken into consideration [4].

We have compared the antigenic properties of A, C, D and K erythrocytes of horse blood group systems with the primary semen properties after cryopreservation for the first time and have proven that high mobility and survival rate of sperm cells after cryopreservation is observed in specimen with a certain set of D system erythrocyte antigens, and absence of A blood group system antigens is accompanied with a certain increase of biological properties of semen after cryopreservation.

The goal of the presented research was to identify the association links between erythrocyte antigens of A, C, D and K stallion blood group systems and properties of their semen after cryopreservation.

Techniques. The research was performed on 1676 fresh ejaculates, of which 1413 displayed cryogenic resistance. The semen was received from 69 stallions of 9 stud farm breeds (Ukrainian Saddle Horse, the Thoroughbred, the Trakehner, the Hanoverian, the Belgian, the Westphalian, the Arabian horse, the Orlov Trotter and Russian Trotter) of stud farms, pedigree breeding units, horse sports club of Kharkiv, Poltava, Zaporizhia, Luhansk, Kyiv, Zhitomir and Dnipropetrovsk region (Ukraine) during 10 years, starting with 2005. The ejaculates were selected onto sterile artificial vagina with a sterile semen receiving unit after sanitary treatment of stallions, and Kharkiv technology was used for selection and cryopreservation of semen [4, 9]. The activity of sperm cells in the de-

frosted semen was determined in points (1 point = 10% of sperm cells with rectilinear translation) visually in Jenaval light microscope (Carl Zeiss, Germany) with lens magnification of ×10-20, sperm cell survival rate (in hours) in the thermostat at 37 °C, the absolute survival rate indicator (in conventional units), preservation of sperm cells as percentage of the initial values in the native seamen using generally accepted methods [25]. The defrosted semen parameters were analyzed individually for alleles from blood group systems (D, A, C and K). The immunogenetic genotyping by erythrocyte antigens of stallion blood group systems was conducted in the direct agglutination immunoassay by application of standard monospecific serum reagents verified by international standard reagents and manufactured in the genetics laboratory of the Animal Breeding Institute of the National Academy of Agrarian Sciences of Ukraine (Aa, Ad, Ca, Da, Db, Dc, Dd, De, Dg, Dk, Ka) using generally accepted methodologies [13]. When determining stallion genotypes by blood groups in each system both alleles were identified, which were inherited from parents. The genotypes were marked delineated by a line: the allele before the line is inherited from a father, the allele after the line is inherited from the mother.

The statistical processing was performed using generally accepted methods of variation statistics, the statistical significance of differences was evaluated according to Student *t*-criterion [26]. The tables show mean (*M*) and mean deviations (±SEM). The dispersion analysis was performed using a specialized application program package SPSS for Windows (nonparametric statistics) (IBM, USA).

Results. The test data regarding identification of associative links between erythrocyte antigens of D blood group system with semen properties after cryopreservation are shown in Table 1.

1. The association of alleles of erythrocyte antigens of D (EA D) **blood group system with stallion semen properties after cryopreservation** (*M*±SEM, *n* = 1413)

EA D	Number of samples	Activity of sperm cells, points	Survival rate of sperm cells		Preservation of sperm cells, %
			at 37 °C, h	absolute, con.unit	
Low semen quality					
ad/bcm	17	2.12±0.23	2.12±0.23	4.62±0.66	42.94±4.57
ad/cgm	12	2.37±0.26	2.79±0.32	5.79±0.67	47.22±5.42
ad/d	16	1.93±0.29	1.93±0.29	4.50±0.71	38.54±5.91
ad/de	36	2.06±0.23	2.01±0.21	5.26±0.68	37.68±4.11
ad/dk	14	1.39±0.27	1.50±0.31	3.32±0.71	27.65±5.42
cgm/ceg	15	1.53±0.33	1.63±0.35	3.53±0.81	28.32±5.72
cgm/dk	45	2.51±0.09	2.84±0.12	7.57±0.47	47.52±1.90
de/cgm	8	2.10±0.29	2.25±0.34	5.1±0.79	40.90±6.10
dg/cgm	14	2.11±0.29	2.46±0.35	5.11±0.75	42.90±5.45
dg/di	16	1.47±0.31	1.50±0.32	3.63±0.85	26.14±5.10
Medium semen quality					
bcm/cgm	103	3.60±0.19**	3.00±0.15**	9.91±0.61**	50.13±2.47*
bcm/de	91	3.53±0.17**	3.11±0.14**	9.59±0.48**	52.57±2.17*
bcm/dg	65	3.38±0.13**	3.43±0.19**	10.40±0.68***	52.84±1.43*
bcm/dk	115	3.59±0.11**	3.70±0.11**	11.80±0.46***	54.30±1.36*
cegm/cgm	27	3.24±0.11**	3.68±0.16**	9.87±0.43**	54.63±1.27**
cegm/d	20	3.25±0.11**	3.55±0.13**	10.70±0.47***	52.60±0.84*
cegm/dg	27	3.18±0.12**	3.67±0.15**	9.67±0.45**	55.50±1.54**
cegm/dk	37	3.33±0.17**	3.54±0.25**	10.90±0.91***	57.31±2.46**
cgm/cgm	72	2.92±0.11*	3.37±0.15**	8.10±0.38**	51.87±1.79*
cgm/d	29	3.93±0.47**	2.17±0.19*	8.27±0.99**	49.80±5.70*
cgm/dg	58	2.98±0.17*	3.24±0.20*	9.49±0.73**	48.18±2.57*
de/d	27	3.80±0.48***	2.02±0.21*	7.59±0.95*	47.20±6.05
de/dk	88	3.18±0.13**	3.47±0.15**	10.24±0.55***	51.90±1.70*
dk/d	67	3.37±0.11**	3.60±0.14**	10.61±0.49***	53.30±1.54**
dk/de	25	3.34±0.12**	3.44±0.16**	11.36±0.73***	58.27±1.77**
dk/dk	30	3.74±0.17***	3.15±0.15**	12.35±0.91***	59.23±2.08**
High semen quality					
bcm/d	27	4.33±0.11***	5.03±0.12***	14.94±2.67***	67.12±0.87***
cgm/de	48	4.67±0.18***	5.19±0.21***	16.45±0.75***	61.90±2.29***
dg/dk	19	4.34±0.33***	4.71±0.36***	15.90±1.32***	56.34±4.30**

*, **, *** Differences with low quality semen are statistically significant respectively at $p < 0.05$; $p < 0.01$ and $p < 0.001$.

Based on the obtained data (see Table 1) it is apparent that erythrocyte antigen alleles *ad/bcm*, *ad/cgm*, *ad/d*, *ad/de*, *ad/dk*, *cgm/ceg*, *cgm/dk*, *de/cgm*, *dg/cgm*, *dg/di* of D blood group system in surveyed horses was accompanied by manifestation of low semen quality after cryopreservation (sperm cell mobility after defrosting below 2.5 points and low survival rate below 2.5 h). The average physiological characteristics of semen (mobility of sperm cells from 2.5 points and survival rate below 4 h) were noted when stallions of Ukrainian selection had alleles of erythrocyte antigens *bcm/cgm*, *bcm/de*, *bcm/dg*, *bcm/dk*, *cegm/cgm*, *cegm/d*, *cegm/dg*, *cegm/dk*, *cgm/cgm*, *cgm/d*, *cgm/dg*, *de/d*, *de/dk*, *dk/d*, *dk/de*, *dk/dk* of D blood group system. High performance of cryopreservation semen (mobility above 4 points and survival rate of 4 h) was observed in stallions with erythrocyte antigen alleles *bcm/d*, *cgm/de*, *dg/dk* of D blood group system.

It has been determined that the degree of impact of erythrocyte antigens of D blood group system on cryogenic resistance of semen was 32.5 % ($p < 0.001$), 18.2 % for mobility of defrosted sperm cells ($p < 0.01$), 25.2% ($p < 0.001$) for their survival rate, 24.5 % ($p < 0.01$) for absolute survival rate of defrosted semen and 12.2 % ($p < 0.05$) for preservation of defrosted semen.

2. The association of alleles of erythrocyte antigens of A (EA D) **blood group system with stallion semen properties after cryopreservation** (M±SEM, n = 1413)

EA A	Number of samples	Activity of sperm cells, points	Survival rate of sperm cells		Preservation of sperm cells, %
			at 37 °C, h	absolute, con.unit	
Test group					
–/–	101	3.42±0.11*	3.45±0.11**	10.88±0.49*	54.97±1.38*
a/–	8	2.06±0.29*	2.25±0.34	5.06±0.78**	40.88±6.09
ad/–	1059	3.25±0.05	3.26±0.05*	9.72±0.17*	50.92±0.60
Control group					
Not determined	245	2.91±0.09	2.71±0.08	8.09±0.31	46.21±1.27

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

The analysis of primary characteristics of cryopreserved semen of Ukrainian selection horses in comparison with alleles of A blood group system (Table 2) allows us to arrive at a conclusion that biological quality of this semen reliably increased when stallions did not have erythrocyte antigens of this system. For instance, the activity of sperm cells was 0.51 points higher ($p < 0.05$) than in the control group, 1.36 points ($p < 0.01$) higher as compared with the activity of carriers of *a/*-allel and 0.17 points higher compared with producers with *ad/*-allels. The survival rate of defrosted semen turned out highest for stallions with no A system antigens, i.e. 0.74 h higher than in the control group ($p < 0.05$), 1.2 h higher ($p < 0.001$) as compared to the carriers with *a/*-allel and 0.19 h higher for carriers of *ad*-allel. The inheritance by stallions of *a/–* erythrocyte antigen of A blood group system was accompanied with the worst preservation rate of sperm cells (below 41 %).

The degree of impact of erythrocyte antigens of A blood group system on cryogenic resistance constituted 3.4 % ($p < 0.05$), 1.2% ($p < 0.05$) on the mobility of sperm cells after defrosting, 2.4 % ($P < 0.05$) on survival rate of defrosted sperm cells, 2.1% ($p < 0.05$) on absolute parameter of survival rate of defrosted semen and 1.4 % ($p < 0.05$) on preservation rate of defrosted semen.

The results of analysis of key physiological characteristics of stallion semen depending on presence of erythrocyte antigens of C blood group system (Table 3) are indicative of the fact that presence of *a/–* accompanied with certain ($p < 0.05$) decline of activity (by 0.33 points), survival rate (by 0.78 h), absolute survival rate parameter (by 1.84 conventional units), preservation rate of sperm cells (by 6.26 %) as compared with similar parameters of stallions with no erythrocyte antigens of this blood group system.

It has been determined that the degree of impact of erythrocyte antigens

of C blood group system on activity of sperm cells of stallions of Ukrainian selection was 1.4 % ($p < 0.05$), 5.6 % ($p < 0.05$) on semen survival rate, 3.1 % ($p < 0.05$) on absolute survival rate parameter and 2.4 % ($p < 0.05$) on preservation of sperm cells.

3. The association of alleles of erythrocyte antigens of C (EA D) **blood group system with stallion semen properties after cryopreservation** ($M \pm$SEM, $n = 1413$)

EA C	Number of samples	Activity of sperm cells, points	Survival rate of sperm cells		Preservation of sperm cells, %
			at 37 °C, h	absolute, con.unit	
		Test group			
–/–	940	3.32±0.05*	3.42±0.05*	10.15±0.17*	52.42±0.57*
a/–	227	2.99±0.12	2.64±0.11	8.31±0.40	46.16±1.61
		Control group			
Not determined	245	2.91±0.09	2.71±0.08	8.09±0.31	46.21±1.27

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

For stallions without K blood group system antigens, the activity of defrosted semen was 0.35 points higher ($p < 0.05$) than of K system antigen *a*/–allel carriers, and 0.40 points ($p < 0.05$) higher than of the control horses whose antigen erythrocyte profile was not determined. The degree of impact of K blood group system erythrocyte antigens on the activity of sperm cells after defrosting was 1.4 % ($p < 0,05$), 2.0 % ($p < 0.05$) on semen survival rate, 1.5 % ($p < 0.05$) on absolute survival rate parameter and 1.2 % ($p < 0.05$) on preservation of sperm cells.

The summarizing dispersion analysis of the obtained data showed that the degree of impact of antigen characteristics of erythrocytes on cryogenic resistance of semen of stallions of Ukrainian selection constitutes 38.7 % ($p < 0.001$) for D blood group system, 1.7 % ($p < 0.05$) for A blood group system, 16.6% ($p < 0.01$) for C blood group system and 12.9 % ($p < 0.01$) for K blood group system.

Therefore, the horses of Ukrainian selection were for the first time surveyed for association link between erythrocyte antigens of A, C, D and K systems with key properties of semen after cryopreservation. It has been observed that presence of erythrocyte antigen alleles *ad*/*bcm*, *ad*/*cgm*, *ad*/*d*, *ad*/*de*, *ad*/*dk*, *cgm*/*ceg*, *cgm*/*dk*, *de*/*cgm*, *dg*/*cgm*, *dg*/*di* of D blood group system in the analyzed animals was accompanied with low mobility of sperm cells (below 2.5 points) and low survival rate (below 2.5 h). The cryopreserved semen of mean quality (mobility from 2.5 points and survival rate up to 4 h) was received from stallions with erythrocyte antigen alleles *bcm*/*cgm*, *bcm*/*de*, *bcm*/*dg*, *bcm*/*dk*, *cegm*/*cgm*, *cegm*/*d*, *cegm*/*dg*, *cegm*/*dk*, *cgm*/*cgm*, *cgm*/*d*, *cgm*/*dg*, *de*/*d*, *de*/*dk*, *dk*/*d*, *dk*/*de*, *dk*/*dk* of D blood group system. The high semen characteristics after cryopreservation (mobility of sperm cells above 4 points and survival rate above 4 h) were observed in stallions with erythrocyte antigen genotype for of D blood group system *bcm*/*d*, *cgm*/*de*, *dg*/*dk*. The percentage of impact of antigen characteristics of D blood group system on cryogenic resistance of sperm cells of stallions was 32.5% ($p < 0.001$), 18.2 % ($p < 0.01$) on mobility, 25.2 % ($p < 0.001$) on survival rate of defrosted semen, 24.5% ($P < 0.01$) on absolute survival rate parameter and 12.2% ($p < 0.05$) on preservation rate of defrosted semen. In absence of K blood group system antigens, the activity of defrosted semen was 0.35 points higher ($p < 0.05$) than activity of *a*/–allel carriers of K system, and 0.40 points higher ($p < 0.05$) than activity of control group producers, whose antigen erythrocyte profile was not determined. The presence of *a*/–allel (C blood group system) was accompanied with certain ($p < 0.05$) reduction of activity (by 0.33 points), survival rate (by 0.78 h), absolute survival rate parameter (by 1.84 conventional units), sperm cell preservation (by 6.26 %) as compared to stallion parameters

without erythrocyte antigen of this blood group system. The absence of A blood group system antigens resulted in certain increase of biological properties of semen after cryopreservation.

REFERENCES

1. Atroshchenko M.M., Kalashnikov V.V., Bragina E.E., Zaitsev A.M. Comparative study of the structural integrity of spermatozoa in epididymal, ejaculated and cryopreserved semen of stallions. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2017, 52(2): 274-281 (doi: 10.15389/agrobiology.2017.2.274eng).
2. Tait B.D., Hudson F., Cantwell L., Brewin G., Holdsworth R., Bennett G., Jose M. Review article: Luminex technology for HLA antibody detection in organ transplantation. *Nephrology (Carlton),* 2009, 14(2): 247-254 (doi: 10.1111/j.1440-1797.2008.01074.x).
3. Kalashnikov V.V., Khrabrova L.A., Zaitsev A.M., Zaitseva M.A., Kalinkova L.V. Polymorphism of microsatellite DNA in horses of stud and local breeds. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2011, 2: 41-45.
4. Tkachev A.V., Sheremeta V.I., Tkacheva O.L., Rossokha V. I. Physiological relationship of erythrocyne antigens with indicators of horse spermogram. *Fiziol. Zh.*, 2017, 63(1): 84-90 (doi: 10.15407/fz63.01.084).
5. Lebedeva L.F., Atroshchenko M.M., Burmistrova S.A. Main factors affecting mare insemination with cryopreserved domestic and foreign sperm. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2015, 50(4): 476-485 (doi: 10.15389/agrobiology.2015.4.476eng).
6. Ellerbrock R.E., Honorato J., Curcio B.R., Stewart J.L., Souza J.A.T., Love C.C., Lima F.S., Canisso I.F. Effect of urine contamination on stallion semen freezing ability. *Theriogenology,* 2018, 1(117): 1-6 (doi: 10.1016/j.theriogenology.2018.05.010).
7. Gibb Z., Lambourne S.R., Curry B.J., Hall E.S., Aitken R.J. Aldehyde dehydrogenase plays a pivotal role in the maintenance of stallion sperm motility. *Biol. Reprod.*, 2016, 94(6): 133 (doi org/10.1095/biolreprod.116.140509).
8. Ducro B.J., Koenen E.P.C., Tartwijk J.M.F.M., Bovenhuis H. Genetic relations of movement and free-jumping traits with dressage and show-jumping performance in competition of Dutch Warmblood horses. *Livestock Science*, 2007, 107(2-3): 227-234 (doi: 10.1016/j.livsci.2006.09.018).
9. Al-Kass Z., Spergser J., Aurich C., Kuhl J., Schmidt K., Johannisson A., Morrell J.M. Sperm quality during storage is not affected by the presence of antibiotics in EquiPlus semen extender but is improved by single layer centrifugation. *Antibiotics*, 2018, 7(1): 1-13 (doi: 10.3390/antibiotics7010001).
10. Ellerbrock R., Canisso I., Feijo L., Lima F., Shipley C., Kline K. Diagnosis and effects of urine contamination in cooled-extended stallion semen. *Theriogenology*, 2016, 85(7): 1219-1224 (doi: 10.1016/j.theriogenology.2015.12.002).
11. Pojprasath T., Lohachit C., Techakumphu M., Stout T., Tharasanit T. Improved cryopreservability of stallion sperm using a sorbitol-based freezing extender. *Theriogenology*, 2011, 75(9): 1742-1749 (doi: 10.1016/j.theriogenology.2011.01.014).
12. Tkachev A.V., Tkacheva O.L., Rossokha V.I. Cytogenetic status of mares (*Equus caballus*) of Ukrainian riding breed influences their fertility. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2018, 53(2): 302-308 (doi: 10.15389/agrobiology.2018.2.302eng).
13. Avanzi B.R., Ramos R.S., Araujo G.H., Fioratti E.G., Trinca L.A., Dell'Aqua J.A., Melo E Oca C.M., Zahn F.S., Martin I., Alvarenga M.A., Papa F.O. Fixed-time insemination with frozen semen in mares: is it suitable for poorly fertile stallions? *Theriogenology*, 2015, 83(9): 1389-1393 (doi: 10.1016/j.theriogenology.2014.07.007).
14. Curik I., Zechner P., Sulkner J., Achmann R., Bodo I., Dovc P., Kavar T., Marti E., Brem G. Inbreeding, microsatellite heterozygosity, and morphological traits in Lipizzan horses. *J. Hered.*, 2003, 94(2): 125-132.
15. Young Y.J., Cho G.J. Factors concerning cell survival. In the same time, the rate of chromosomal early embryonic death in thoroughbred mares in aberrations was relatively low which is insufficient alone South Korea. *J. Vet. Med. Sci.*, 2007, 69: 787-792.
16. Ezzo O.H., Farghaly A.A., El-Maaty A.M.A., Mahmoud K.Gh.M. Hormonal and cytogenetic investigations in mares with early embryonic death. *Global Veterinaria*, 2011, 7(3): 211-218.
17. Aitken R.J., Whiting S., DeIuliis G.N., McClymont S., Mitchell L.A., Baker M.A. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *J. Biol. Chem.*, 2012, 287: 33048-33060 (doi: 10.1074/jbc.M112.366690).
18. Dias G.M., Lopez M.L., Ferreira A.T., Chapeaurouge D.A., Rodrigues A., Perales J., Retamal C.A. Thiol-disulfide proteins of stallion epididymal spermatozoa. *Anim. Reprod. Sci.*, 2014, 145: 29-39 (doi: 10.1016/j.anireprosci.2013.12.007).
19. Koppers A.J., Mitchell L.A., Wang P., Lin M., Aitken R.J. Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxida-

tive DNA damage in human spermatozoa. *Biochem. J.,* 2011, 436(3): 687-698 (doi: 10.1042/BJ20110114).
20. Baker M.A., Weinberg A., Hetherington L., Villaverde I., Velkov T., Baell J., Gordon C.P. Defining the mechanisms by which the reactive oxygen species by-product, 4-hydroxynonenal, affects human sperm cell function. *Biol. Reprod.*, 2015, 92(4): 108-115 (doi: 10.1095/biolreprod.114.126680).
21. Auger J., Eustache F., Maceiras P., Broussard C., Chafey P., Lesaffre C., Vaiman D., Camoin L., Auer J. Modified expression of several sperm proteins after chronic exposure to the antiandrogenic compound vinclozolin. *Toxicol. Sci.,* 2010, 117(2): 475-484 (doi: 10.1093/toxsci/kfq199).
22. Gibb Z., Lambourne S.R., Aitken R.J. The paradoxical relationship between stallion fertility and oxidative stress. *Biol. Reprod.,* 2014, 91(3): 77, 1-10 (doi: 10.1095/biolreprod.114.118539).
23. Arnold M.L., Dechant M., Doxiadis I.I., Spriewald B.M. Prevalence and specificity of immunoglobulin G and immunoglobulin A non-complement-binding anti-HLA alloantibodies in retransplant candidates. *Tissue Antigens*, 2008, 72(1): 60-66 (doi: 10.1111/j.1399-0039.2008.01067.x).
24. Lazary S., de Weck A.L., Bullen S., Straub R., Gerber H. Equine leukocyte antigen system. I. Serological studies. *Transplantation*, 1980, 30(3): 203-209.
25. Filannino A., Stout T., Gadella B.M. Dose-response effects of estrogenic mycotoxins (zearalenone, alpha- and beta-zearalenol) on motility, hyperactivation and the acrosome reaction of stallion sperm. *Reproductive Biology and Endocrinology*, 2011, 9: 134-140 (doi: 10.1186/1477-7827-9-134).
26. Plokhinskii N.A. *Rukovodstvo po biometrii dlya zootekhnikov* [Biometry in animal science]. Moscow, 1969 (in Russ.).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 743-752
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.2:636.082:577.17

doi: 10.15389/agrobiology.2018.4.743eng
doi: 10.15389/agrobiology.2018.4.743rus

ENDOGENOUS HORMONE LEVEL IN BULL SIRES IN RELATION TO AGE, AUTOIMMUNE STATUS, AND PRODUCTION PERFORMANCE OF MATERNAL ANCESTORS

A.I. ABILOV[1], O.S. MITYASHOVA[1], S.V. MYMRIN[2], A.A. GUDILINA[2], E.A. PYZHOVA[3], N.A. KOMBAROVA[1, 4], G.N. LEVINA[1]

[1]*Ernst Federal Science Center for Animal Husbandry,* Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail ahmed.abilov@mail.ru (✉ corresponding author), mityashova_o@mail.ru, gnlevina@yandex.ru;
[2]*Uralplemcentr OAO*, 21 km, Siberian tract, Ekaterinburg, 620913 Russia, e-mail uralplem@mail.ru;
[3]*Russian Academy of Livestock Management*, 9, ul. Akademicheskaya, pos. Bykovo, Podolsk Region, Moscow Province, 142143 Russia, e-mail elena@ramj.ru;
[4]*Head Center for Reproduction of Farm Animals AO*, 3, ul. Tsentralnaya, pos. Bykovo, Podolsk Region, Moscow Province, 142143 Russia, e-mail komnina@list.ru
ORCID:
Abilov A.I. orcid.org/0000-0001-6236-8634
Mityashova O.S. orcid.org/0000-0002-0401-5088
Mymrin S.V. orcid.org/0000-0001-5173-984X
Gudilina A.A. orcid.org/0000-0002-7962-5315
Pyzhova E.A. orcid.org/0000-0001-5719-3075
Kombarova N.A. orcid.org/0000-0003-3861-4465
Levina G.N. orcid.org/0000-0002-7978-6696
The authors declare no conflict of interests
Received April 11, 2018

Abstract

The role of sex steroid hormones, their physiological functions in the bull sires, and mechanism of action are not still completely elucidated. This paper is the first report of a large-scale survey that we carried out among bull sires under the ecological conditions of Ural region to estimate production of endogenous hormones estradiol and testosterone, and their precursor cholesterol, as depending on the bull age, origin (Denmark, Netherlands, Russia, the USA, France, and Germany), the milk performance of their maternal ancestors, and the titers of anti-sperm antibodies. The relationship between the endogenous hormones and the studied parameters is ascertained, and it is found that the synthesis of steroid sex hormones is sustainable even at low level of blood cholesterol. Our objective was to assay concentration of blood hormones and the titers of anti-sperm antibodies in 56 bull sires including 49 Black-and-White Holsteins and 7 Black-and-White animals of different origin aged from 24 to 91 months which are exploited in the Ural Regional Information and Breeding Center (UralPlemCenter, Sverdlovsk Province, 2016-2017). The blood serum hormones were measured by ELISA with testosterone Immuna-FA-TC and Immuna-FA-Estradiol kits (Immunotech, Russia). The ratios of testosterone to estradiol were calculated. The cholesterol level was assayed with a ChemWell 2902 automated analyzer (Awareness Technology, Inc., USA). The autoantibody titers were detected in the sperm immobilization test with blood auto serum of the bull sires and guinea pig serum complement. The obtained results were processed depending on the country of origin, the bull age, and the retrospective data on milk productivity of the bulls' mothers (M), the mothers' mothers (MM), and the fathers' mothers (FM). The highest (30.7 nmol/l) and the lowest (13.4 nmol/l and 10.7 nmol/l) concentrations of testosterone had the Danish, Holland and French bull sires, respectively. It is found that the testosterone level was rising to five-year age and eventually reached 26.9 nmol/l vs. 9.6 nmol/l in two-year old animals. The blood concentrations of testosterone and estradiol inversely correlated. The testosterone to estradiol ratio significantly varied ($P < 0.001$). The lowest testosterone concentration (15.4 nmol/l) was in hyperestrogenization, and the indicator value reached 25.1 nmol/l in the animals with low estrogens. The testosterone to estradiol ratio (T/E) increased with the age, from 0.4 at 28 months of age up to 19.3 at 50 months, by a statistically significant value ($P < 0.001$). No significant relationship was revealed between the endogenous hormone concentrations in the bulls and the milk performance of their maternal ancestors, except some effect of the father's mothers with milk yield of 12000 to 16000 kg. An increase in the sperm autoantibody titer was accompanied by the decreases in the serum testosterone and estradiol levels by 37.9 % and 4.6 %, respectively. The cholesterol level increased by 13.7 %. Changes between the groups were within the normative range. Therefore, the concentration of the endogenous hormones (testosterone and estradiol) depends on the sire age and origin. The test for anti-sperm antibodies during clinical andrological examination is diagnostically important to indirectly characterize the hormonal status of sires.

Keywords: bull sires, testosterone, estradiol, cholesterol, anti-sperm antibodies

The chemical composition of endogenous testosterone and androsterone hormones is similar to ovarian hormones estrogen and progesterone [1]. The bodies of males produce both androgenic hormones and estrogenic hormones [2]. The androgenic hormones ensure gender differentiation and determine the function of male reproductive organs (ovaries, testicles and prostate) [3]. The synthesis of steroid hormones and spermatogenesis in testicles is controlled by gonadotropic hormones and testosterone [4]. In turn, spermatogenesis is controlled by peptide hormones and steroid hormones: the follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone and estradiol [5]. About 2-3% of androgens are biologically active and circulate freely, the rest remain in blood plasma and form a complex with testosterone-estradiol-binding globulin (TEBG) [6, 7]. Hyperestrogenism and hyperthyrosis result in decline of the free testosterone function and increase of testosterone-estradiol-binding globulin content [8]. The reduced testosterone levels in blood plasma with increased levels of luteinizing hormone and follicle-stimulating hormone can result in primary and secondary testicular failure [9, 10] and changes mobility of sperm cells in ejaculate. The involvement of testosterone in activation of mitosis protein synthesis, formation of androgen-dependant enzymes, structural changes of chromatin in sperm cells, amplification of DNA and RNA synthesis [4, 11] has been proven. Testosterone affects libido, endurance, improves muscle capacity and oxygen capacity of blood [12].

Male estrogens are represented by estrone, estriol and 17β-estradiol; 20% of endogenous estrogens are synthesized directly in testicles, 80% are synthesized in peripheral tissues on account of aromatization with predominant formation of 17β-estradiol [13, 14]. The largest amount of estradiol in blood plasma is associated with testosterone-estradiol-binding globulin and albumins [7], where biologically active fraction constitutes 1-2%. The main purpose of functions of male estrogens is to enable the mechanism of negative feedback via gonadotropins (luteinizing hormones and follicle-stimulating hormones) and to regulate testosterone synthesis in testicles [13, 14]. In most cases, the increased levels of estradiol are caused by various functional disorders of estradiol metabolism. The estrogen receptors (α and β are coded with *ESR1* and *ESR2* genes) are 44 % homological and contain domains characteristic for intracellular receptors. The estrogen receptors of α-type are located in adenohypophysis, testicles, liver, kidneys, bones and brain, the receptors of β-types are located in bones, cartilages, gastrointestinal tract, thyroid gland, prostate, skin and bladder, and estrogen effect manifests itself via them [15]. The luteinizing hormone causes proliferation of seminiferous tubules and stimulates the initial stages of spermatogenesis [16], and progestin is crucial for meiosis initiation [17]. The impact of estrogens on spermatogenesis via hypothalamus is known [8]. The impact of estradiol on behavior, qualitative and quantitative characteristics of semen of bull sires [18] has been identified. The maximum amount of ejaculate was registered at minimal levels of estradiol, and the lower were the levels on the day of semen extraction, the better were the results of cow insemination [19].

In males, the volumes of testosterone and estradiol, as well as balance of their free fractions strictly correlate. The balance of sex hormones is upset in case of overweight: the content of estrogens increases [20] resulting in reduced sperm concentration [21]. Fat tissue increases [23] in case of physiological activity increase of aromatase enzyme [14, 22] converting testosterone into estrogen (estradiol) during normal ageing of males. In case of insufficient body weight the quality of semen also deteriorates [24].

It has been determined that for Holstein cattle the levels of testosterone

in blood plasma positively correlate with the quantity of sperm cells carrying Y-chromosome [25]. In Simmental bulls a positive correlation between semen concentration in ejaculate and testosterone levels has been observed, and negative correlation has been observed between semen pH and blood plasma testosterone depending on season [26, 27]. It has been mentioned that for bulls at the age of 2-4 months to 2 years the levels of blood plasma testosterone are statistically significantly different, and for older animals this parameter tends to increase [28]. We have shown the dependency of endogenous testosterone levels in the blood of bull sires on age and season [29]. It is presumed that seasonal fluctuations of testosterone are related with seasonal factors (pesticides, exogenous estrogenic hormones, physical and other phenomena) that affect the survivability of sperm cells detrimentally by producing structural and genetic changes and damaging chromatin integrity [30].

Modern industrial countries tend to accumulate synthetic endocrine-active compounds in their biosphere (with ultimate estrogen and/or antiandrogenic activity) such as ecological estrogens (xenoestrogens) and antiandrogens capable of affecting the reproductive function, nervous, immune and endocrine systems of animals and humans [15, 31-33].

Similar to glucocorticoids, testosterone causes immunodepressive effect [34, 35]. There is a connection between endogenous hormone levels (thyroxine, estradiol, and testosterone) and presence of spermatic autoantibodies in blood plasma of bull sires [36]. In the context of autoimmune response development the new data about sex steroid levels in blood (androgens, gestagens, and estrogens) and spermatogenesis will help better understand the physiological and pathophysiological function of so-called female sex hormones in a male body, whose mechanism is still unclear.

In the course of a wide-scale survey of bull sires first performed by us in the environmental conditions of the Sverdlovsk region (the region with dominating ferrous metal industry, nonferrous metal industry and ore dressing plants) we identified a correlation between the titres of spermatic autoantibodies with estradiol and testosterone levels in blood plasma, and correlation between the levels of estradiol, testosterone and cholesterol in blood, as well as age and origins of animals (Denmark, the Netherlands, Russia, USA, France, Germany) and milk productivity of their maternal ancestors. It has been observed that sex steroid hormones were synthesized in bulls even with low cholesterol levels in blood.

Our goal was to conduct qualitative evaluation of endogenous serum hormones and antibodies regarding the antibodies and spermatic antigens in bulls depending on age, origins and productivity of maternal ancestors.

Techbiques. The sampling included 56 bull sires of different origins at the age of 2-9 years (49 black-and-white Holstein animals, 7 black-and-white animals) (OJSC Uralplemcentre, Sverdlovsk region, 2016-2017). Animal breeding and maintenance conditions complied with the national technological requirements for freezing and use of semen of bull sires.

The blood was taken from jugular vein (December of 2016). After separation of blood plasma from formed elements the levels of endogenous hormones were identified using the enzyme-linked immunosorbent assay method twice using laboratory reagents (CJSC Immunotech, Russia): for testosterone Immuna-FA-TC, for estradiol Immuna-FA-estradiol. The testosterone and estradiol ratio was calculated. The cholesterol levels were evaluated using an automatic analyzer Chem Well 2902 (Awareness Technology, Inc., USA).

The ratio of autoantibody titres and own sperm cells was evaluated in a sperm immobilization test [36].

The obtained results were compared by taking into consideration the

country of origin and age of producers (24-91 months), as well as data about milk productivity of maternal ancestors, i.e. the mothers of bulls (M), mothers of mothers (MM) and mothers of fathers (MF).

Microsoft Excel was used for statistical processing of data. The tables display mean (*M*) and errors in mean (±SEM) values. The statistical significance of differences was evaluated according to Student *t*- criterion with statistical significance at $p < 0.05$, $p < 0.01$, $p < 0.001$.

Results. The highest and lowest testosterone levels (30.7 and 13.4 nmol/l) at sampling size $n > 6$ were registered for producers of the Danish and Dutch selection respectively (table 1). Testosterone levels directly depended on age because by the time of the survey the age of producers of Danish selection was 3.3 years, whereas bulls imported from the Netherlands were only 2 years of age. The lowest estradiol levels were characteristic for American selection bulls (3383.3 pmol/l), whereas for other animals this parameter was in the range of 4052-4956 pmol/l. The bulls of different origin did not differ significantly in terms of cholesterol content.

1. The levels of endogenous hormones and cholesterol in blood plasma of bull sires of different origins (*M*±*m*, OJSC Uralplemcenter, Sverdlovsk region, 2016-2017)

Origin	*n*	Breed	Testosterone, nmol/l	Estradiol, pmol/l	Cholesterol, mol/l	T/E, units
Denmark	11	H	30.7±7.7*	4052.0±477.6	3.2±0.4	4.0±1.3
the Netherlands	11	H	13.4±5.2	4786.4±341.7	3.1±0.3	2.8±1.0
Russia	13	H	15.7±4.4	4254.3±510.1	3.5±0.1	4.4±1.4
Russia	7	BW	27.4±10.1	4593.4±324.2	3.2±0.6	6.9±2.8
USA	6	H	21.9±8.0	3383.3±822.7	2.9±0.2	5.4±1.7
France	3	H	10.7±6.4	4166.9±1031.8	2.8±0.4	4.3±3.4
Germany	4	H	15.7±6.6	4956.5±444.0	4.1±0.3	2.2±1.4
(France+ Germany)	(3 + 4)	H	13.5±4.4	4618.1±483.5	3.6±0.3	3.6±1.6
Total	55					

N o t e. H — black-and-white Holstein breed, BW— black-and-white breed. T/E — quantitative testosterone to estradiol ratio.
* Differences with a group of bulls from Russia ($n = 13$) are statistically significant at $p < 0.05$.

2. The levels of endogenous hormones and cholesterol in blood plasma of bull sires of black-and-white Holstein breed and black-and-white breed depending on age (*M*±*m*, OJSC Uralplemcenter, Sverdlovsk region, 2016-2017)

Age group, months	*n*	Testosterone, nmol/l	Estradiol, pmol/l	Cholesterol, mol/l	T/E, units
До 24	13	9.6±2.9	5044.2±147.1	3.0±0.3	1.7±0.5
25-36	13	19.7±5.8	4559.5±321.6	3.4±0.3	4.8±1.6
37-60	23	26.9±4.7*	3955.4±384.2**	3.6±0.1*	5.4±1.1*
61 и старше	7	16.0±7.5	3426.5±549.2*	2.6±0.4*	5.7±2.4

N o t e. T/E — quantitative testosterone to estradiol ratio.
*, ** Differences with a group below 24 months are statistically significant at $p < 0.05$ and $p < 0.01$, respectively.

The analyzed bulls were divided into four age groups: below 2 years of age, 2-3 years of age, 4-5 years of age and above 5 years of age. The most numerous group ($n = 23$) was the group of animals from 3 to 5 years of age. The breeding farms primarily use producers of this age. Testosterone levels in bulls was dynamically increasing until the age of 5 (table 2). Below 2 years of age this parameter was 9.6 nmol/l, by 25-36 months it increased, at the age of 37-60 months it reached its maximum level (26.9 nmol/l). After the age of 7-8 years it was declining (down to 16.0 nmol/l), most probably due to commencement of physiological aging. Alternatively, the levels of estradiol below the age of 2 years were the highest, and in animals older than 5 years of age this parameter declined by 32 %. The levels of cholesterol in all groups corresponded to the lower limit of normal, and after the age of 5 it did not reach the normal level. The animal origin feeds served as source of cholesterol, and, consequently, a corresponding adjustment of the ratios of bull sires is required: cholesterol is the precursor of more

than 40 hormones and its deficit can result in a lack of sex steroids.

The bull sires at the age below 24 months and at the age of 37-60 months displayed reliably ($p < 0.01$) differing levels of testosterone and estradiol in blood plasma. These parameters were statistically significant ($p < 0.05$) for young (below 2 years of age) bull sires and bull sires at the beginning of physiological aging (above 5 years of age). In terms of cholesterol levels, the 37-60 month group, 61 month group and older ($p < 0.05$) groups differed reliably, and in terms of testosterone and estradiol levels ($p < 0.01$) the groups below 24 months and groups of 37-60 months of age differed reliably.

We conditionally divided the animals by their testosterone levels into three groups: with low (below 10.0 nmol/l), average (10.1-30.0 nmol/l) and high (30.0 and above nmol/l) levels of this hormone. The testosterone levels reversely depended on the levels of estradiol in blood plasma. Depending on testosterone levels, the testosterone and estradiol ratio increased by a statistically reliable value ($p < 0.001$). The levels of cholesterol in all groups remained completely identical and within limits of normal (Table 3).

3. The levels of estradiol and cholesterol in blood plasma of bulls sires of black-and-white Holstein breed and black-and-white breed depending on testosterone levels (*M*±*m*, OJSC Uralplemcenter, Sverdlovsk region, 2016-2017)

Groups by testosterone levels, nmol/l	*n*	Testosterone, nmol/l	Estradiol, pmol/l	Cholesterol, mol/l	T/E, units
Below 10.0	28	4.8±0.5	4688.1±207.5	3.0±0.3	1.1±0.1
10.1-30.00	15	22.2±1.5*	3837.7±470.5	3.2±0.3	6.9±0.9*
30 and above	13	50.6±5.5*	3998.1±435.4	3.3±0.3	18.9±4.9*

N o t e. T/E — quantitative testosterone to estradiol ratio.
* Differences with a group with testosterone levels below 10.0 nmol/l are statistically significant $p < 0.001$.

The bull sires were divided into four groups by estradiol levels in blood plasma: from 3000 pmol/l with a difference of 1000 pmol/l (Table 4). The testosterone levels changed in reverse proportion in groups with estradiol levels from 3001 to 5,001 pmol/l and higher (the difference from the group with estradiol levels below 3000 pmol/l are statistically significant at $p < 0.001$). No significant difference was observed in cholesterol level dynamics.

4. The levels of testosterone and cholesterol in blood plasma of bulls sires of black-and-white Holstein breed and black-and-white breed depending on estradiol levels (*M*±*m*, OJSC Uralplemcenter, Sverdlovsk region, 2016-2017)

Groups by estradiol levels, pmol/l	*n*	Estradiol (actual), pmol/l	Estradiol, pmol/l	Cholesterol, mol/l	T/E, units
Below 3000	8	1887.3±293.0	25.1±10.0	2.8±0.4	18.8±8.8
3001-4000	9	3588.6±104.7*	30.8±7.3	3.3±0.4	8.8±2.4
4001-5000	11	4495.4±72.8*	16.9±6.6	3.7±0.3	2.8±1.0
5001 and above	28	5401.2±45.8*	15.4±2.9	3.3±0.2	3.3±0.7

N o t e. T/E — quantitative testosterone to estradiol ratio.
* Differences with a group with estradiol levels below 3000 pmol/l are statistically significant at $p < 0.001$.

5. The levels of endogenous hormones and cholesterol in blood plasma of bull of black-and-white Holstein breed and black-and-white breed depending on testosterone to estradiol levels (*M*±*m*, OJSC Uralplemcenter, Sverdlovsk region, 2016-2017)

Groups by T/E ratio, units	*n*	T/E (actual), units	Testosterone, nmol/l	Estradiol, pmol/l	Cholesterol, mol/l
Below 1.0	10	0.4±0.1	2.3±0.4	4604.3±492.0	3.3±0.4
1.1-5.0	24	2.1±0.3*	9.2±1.4*	4673.3±247.0	3.1±0.2
5.1-10.0	9	6.3±0.3*	25.7±2.1*	4299.7±449.9	3.6±0.1
10.1 and above	13	19.3±4.9*	43.3±6.8*	3069.8±493.7	3.1±0.4

N o t e. T/E — quantitative testosterone to estradiol ratio.
* $p < 0.001$ as compared to the group with T/E ratio below 1.0 units.

In order to identify the impact of testosterone and estradiol ratio and as-

sociation of this parameter with the levels of hormones analyzed, bull sires were divided into groups with a variance of 5 T/E units (Table 5). Among T/E groups the actual value of this ratio differed by a statistically significant value ($p < 0.001$). As T/E ratio increased, so did testosterone levels in blood plasma. The levels of cholesterol did not depend on T/E and remained at the levels of 3.1-3.6 mol/l. Furthermore, the T/E ratio increased depending on age. For instance, at the age of 28 months the actual T/E ratio constituted 0.4 units, and at the age of 50 months and more the ratio was 19.3 units.

Knowing that cholesterol acts as precursor of sex hormones in the bodies of bull sires, we analyzed the connection between testosterone and estradiol levels and cholesterol levels (Table 6). In 41 animals cholesterol levels remained within limits of normal and on the average was 3.8 mmol/l, in 14 bull sires this parameter was lower than normal (2.5 mol/l) with average age in both groups of 36 months. The levels of testosterone and estradiol were within physiological range. Apparently, the synthesis of sex hormones is prioritized and is enabled even with low cholesterol levels.

6. The levels of endogenous hormones in blood plasma of bull of black-and-white Holstein breed and black-and-white breed depending on cholesterol levels (*M*±*m*, OJSC Uralplemcenter, Sverdlovsk region, 2016-2017

Groups by cholesterol levels, mol/l	*n*	Cholesterol (actual), mol/l	Testosterone, nmol/l	Estradiol, pmol/l	T/E, units
Normal	41	3,8±0,1	22,6±3,2	4382,3±220,6	5,3±0,8
Below normal	14	2,5±0,2*	25,2±9,5	4025,3±463,4	2,7±1,1
Above normal	1	5,6	2,6	4780,3	0,5

N o t e. T/E — quantitative testosterone to estradiol ratio.
* Differences with the parameters in a group with normal cholesterol levels are statistically significant at $p < 0.05$. In a group with reliable reduction of cholesterol levels below normal the T/E difference with the group that had normal cholesterol levels was close to significant.

7. The levels of endogenous hormones and cholesterol in blood plasma of bull of black-and-white Holstein breed and black-and-white breed depending on milk productivity of maternal ancestors (*M*±*m*, OJSC Uralplemcenter, Sverdlovsk region, 2016-2017)

Groups by productivity, kg	*n*	Testosterone, nmol/l	Estradiol, pmol/l	Cholesterol, mol/l	T/E, units
Productivity of mothers (M)					
Below 12000	21	20.8±5.1	4687.2±204.0	3.6±0.2	4.6±1.2
12001-14000	22	18.9±4.2	4108.9±325.2	3.4±0.1	3.4±2.8
14001-16000	13	23.9±5.3	4537.1±381.2	3.5±0.1	8.7±3.9
Productivity of fathers' mothers (MF)					
Below 12000	6	9.5±2.8	4405.4±480.1	3.2±0.3	2.3±0.8
12001-14000	27	24.8±4.0**	4580.3±230.1	3.4±0.2	7.4±2.0**
14001-16000	16	22.1±5.7*	4096.6±349.9	3.4±0.2	7.2±3.8
16001 and above	5	28.3±11.1	4423.5±890.9	3.7±0.2	8.1±2.9
Productivity of mothers' mothers (MM)					
Below 12000	23	17.8±4.1	4338.6±256.1	3.6±0.1	5.1±1.2
12001-14000	11	27.4±8.1	4114.7±454.1	3.5±0.2	10.3±5.4
14001-16000	5	24.8±2.8	4417.2±551.3	2.7±0.3	6.0±2.8
16001 and above	8	18.2±5.8	4720.7±406.9	3.8±0.2	4.2±1.5

N o t e. T/E — quantitative testosterone to estradiol ratio. M, MM and MF — milk productivity of mothers of bull sires, mothers of mothers and mothers of fathers, respectively.
*, ** Differences with the group in terms of productivity of mothers of fathers 12000 kg are statistically significant at $p < 0.05$ and $p < 0.001$, respectively.

The milk productivity of maternal ancestors can affect sex hormone levels of bull sires; therefore, we analyzed the dependency of hormone levels on productivity of bull sire maternal ancestors (M), mothers of mothers (MM) and mothers of fathers (MF). The milk productivity M and MM did not have a significant impact on endogenous hormones of bill sires, whereas MF reliably affected ($p < 0.05$ and $p < 0.01$) this parameter. No significant differences were

identified in terms of estradiol and cholesterol levels (Table 7).

A sufficient amount of information has been accumulated about mutual regulation of endocrine and immune systems, because it is known that testosterone suppresses the immune response (34, 35). Keeping this in mind, we have studied the dependency between endogenous hormone levels and autoimmune response of bill sires to own sperm cells (Table 8). As sperm immobilization test titers increased, testosterone levels in blood plasma declined by 37.9%, estradiol levels in blood plasma declined by 4.6%, and T/E ratio declined by 48.9%. At the same time, we registered increase of cholesterol levels by 13.7%, which, however, remained within normal ranges. We have obtained similar results in previous studies where we identified the connection between the immune system function with endogenous hormones [36].

8. The levels of endogenous hormones and cholesterol in blood plasma of bull of black-and-white Holstein breed and black-and-white breed depending on autoimmunity titre in sperm immobilization tests (*M*±*m*, OJSC Uralplemcenter, Sverdlovsk region, 2016-2017)

Titer in sperm immobilization tests	Testosterone, nmol/l	Estradiol, pmol/l	Cholesterol, mol/l	T/E, units	Age, months
Total for sampling (n = 34)	17,5±3,1	4452,2±212,3	3,5±0,1	5,217±1,6	35,1±3,1
Including:					
in a group with the titer 0-1:2 (n = 23)	20,0±3,8	4528,9±242,6	3,270±0,2	6,338±2,3	38,1±4,1
in a group with the titer 1:4-1:8 2 (n = 11)	12,4±4,9	4319,1±438,1	3,718±0,2	3,236±1,1	28,0±4,6

N o t e. T/E — quantitative testosterone to estradiol ratio.

The results obtained in this study correlate with our previous data and medical studies of other authors. For instance, autoimmune orchitis is characterized with increased levels of autoantibodies compared with the steroid-producing testicular cells, reliable reduction of total and free testosterone in blood and disruption of spermatogenesis, which manifests itself in significant reduction of sperm cell levels and percentage of progressively mobile and morphologically normal forms of sperm cells in ejaculate [37].

We have analyzed the levels of female estradiol hormone in blood plasma of bulls in the conditions of the Sverdlovsk region and its connection with the other parameters. In similar works [38, 39] producers were examined at the age of 16-35 months. We believe that the data received at the age below 3 years are insufficient for valid conclusions because during this period the physiological development is not complete. Therefore, we compared the analyzed parameters in all age groups of bull sires, from 12 to 91 months old (from young to adult and senior). As the immune method we used a specific sperm immobilization test, whereas in the aforementioned publications the authors evaluated the levels of globulin and neutrophil phagocytic activity.

To summarize, testosterone levels in blood plasma of surveyed bull sires depended on origins and age ($p < 0.05$), and estradiol and cholesterol levels depended on age ($p < 0.05$). The qualitative testosterone and estradiol ratio (T/E) increases proportional to age by a reliable value. 25% animals displayed cholesterol levels (2.5 mol/l) at the lower limit of normal; however, testosterone and estradiol levels did not differ significantly from those in groups with normal cholesterol levels, which can be indicative of priority of synthesis of sex steroid hormones. The impact of milk productivity of mothers of fathers on testosterone synthesis and T/E ($p < 0.05$, $p < 0.01$) value has been observed. It has been established that upon increase of titres of spermatic autoantibodies the synthesis of endogenous steroid hormones is reduced: testosterone levels dropped by 37.9 %, estradiol levels dropped by 4.6 % and T/E value dropped by 48.9%. These data validate the necessity of monitoring spermatic autoantibodies, which can act as indirect markers of endogenous hormones levels in blood plasma in bull sires.

REFERENCES

1. Dluzen D.E. Estrogen, testosterone, and gender differences. *Endocrine*, 2005, 27(3): 259-267 (doi: 10.1385/ENDO:27:3:259).
2. Hess R.A. Estrogen in the adult male reproductive tract: a review. *Reprod. Biol. Endocrinol.*, 2003, 1: 52 (doi: 10.1186/1477-7827-1-52).
3. Köhn F.M. Testosterone and body functions. *Aging Male*, 2006, 9(4): 183-188 (doi: 10.1080/13685530601060396).
4. Walker W.H. Testosterone signaling and the regulation of spermatogenesis. *Spermatogenesis*, 2011, 1(2): 116-120 (doi: 10.4161/spmg.1.2.16956).
5. Kumanov P., Nandipati K., Tomova A., Agarwal A. Inhibin B is a better marker of spermatogenesis than other hormones in the evaluation of male factor infertility. *Fertil. Steril.*, 2006, 86(2): 332-338 (doi: 10.1016/j.fertnstert.2006.01.022).
6. Hammond G.L. Diverse roles for sex hormone-binding globulin in reproduction. *Biol. Reprod.*, 2011, 85(3): 431-441 (doi: 10.1095/biolreprod.111.092593).
7. Grasa M.D., Gulfo J., Camps N., Alcalá R., Monserrat L., Moreno-Navarrete J.M., Ortega F.J., Esteve M.3, Remesar X., Fernández-López J.A., Fernández-Real J.M., Alemany M. Modulation of SHBG binding to testosterone and estradiol by sex and morbid obesity. *Eur. J. Endocrinol.*, 2017, 176(4): 393-404 (doi: 10.1530/EJE-16-0834).
8. Rochira V., Madeo B., Diazzi C., Zirilli L., Daniele S., Carani C. Estrogens and male reproduction. Available https://www.ncbi.nlm.nih.gov/books/NBK278933/. Accessed August 11, 2018.
9. Nieschlag E., Nieschlag S. Testosterone deficiency: a historical perspective. *Asian J. Androl.*, 2014, 16(2): 161-168 (doi: 10.4103/1008-682X.122358).
10. McBride J.A., Carson C.C., Coward R.M. Diagnosis and management of testosterone deficiency. *Asian. J. Androl.*, 2015, 17(2): 177-186 (doi: 10.4103/1008-682X.143317).
11. Okuda Y., Fujisawa M., Matsumoto O., Kamidono S. Testosterone dependent regulation of the enzymes involved in DNA synthesis in the rat ventral prostate. *The Journal of Urology*, 1991, 145(1): 188-191 (doi: 10.1016/S0022-5347(17)38288-5).
12. Bain J. The many faces of testosterone. *Clin. Interv. Aging*, 2007, 2(4): 567-576.
13. Kalinchenko S.Yu., Tyuzikov I.A. *Prakticheskaya andrologiya* [Practical andrology]. Moscow, 2009 (in Russ.).
14. Tyuzikov I.A., Kalinchenko S.Yu., Vorslov L.O., Tishova Yu.A. *Andrologiya i genital'naya khirurgiya*, 2014, 4: 8-12 (in Russ.).
15. McLachlan J.A. Environment signaling: what embryos and evolution teach us about endocrine disrupting chemicals. *Endocr. Rev.*, 2001, 22(3): 319-341 (doi: 10.1210/edrv.22.3.0432).
16. Araujo A.B., Wittert G.A. Endocrinology of the aging male. *Best Pract. Res. Clin. Endocrinol. Metab.*, 2011, 25(2): 303-319 (doi: 10.1016/j.beem.2010.11.004).
17. Miura T., Higuchi M., Ozaki Y., Ohta T., Miura C. Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. *PNAS USA*, 2006, 103(19): 7333-7338 (doi: 10.1073/pnas.0508419103).
18. Brockett C.C., Presicce G.A., Foote R.H., Kaproth M.T., Rycroft H.E. Semen quality and behavior of Holstein bulls exposed to estradiol-treated bulls for mounts. *J. Dairy Sci.*, 1994, 77(1): 124-131.
19. Abilov A.I., Eskin G.V., Kombarova N.A. Blood estradiol level in bull sires influences sperm count and effectiveness of artificial insemination. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2016, 51(6): 830-836 (doi: 10.15389/agrobiology.2016.6.830eng).
20. Gates M.A., Mekary R.A., Chiu G.R. Ding E.L., Wittert G.A., Araujo A.B. Sex steroid hormone levels and body composition in men. *J. Clin. Endocr. Metab.*, 2013, 98(6): 2442-2450 (doi: 10.1210/jc.2012-2582).
21. Fejes I., Koloszár S., Závaczki Z., Daru J., Szöllösi J., Pál A. Effect of body weight on testosterone/estradiol ratio in oligozoospermic patients. *Arch. Androloigy,* 2006, 52(2): 97-102 (doi: 10.1080/01485010500315479).
22. Kadioglu P., Oral G., Sayitoglu M., Erensoy N., Senel B., Gazioglu N., Sav A., Cetin G., Ozbek U. Aromatase cytochrome P450 enzyme expression in human pituitary. *Pituitary*, 2008, 11(1): 29-35 (doi: 10.1007/s11102-007-0065-3).
23. Braunstein G.D. Gynecomastia. *New Engl. J. Med.*, 2007, 357(12): 1229-1237 (doi: 10.1056/NEJMcp070677).
24. Qin D.-D., Yuan W., Zhou W.-J., Cui Y.-Q., Wu J.-Q., Gao E.-S. Do reproductive hormones explain the association between body mass index and semen quality? *Asian J. Androl.,* 2007, 9(6): 827-834 (doi: 10.1111/j.1745-7262.2007.00268.x).
25. Heidary F., Kholghi M., Rostam Zade Y. Analysis of the effect of blood testosterone on sex chromosome ratio in Holstein cattle. Reproduction in domestic animals. *Proc. 18 Annual Conference of the European Society for Domestic animal reproduction (ESDAR)*. Helsinki, Finland, 2014, V. 49, Supplement 3: 71.
26. Chacur M.G.M., Mizusaki K.T., Gabriel Filho L.R.A., Oba E., Ramos A.A. Seasonal effects on semen and testosterone in Zebu and Taurine bulls. *Acta Scientiae Veterinariae*, 2013, 41: pub. 1110.

27. Javed M.T., Abrar K., Mumtaz A. Influence of season on seminal plasma testosterone and oestrogen in healthy and abnormal bulls and their relationship with other semen parameters. *Veterinary Archive*, 2000, 70(3): 141-149.
28. Kozumplik J. The level of plasma testosterone during the prenatal and postnatal period of development in bulls. *Acta Vet. Brno*, 1981, 50: 27-32 (doi: 10.2754/avb198150010027).
29. Amerkhanov Kh.A., Abilov A.I., Eskin G.V., Kombarova N.A., Turbina I.S., Fedorova E.V., Varenikov M.V., Gusev I.V. Concentration of testosterone and cholesterol in blood serum of servicing bulls depending on their type of productivity, age and the season. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2014, 2: 59-66 (doi: 10.15389/agrobiology.2014.2.59eng) (in Russ.).
30. Delbes G., Hales B.F., Robaire B. Toxicant and human sperm chromatin integrity. *Mol. Hum. Reprod.*, 2010, 16(1): 14-22.
31. Amstislavskii S.Ya., Eroshenko V.P. *Ontogenez*, 2000, 31: 165-177 (in Russ.).
32. Sharpe R.M., Skakkebaek N.E. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet*, 1993, 341(8857): 1392-1395 (doi: 10.1016/0140-6736(93)90953-E).
33. Davis D.L., Cottlieb M.B., Stampnitzky J.R. Reduced ratio of male to female births in several industrialised countries. A sentinel health indicator? *JAMA*, 1998, 279(13): 1018-1023 (doi: 10.1001/jama.279.13.1018).
34. Roberts M., Peters A. Is testosterone immunosuppressive in a condition-dependent manner? An experimental test in blue tits. *J. Exp. Biol.*, 2009, 212(Pt 12):1811-1818 (doi: 10.1242/jeb.031047).
35. Roberts M.L., Buchanan K.L., Evans M.R., Marin R.H., Satterlee D.G. The effects of testosterone on immune function in quail selected for divergent plasma corticosterone response. *J. Exp. Biol.*, 2009, 212(19): 3125-3131 (doi: 10.1242/jeb.030726).
36. Abilov A.I., Amerkhanov Kh.A., Eskin G.V., Fedorova E.V., Zhavoronkova N.V., Kombarova N.A., Varennikov M.V. *Zootekhniya*, 2013, 9: 25-28 (in Russ.).
37. Gzgzyan A.M. *Autoimmunnyi gipogonadizm (patogenez, diagnostika, printsipy lecheniya). Avtoreferat doktorskoi dissertatsii* [Autoimmune hypogonadism (pathogenesis, diagnosis, treatment principles). PhD Thesis]. St. Petersburg, 2008 (in Russ.).
38. Mymrin V.S., Khalturina L.V., Shkuratova I.A., Ryaposova M.V. *Molochnoe i myasnoe skotovodstvo*, 2012, 4: 7-9 (in Russ.).
39. Khalturina L.V. *Agrarnyi vestnik Urala*, 2012, 9(101): 22-21 (in Russ.).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 753-761
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.2:636.082:618

doi: 10.15389/agrobiology.2018.4.753eng
doi: 10.15389/agrobiology.2018.4.753rus

FORECASTING THE EMBRYO PRODUCTIVITY OF DONOR COWS ON THE BASIS OF ECHOGRAPHIC CHARACTERISTICS OF THE OVARIES

A.V. BRIGIDA[1], V.I. SOROKIN[2], S.N. KOVALCHUK[1], K.S. PANTIUKH[3], I.V. RUKIN[3, 4], K.A. ROZHIN[2]

[1]*Center for Experimental Embryology and Reproductive Biotechnologies,* Federal Agency of Scientific Organizations, 12/4, ul. Kostyakova, Moscow, 127422 Russia, e-mail brigida_86@mail.ru, s.n.kovalchuk@mial.ru;
[2]*Orenburg State Agrarian University,* 18, ul. Cheluskintsev, Orenburg, 460014, Russia, e-mail vis5256@yandex.ru, info-ceerb@mail.ru;
[3]*ZAO Genoanalitika,* 77/vl. 1, ul. Leninskie gori, Moscow, 119991 Russia, e-mail pantiukh@i-gene.ru;
[4]*Lomonosov Moscow State University,* 1, ul. Leninskie gori, Moscow, 119991 Russia, e-mail ruki.n.ilya@gmail.com (✉ corresponding author)
ORCID:
Brigida A.V. orcid.org/0000-0002-0139-8087
Sorokin V.I. orcid.org/0000-0003-0057-2976
Kovalchuk S.N. orcid.org/0000-0002-5029-0750
Pantiukh K.S. orcid.org/0000-0002-2595-0673
Rukin I.V. orcid.org/0000-0003-4093-3254
Rozhin K.A. orcid.org/0000-0002-4034-7434
The authors declare no conflict of interests
Acknowledgements:
Authors thank employees of the farm Plemzavod Dimitrovsky (Orenburg region) for their assistance in the research. Supported financially by Ministry of Education and Science of the Russian Federation for applied projects, Subsidy Agreement № 14.579.21.0147, UID RFMEFI57917X0147
Received December 25, 2017

Abstract

The main problem restricting the wide use of reproductive biotechnology in animal husbandry is insufficiently developed methods for selection of donor cows for embryo transfer. The objective reason is the variability of the ovarian response to gonadotropins injections. Until now, there is no reliable information about possibility of forecasting the embryo productivity of donor cows before gonadotropin stimulation, which affects substantively the economic feasibility of embryo transfer as a method of accelerated cattle reproduction. We have applied the post-pressing analysis of ovaries echograms for forecasting the embryo productivity of donor cows on the basis of comparisons of quantitative and qualitative indicators of ovaries after induced superovulation and its' echographic characteristics. We carried out morphofunctional study of ovaries in donor cows (n = 30) on day 10 of estrous cycle, before artificial insemination (estrus) and on day 7 of the inducted estrous cycle, immediately before the extraction of embryos using data on post-pressing ovarian morphometry. Animals were divided into three groups (I, II и III, n = 10 for each group) with yellow body length of 2.5 cm, 1.5-2.5 cm and 1.5 cm, respectively. Echographic visualization of the ovaries was performed using endorectal ultrasonography. Polyovulatory response of ovaries was induced with FSH-super (Russia) injected eight times, with 12 h interval, at decreasing doses. The embryos were recovered on day 7 after artificial insemination. Optimal criteria for predicting the polyovulatory response of ovaries and the quantity of embryos were determined on the basis of the ovarian morphometry. Statistically significant differences with the control were assessed by the Student's t-test. It was found that the average areas of the ovaries on the echograms were 7.9±0.94, 5.7±0.78 and 3.5±0.06 cm^2 for group I, group II and group III, respectively. The area of the yellow body in group I averaged 4.5±1.21 cm^2, was 2.08 cm^2 higher ($P \leq 0.05$) than in group II, and exceeded the corresponding parameter in group III by 3.43 cm^2 ($P \leq 0.05$). A comparative evaluation of the ratio of the yellow body areas to the ovaries area of each animal and on average along the groups showed that in group I with the ratio of 57.1±3.01 % the number of yellow bodies was 11.6±1.26 and the average yield of the embryos was 9.3±1.23 per animal. In group II with the ratio of the areas of yellow bodies and ovaries of 42.1±2.9 % the number of yellow bodies before embryos recovery was 5.7±1.24, and 4.6±1.01 embryos were recovered per procedure. The lowest embryo recovery (less than one embryo per procedure) was observed in group III with the relative area of yellow bodies of 30.2±2.56 % and the average number of the yellow bodies of 1.8±0.18. Comparison of the size of the yellow bodies before the induction of polyovulation and data characterizing the efficiency of induction of superovulation and recovery of embryos showed that in animals with a ratio of the areas of the yellow body and ovary more than 50 %, high response can be obtained resulting in 11.6±1.26 yellow bodies and 9.3±1.23 embryos per extraction.

Keywords: cow, ovaries, estrus, echography, morphometric parameters, polyovulation, embryos, embryo productivity

Increase of cattle livestock severely curtailed by extremely low frequency of tweens and long pregnancy. Though the female generative potential in cattle is enormous, i.e. hundreds of thousands of ova, with only a small portion used before the end of the economic use of the animal [1].

Embryo transfer biotechnology of accelerated reproduction opens huge opportunities in the realization of reproductive and biological potential of the animals with the given phenotypic and genotypic characteristics and for its subsequent reproduction in the least valuable recipient herds [2-6]. Despite the advances in technology, the search is relevant for methods to select donors and recipients, to induce superovulation with a higher output of embryos, to decrease the process efforts, and to lower animal stress [7-9].

Embryo transfer technology includes many stages [10]. The first requires the selection of donor cows [11] for the subsequent induction of the polyovulatory response of ovaries [12, 13]. The focus at this point is usually put on the breeding value and the gynaecological health of the animal, but not to the prediction of individual ovarian response on the gonadotropins injected. At the same time, the donor embryos selection is critical for the effectiveness of the following stages and economic expediency of the transfer procedure in general [14].

Variability of cows' ovaries response to exogenous gonadotropins leads to exclusion of approximately 30% of the treated animals from the donor group because of lack of the polyovulatory response to gonadotropins [15]. It is the high variability that makes the polyovulatory response unpredictable [16, 17] and serves as the main limitation of the practical application of the embryo transfer technology. Currently, no reliable information is still present on the possibility of predicting embryo productivity in donor cows prior to the introduction of gonadotropins that significantly affects the cost-effectiveness of such an expedited reproduction of the cattle.

Analysis of reported dataindicates that the effectiveness of the response of the ovaries of embryo donor cows to the exogenous gonadotropin is influenced by many factors [18]. The major are the breed and physiological state of the animals [19, 20], especially metabolic [21] and hormonal status [22-25], genetic predisposition [26-30], ecological and climatic conditions [31, 32]. Given the multiplicity of reasons, the ovaries are the most convenient object to predict embryo productivity of a cow [33-35].

Ultrasonograhy (US) is considered as a promising method to evaluate the ovarian morphofunctional state for embryo transfer [36]. The examination is easy to reproduce in a production environment, it is minimally invasive, and allows one to get the information, complete and objective enough, in real time.

We were the first to use the postpressing morphometry of the ovarian structures for prediction of superovulatory response prior to the introduction of gonadotropins and therefore provided a fast method of selection of cows for embryos recovery. The method testing showed its perspectivity for the cows' embryo productivity estimation.

The aim of the study was to compare the ovarian US study data, embryo productivity, and embryo recovery to develop ways to assess the expected superovulatory reaction and identify the promising donors of embryos.

Techniques. A study is made on the cows from breed Kazakh whiteheaded (LLC Plemzavod Dimitrovskij, Ileksky region, Orenburg Province). As donors, clinically healthy animals were selected (n = 30) with no signs of metabolic disorders (obesity, dystrophy, etc.) with data on the origin of at least three rows of ancestors, with a strong constitution and the exterior score no less than 8

points, body weighing not below the standard of the breed, aged 3-6 calvings, with certainty of origin (by blood groups), with the first manifestations of estrus within 50 days after calving, easy calving and uncomplicated postpartum period, insemination index of 1.2-1.5, and normal condition of the uterus and ovaries (according to rectogenital US as a real-time visualization, and to the endorectal palpation of reproductive organs). All cows have been screened for infectious diseases (brucellosis, tuberculosis, viral respiratory infections, leukemia, trichomoniasis, including foot and mouth disease, etc.). According to the results, the animals were divided into three groups (n = 10 each) depending on the length of the yellow body (I, II and III, respectively from 2.5; 1.5-2.5; and less than 1.5 cm).

Morphofunctional evaluation of ovaries of the cows was conducted with the follow-up (considering also the postpressing ovaries morphometry) on day 10 of the sexual cycle prior to the introduction of follicular stimulators, prior to insemination if multiple mature dominant follicles were present, and at day 7 of the induced sexual cycle with the polyovulatory response of ovaries and the presence of multiple yellow bodies on the surface of the ovaries (directly before removing the embryos).

For endorektal sonogaphic ovarian visualization, the Tringa Linear (ESA OTES p. a., Italy) and Kaixin KX5200 (Xuzhou Kaixin Electronic Instrument Co., Ltd, China) ultrasound scanners were used with linear sensors (frequency 7.5 MHz); the black and white US images of reproductive organs were recorded. When postpressing morphometry, the US images were treated using the ImageJ graphical editor (National Institute of Health, USA). The length, width, and size of ovaries and yellow bodies as parameters of functional activity were determined. The values were calculated taking into account the length of the straight line, polyline, irregular circumference line, the area of geometric figure, and the angle of projection of the two sections. The projections of the structures at the US image were displayed schematically. Ovarian and yellow body areas were determined by the formula for the ellipsoid: $S = \pi Rr$, where R and r are, respectively, the major and minor semiaxis.

The sexual cycle of cows was induced by Estrofan (Bioveta, Czech Republic), a prostaglandin F2a drug, 2 ml per animal (one intramuscular injection), polyovulation — by the FSH-super (Russia) injected eight times with 12 hours intervals (morning-evening) in decreasing doses (50 AE, 3; 3; 2.5; 2.5; 2; 2; 1.5; 1.5 ml per animal).

For artificial insemination the fresh semen of outstanding bulls was used, tested for offspring quality and recognized as grades based on such factors as ease of calving and milk yield. Semen was consistent with RF Standard GOST 26030-2015 (main indices: the percentage of sperm with straight-forward movement is not less than 40%, their number in a single dose is 15 mln; the amount of doses for artificial insemination is not less than 0.2 cm^3; the viability of sperm at 38 °C is no less than 5 hours). Selection of sires and donor cows was in accordance with the plan of breeding work at the farm.

Embryos were harvested using the extrusion method (liquid injection in an isolated part of the uterine horn with a syringe connected to a 2-channel catheter previously entered in the uterus horn) on day 7 after insemination. Flexible cleaning catheters Neustadt/Aisch Rusch SN18 were used with four ports (Minitube, Germany). Fluid filtration and collection of embryos, the filters for cattle Em Guard (Minitube, Germany) were used.

To define the morphological condition of the embryos, they were examined under a Biolam microscope (JSC LOMO, Russia, × 60-100 magnification). The following was considered: the compliance with stages of development, the

shape of the zone pellucida and its integrity, the uniformity of crushing blastomeres, and the general condition of the cytoplasm.

When aggregating results, the medium (*M*) and their standard errors (± SEM) were calculated. The data was processed using Microsoft Excel 2010 and Statistica 6 (StatSoft, Inc., USA). Reliability of differences was assessed by *t*-test, deeming them statistically significant at $p \leq 0.05$.

Results. An example of echographic images of the cow ovarian morphometry is provided on Figure 1.

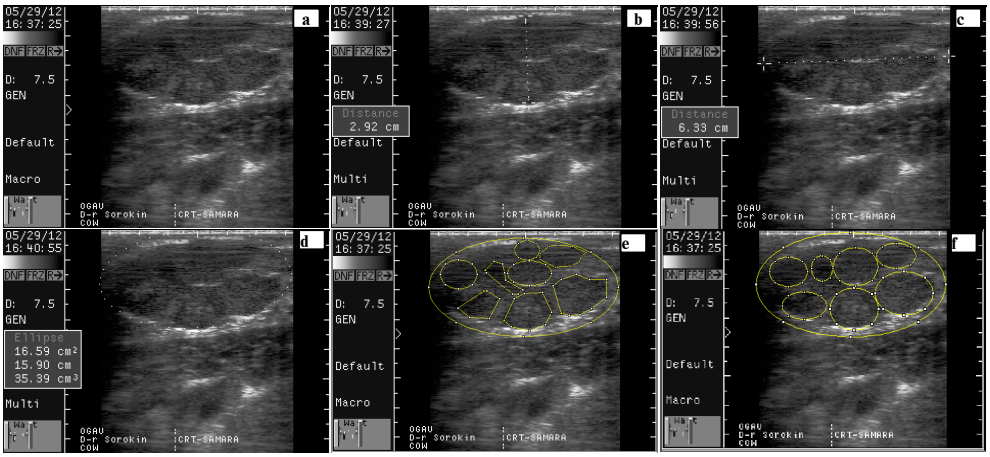


Fig 1. An example of US image processing of the embryo donor cow ovary No. 12764 (Kazakh white-headed breed) when assessing the polyovulatory response: a — original image; b— the height of the ovary, c — the width of the ovary, d — the circumference of the ovary, e — the border of the structures depicted expressed by irregular circlular polylines, f — the projection of the structures shown on the US image (sagittal scanning plane, b-f are image processing with graphics processing ImageJ editor).

For the prediction of ovarian response to gonadotropin drugs, we evaluated the embryo productivity of the donor cows by analyzing the morphometric indices of ovaries and yellow bodies with subsequent postpressing processing of US images of reproductive organs of the cows at day 10 of the sexual cycle. Measuring the length and width of the ovaries and yellow bodies allowed us to calculate their area, the ratio of the size of the yellow body and the ovary.

The results of the morphometry of the linear dimensions and area of ovaries and yellow bodies in donor cows are presented in Table 1.

1. Morphometric indices of ovaries and yellow body in embryo donor cows at day 10 of sexual cycle without polyovulation induction (*M*±SEM, Kazakh white-headed breed, LLC Plemzavod Dimitrovskij, Orenburg Province, 2017)

Average for group	Ovaries			Yellow body		
	length, cm	width, cm	area, cm^2	length, cm	width, cm	area, cm^2
I (n = 10)	4.0±0.09	2.5±0.11	7.9±0.94	2.7±0.02	2.1±0.14	4.5±1.21
II (n = 10)	3.2±0.04	2.3±0.34	5.7±0.78*	2.0±0.90	1.5±0.08	2.4±0.24*
III (n = 10)	2.4±0.12	1.8±0.04	3.5±0.06**	1.4±0.08	1.0±0.00	1.1±0.25*

N o t e. Groups I, II, and III are animals with the yellow body length, respectively, up to 2.5, 1.5-2.5, and less than 1.5 cm.

*, * Differences in ovarian area in groups II and III as compared to group I are statistically significant at $p \leq 0.05$ and $p \leq 0.001$, respectively.

The largest ovarian area was in group I, the lowest was in group III. This rate in cows of group I exceeded that of individuals from group II (2.2 cm^2, $p \leq 0.05$) and group III (4.3 cm^2, $p \leq 0.001$). The average size of yellow bodies also varies by group. Thus, in the group I the value was higher than in the group II by 2.08 cm^2 ($p \leq 0.05$) and group III by 3.43 cm^2 ($p \leq 0.05$).

2. Areas of ovaries and yellow body in embryo donor cows at day 10 of sexual cycle without polyovulation induction (*M*± SEM, Kazakh white-headed breed, LLC Plemzavod Dimitrovskij, Orenburg Province, 2017)

Average for group	Area, cm^2		Area of the yellow body of the ovary area, %
	ovary	yellow body	
I (n = 10)	7.9±0.94	4.5±1.21	57.1±3.01**
II (n = 10)	5.7±0.78	2.4±0.24	42.1±2.95*
III (n = 10)	3.5±0.06	1.1±0.25	30.2±2.56

Note. Groups I, II, and III are animals with the yellow body length, respectively, up to 2.5, 1.5-2.5, and less than 1.5 cm.
*, ** Differences of ratios and areas of yellow bodies and ovaries in groups I and II as compared to group III are statistically significant at $p \le 0.01$ and $p \le 0.001$, respectively.

We compared the ratio of areas of yellow bodies and the ovaries of each animal and the average for the groups (Table 2). This estimate, characterizing the size of the functionally active yellow body before induction of polyovulation, shows the size of luteal tissue being the determinator of the size of the ovaries. In animals with the smallest size of yellow body its lutein structures amounted to 30.2% of the total area of the ovaries that is reliably less than the relevant indices in animals in groups II and III by 12.0 ($p \le 0.01$) and 27.0 cm^2 ($p \le 0.001$), respectively.

Further ovarian US visualization conducted during estrus before insemination (0 day of sexual cycle) and before removing embryos at day 7 after insemination. The postpressing US images received were processed to determine the changes in the ovaries in response to FSH stimulation and exogenous luteolytic drugs of the F2a prostaglandin type. In doing so, we considered also the ovaries morphometry and the quantitative and qualitative composition of follicles and yellow bodies, reflecting the polyovulatory response of ovaries (Fig. 2).

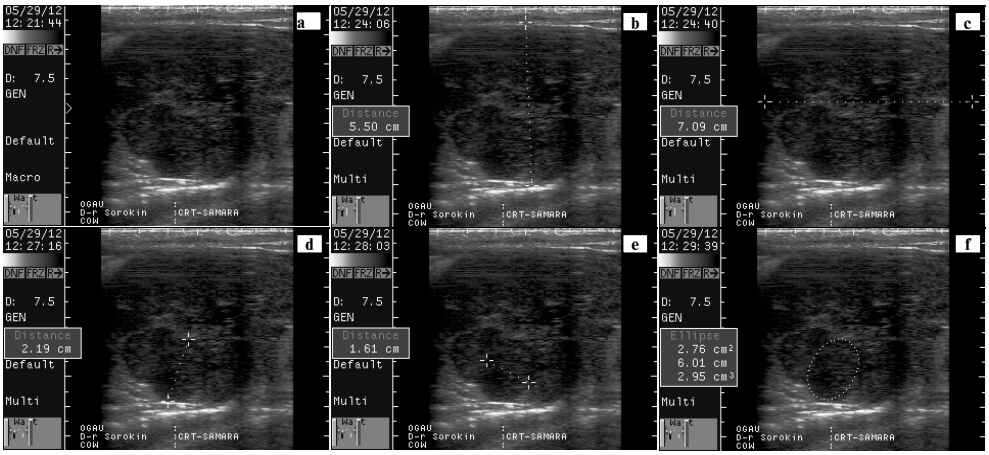


Fig. 2. The US display of polyovulatory response of ovaries of embryo donor cows (Kazakh white-headed breed) with a length of yellow body of 1.5-2.5 cm: a — the original image; b — the height of an ovary; c — the width of the ovary; d — the height of the yellow body; e — the width of the yellow body; f — the circumference of the yellow body (sagittal plane scan, image processing with graphics processing ImageJ editor).

The results of the rectopalpatory ovaries exam, as well as the data obtained by ultrasound scanning and postpressing US processing, demonstrate that the polyovulatory response of ovaries and conception oocyte fertilization depend on the quality of the yellow body in the middle of L-phase (during the period prior to the beginning of the gonadotrophic stimulation of the donor cows) (Table 3). In groups I and II the number of yellow bodies and resultant embryos differed nearly twice, while in the group III were almost an order of magnitude lower (see Table 3).

We used the postpressing US images processing for the morphometric evaluation of ovaries and structures involved in folliculogenesis and luteogenesis in

cows. By the report of V. Kayacik et. al. [36], postpressing US images processing indicates high efficiency of echography method in the morphometric studies.

3. Stimulation of polyovulation and embryo productivity in embryo donor cows, depending on the ratio of the yellow body and ovary area (*M*±SEM, Kazakh white-headed breed, LLC Plemzavod Dimitrovskij, Orenburg Province, 2017)

Average for group	Area of the yellow body of the ovary area, %	Number of	
		yellow bodies	embryos
I (n = 10)	57.1±3.01	11.6±1.26	9.3±1.23
II (n = 10)	42.1±2.95	5.7±1.24	4.6±1.01
III (n = 10)	30.2±2.56	1.8±0.18	0.5±0.02

Note. Groups I, II, and III are animals with the yellow body length, respectively, up to 2.5, 1.5-2.5, and less than 1.5 cm.

The comparison of data on the size of yellow bodies before induction of polyovulation and the results characterizing the efficiency of superovulation and embryos recovery show that cows with the more than 50% ratio of the yellow body to ovary area are appropriate for ovarian induction to get the number of yellow bodies 11.6±1.26 on average and embryos 9.3±1.23 per extraction. Note, the recovery of average 5-6 embryos per one handled animal is usually reported [3, 8, 15, 34].

Thus, the US ovarian morphometry is highly informative and allows breeder to objectively evaluate ovarian functional status and predict the embryo productivity in cattle. Processing of US images in ImageJ editor significantly improves the performance of US imaging. It is shown that the response to the FSH stimulation and embryo recovery depend on the size of the yellow body in the potential donors at day 10 of sexual cycle (without the induction of polyovulation). This can be used as a criterion in the selection of animals to a group of potential donors of embryos. So, when the ratio of the yellow body and the ovary area is more than 50%, the numbers of ovulated follicles and quality embryos are, respectively, 11.6±1.26 and 9.3±1.23 per cow.

REFERENCES

1. Anzorov V.A., Shiriev V.M., Sergeev N.I., Titov V.A., Nasibov F.N., Baitlesov E.U. Comparative characteristic and estimation of viability in embryos obtained from healthy and problem cows-donors with various level of dairy productivity. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2005, 6: 37-41 (in Russ.).
2. Kononov V.P., Chernykh V.Ya. *Biotekhnika reproduktsii v molochnom skotovodstve* [Biotechnics of reproductions in dairy cattle breeding]. Moscow, 2009 (in Russ.).
3. Baruselli P.S., Vieira1 L.M., Batista E.O.S., Ferreira R.M., Sales J.N.S., Gimenes L.U., Torres-Junior J.R.S., Martins C.M., SáFilho M.F., Bo G.A. Updates on embryo production strategies. *Anim. Reprod.*, 2015, 12(3): 375-382.
4. Hopper R.M. *Bovine reproduction.* Wiley Blackwell, 2015: 696-733.
5. Kugonza D.R., Kayitesi A., Semahoro F., Ingabire D., Manzi M., Hirwa C.A., Gahakwa D. Factors affecting suitability of surrogate dams for embryo transfer in cattle. *Journal of Animal Science Advances*, 2013, 3(4): 203-210 (doi: 10.5455/jasa.20130430123202).
6. Vasconcelos J.L.M., Dementrio D.G.B., Santos R.M., Chiari J.R., Rodrigues C.A., Filho O.G. Factors potentially affecting fertility of lactating dairy cow recipients. *Therlogenology*, 2006, 65: 192-200 (doi: 10.1016/j.theriogenology.2005.09.030).
7. Ptashinskaya M. *Kratkoe rukovodstvo po reproduktsii zhivotnykh* [A brief guide to animal reproduction]. Intervet International BV, 2009 (in Russ.).
8. Ernst L.K., Varnavskii A.N. *Reproduktsiya zhivotnykh* [Animal reproduction]. Moscow, 2007 (in Russ.).
9. Mapletoft J., Garcia Guerra A., Dias F.C.F., Singh J., Adams G.P. In vitro *and* in vivo embryo production in cattle superstimulated with FSH for 7 days. *Anim. Reprod.,* 2015, 12 (3): 758-388.
10. Petroman I., Pacala N., Petroman C., Bencsik I., Orboi M.D., Dronca D., Băneş A. Utilization of gestagen hormones and pituitary FSH extracts in inducing the superovulation at embryo donor cows. *Journal of Food Agriculture & Environment,* 2009, 7(2): 193-195.
11. Voronin E.S., Petrov A.M., Serykh M.M., Devrishov D.A. *Immunologiya* [Immunology]. Moscow, 2002 (in Russ.).
12. Kosovskii G.Yu., Popov D.V., Brigida A.V. *Veterinariya i kormlenie*, 2016, 5: 29-31 (in Russ.).
13. Kosovskii G.Yu., Popov D.V., Brigida A.V., Volkolupov G.V. *Izvestiya Nizhnevolzhskogo agrouniversitetskogo kompleksa: nauka i vysshee professional'noe obrazovanie*, 2015, 3(39): 106-108 (in Russ.).
14. Sanchez Z., Lammoglia M.A., Alarcon M.A., Romero J.J., Galina C.S. Is the production of

embryos in small-scale farming an economically feasible enterprise? *Reprod. Domest. Anim.*, 2015, 50(4): 574-579 (doi: 10.1111/rda.12526).
15. Kosovskii G.Yu., Popov D.V., Brigida A.V., Volkolupov G.V. *Izvestiya Nizhnevolzhskogo agrouniversitetskogo kompleksa: nauka i vysshee professional'noe obrazovanie*, 2015, 2(38): 148-152 (in Russ.).
16. Liang A., Salzano A., D'Esposito M., Comin A., Montillo M., Yang L., Campanile G., Gasparrini B. Anti-Mullerian hormone (AMH) concentration in follicular fluid and mRNA expression of AMH receptor in granulose cell as predictive markers of good buffalo (*Bubalus bubalis*) donors. *Theriogenology*, 2016, 86(4): 963-970 (doi: 10.1016/j.theriogenology.2016.03.020).
17. Rao M.M., Uma Mahesh Y., Misra A.K. Evaluation of ovarian response and embryo production pattern in Ongole cows. *Indian Journal of Animal Science*, 2010, 80(10): 973-975.
18. Baruselli P.S., Batista E.O.S., Vieira L.M., Sales J.N.S., Gimenes L.U., Ferreira R.M. Intrinsic and extrinsic factors that influence ovarian environment and efficiency of reproduction in cattle. *Anim. Reprod.*, 2017, 14(1): 48-60 (doi: 10.21451/1984-3143-AR907).
19. Ali M.S., Khandoker M.A.M.Y., Afroz M.A., Bhuiyan A.K.F.H. Ovarian response to different dose levels of follicle stimulating hormone (FSH) in different genotypes of Bangladeshi cattle. *Asian-Australasian Journal of Animal Science,* 2012, 25(1): 52-58 (doi: 10.5713/ajas.2011.11167).
20. Villaseron Gonzaleza F., de la Torre Sanchez J.F., Martinez Velazques G., Gallardo H.A., Reynozo S.P., Fránquez J.A.P., Sojo R.P., Bermúdez M.M. Characterization of the ovarian response to superovulation in Creole Coreno cattle using reduced doses of FSH. *Revista Mexicana De Ciencias Pecuarias*, 2017, 8(3): 225-232.
21. Sartori R., Monteiro P.L.J. Jr., Wiltbank M.C. Endocrine and metabolic differences between *Bos taurus* and *Bos indicus* cows and implications for reproductive management. *Anim. Reprod.*, 2016, 13(3): 168-181 (doi: 10.21451/1984-3143-AR868).
22. Aziz R.L.A., Khalil A.A.Y., Abdel-Wahab A., Hassan N.Y., Abdel-Hamied E., Kasimanickam R. Relationship among circulating anti-Mullerian hormone, insulin like growth factor 1, cadmium and superovulatory response in dairy cows. *Theriogenology*, 2017, 100: 72-79 (doi: 10.1016/j.theriogenology.2017.06.007).
23. Baruselli P.S., Batista E.O.S., Vieira L.M., Souza A.H. Relationship between follicle population, AMH concentration and fertility in cattle. *Anim. Reprod.*, 2015, 12(3): 487-497.
24. Mossa F., Jimenez-Krassel F., Scheetz D., Weber-Nielsen M., Evans A.C.O., Ireland J.J. Anti-Mullerian Hormont (AMH) and fertility management in agricultural species. *Reproduction,* 2017, 154(1): R1-R11 (doi: 10.1530/REP-17-0104).
25. Redhead A.K., Siew N., Lambie N., Carnarvon D., Ramgattie R., Knights M. The relationship between circulating conception of AMH and LH content in the follicle stimulating hormone (FSH) preparations on follicular growth and ovulatory response to superovulation in water buffaloes. *Animal Reproduction Science*, 2018, 188: 66-73 (doi: 10.1016/j.anireprosci.2017.11.010).
26. Yang W.C., Tang K.Q., Li S.J., Yang L. Association analysis between variants in bovine progesterone receptor gene and superovulation traits in Chinese Holstein cows. *Reprod. Domest. Anim.*, 2011, 46(6): 1029-1034 (doi: 10.1111/j.1439-0531.2011.01780.x).
27. Yang W.C., Li S.J., Tang K.Q., Hua G.H., Zhang C.Y., Yu J.N., Han L., Yang L.G. Polymorphisms in 5′ upstream region of the FSH receptor gene, and their association with superovulation traits in Chinese Holstein cows. *Animal Reproduction Science*, 2010, 119(3-4): 172-177 (doi: 10.1016/j.anireprosci.2010.02.004).
28. Yang W.C., Tang K.Q., Li S.J., Chao L.M., Yang L.M. Polymorphisms of the bovine luteinizing hormone/choriogonadotropin receptor (*LHCGR*) gene and its association with superovulation traits. *Molecular Biology Reports,* 2012, 39(3): 2481-2487 (doi: 10.1007/s11033-011-0999-4).
29. Yang W.C. Li S.J., Xie Y.H., Tang K.Q., Hua G.H., Zhang C., Yang L.G. Two novel SNPs of the type I gonadotropin releasing hormone receptor gene and their associations with superovulation traits in Chinese Holstein cows. *Livestock Science*, 2011, 136: 164-168 (doi: 10.1016/j.livsci.2010.08.012).
30. Yu Y., Pang Y., Zhao H., Xu X., Wu Z., An L., Tian J. Association of a missense mutation in the luteinizing hormone/choriogonadotropin receptor gene (*LHCGR*) with superovulation trains in Chinese Holstein heifers. *Journal of Animal Science and Biotechnology*, 2012, 3(1): 35 (doi: 10.1186/2049-1891-3-35).
31. Bezdicek J., Makarevich A., Stadnik A., Kubovičová E., Louda F., Hegedüšová Z., Holásek R., Ducháček J., Stupka R. Analysis of factors affecting the quantity and quality of embryo production in superovulated cows. *Zuchtungskunde*, 2015, 87(4): 249-264.
32. Mehmood M.U., Mehmood S., Riaz A., Ahmad N., Sattar A. Superovulatory response in Summer anestrus Buffaloes and cattle treated with estrus synchronization protocol. *Journal of Animal and Plant Science*, 2012, 22(4): 888-893.
33. Glazko T.T., Kosovskii, G.Yu., Popov D.V., Brigida A.V. *Veterinariya Kubani*, 2015, 6: 9-11 (in Russ.).
34. Kosovskii, G.Yu., Popov D.V., Brigida A.V. *Veterinariya Kubani*, 2015, 5: 15-17 (in Russ.).
35. Tomac J., Cekinovich D., Arapovic J. Biology of the corpus luteum. *Periodicum Biologorum*, 2011, 113(1): 43-49.
36. Kayacik V., Salmanoglu M.R., Polat B., Ozluer A. Evaluations of the corpus luteum size throughout the cycle by ultrasonography and progesterone assay in cows. *Turk. J. Vet Anim Sci.*, 2005, 29: 1311-1316.

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 762-768
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Aquaculture

UDC 639.3.034.2

doi: 10.15389/agrobiology.2018.4.762eng
doi: 10.15389/agrobiology.2018.4.762rus

REPRODUCTION OF RUSSIAN STURGEON (*Acipenser gueldenstaedtii*) VIABLE JUVENILES USING CRYOPRESERVED SPERM AND BEHAVIORAL REACTIONS OF THE CRYO-PROGENY

A.A. KRASILNIKOVA[1], A.M. TIKHOMIROV[2]

[1]*Federal Research Centre Southern Scientific Center RAS,* Federal Agency of Scientific Organizations, 41, ul. Chekhova, Rostov-on-Don, 344006 Russia, e-mail alexandra.kras@yandex.ru (✉ corresponding author);
[2]*Astrakhan State Technical University,* 16, ul. Tatishcheva, Astrakhan, 414056 Russia, e-mail tikhomirov41@mail.ru
ORCID:
Krasilnikova A.A. orcid.org/0000-0002-7186-8849 Tikhomirov A.M. orcid.org/0000-0002-4087-818X
The authors declare no conflict of interests
Acknowledgements:
This work was carried out using devices of Unique Scientific Installation Module Complex (USI-MC, SSC RAS) and Bio-resource Collection of Rare and Endangered Fish Species (SSC RAS) № 73602.
Supported financially by Federal Program of Russian State Academies for 2013-2020, State Registration No 01201354245 (0256-2018-0007), and by the grant of the President of the Russian Federation (MK-115.2017.11)
Received June 9, 2016

Abstract

Cryopreservation of male reproductive cells is an important issue of genetic biodiversity conservation strategy and the development of fisheries and aquaculture. The use of cryopreserved semen in artificial reproduction and aquaculture will provide genetically diverse progeny, reduce area and the cost of maintaining male fishes, and, thereby, will allow rise in female herd abundance. Cryopreserved sperm can be used at any time, without risk of untimely maturing or improper quality. The data on the low-temperature preservation indicates that the long-term storage in liquid nitrogen does not significantly affect the safety of cells after freezing and thawing. The viability of cryopreserved sperm is usually confirmed by laboratory fertilization of caviar, without further observations of the obtained juveniles. This paper reports for the first time the behavioral response of pre-larvae, larvae, and juveniles of Russian sturgeon (*Acipenser gueldenstaedtii* von Brandt & Ratzeburg 1833), obtained with the use of frozen-thawed sperm, and shows physiological fullness of the resulting progeny. The aim of this work was to compare quality of Russian sturgeon offspring as influenced by cryopreserved and native semen. In using frozen-thawed sperm stored 2 years in liquid nitrogen, the conception was 50 % compared to 80 % in the control insemination with native semen. The mortality rate for the whole period was 7 and 5 % in the test and the control groups, respectively. The obtained cryo-progeny was viable. One-day old pre-larvae, 8-day old larvae and 15-day old juveniles of the test group were superior in size and weight as compared to the control group. Behavioral responses of the offspring were evaluated in «open field» test which was carried out individually by placing an individual (pre-larvae, larvae, juveniles) in a special installation with coordinate grid. The pre-larvae activity was somewhat, but not significantly, higher in cryo-progeny. No differences were found between 8-day old larvae in the response to irritants, except bright light ($p \leq 0.05$). In 15-day old juveniles, the response was adequate in both groups. Basal activity and reactivity differed significantly ($p \leq 0.05$) in both groups. So, cryopreserved sperm led to some morphometric advantage in the progeny compared to the individuals produced by conventional methods, and some advantages were found in the response to irritants, however, on the whole, the differences were not significant. A small alteration may be due to the difference in frozen cell subpopulations. Thus, frozen semen contributes to young fish vitality and may be recommended for artificial reproduction and aquaculture.

Keywords: sturgeons, Russian sturgeon, *Acipenser gueldenstaedtii*, sperm, cryopreservation, cryo-progeny, pre-larvae, larvae, juveniles, behavior

Today, preservation of a genetic pool of rare and endangered populations and species of fish, specifically those that are of practical interest for increase of catch in natural water bodies or for their introduction to the aquaculture as promising fish farming objects is particularly relevant [1-3[. One of the main sources of generation and maintenance of stock of rare and endangered and commercially

valuable fish species is their artificial reproduction [4, 5]. However, the approach of fisheries to generation of broodstocks is simplified due to deficit of producers. The usage of closely related pairs for breeding is fraught with inbreeding and loss of natural genetic polymorphism resulting in significant reduction of adaptive potential of the population [6-8].

The cryogenic technologies are deemed strategically important and anti-crisis for preservation of biological diversity of fish species [9, 10]. The advancement in cryopreservation technologies will expand the fields of application of cryogenic technologies in fishery and aquaculture, will enable maintaining genetic diversity of production broodstocks, will stabilize their reproduction and thus will stimulate stable fishing, and will lay the groundwork for growth of production of fish and other aquatic organisms at aquaculture farms [11-13]. The usage of cryopreserved semen will provide genetically diverse young fish, reduce the costs and surface areas of male fish maintenance. Consequently, the quantities of females in the broodstock will be increased [14-16]. The usage of cryopreserved semen is possible at any season, which eliminates the risk of untimely maturing of males or the risk of obtaining low quality ejaculate from them [17, 18].

The accumulated biological object cryopreservation data point to the fact that protracted storage of genetic biomaterial in a cryobank does not have a significant impact on cell preservation [19-21]. Furthermore, the viability of cryopreserved semen is usually confirmed by fish ovum fertilization in laboratory conditions [22-24], but no further observation of young fish is conducted.

In this work we have analyzed for the first time the behavioral response of sac fry, fish larvae and young Russian sturgeon specimens (*Acipenser gueldenstaedtii* von Brandt & Ratzeburg 1833) obtained when using cryopreserved semen, and have proven the physiological adequacy of developing specimen.

The goal of the work was to apply unfrozen semen in artificial reproduction of Russian sturgeon and to evaluate behavior of cryo-progeny.

Techniques. The work was performed during spawning campaign at the Alexandrovskiy sturgeon fishery (Astrakhan region). The roe was obtained from female Russian sturgeons (*Acipenser gueldenstaedtii*) weighing 20 kilos and 142 centimeters long, working fertility 229500 fish eggs. One part of the roe was fertilized by defrosted semen of Russian sturgeon stored in a cryobank for 2 years, the other was fertilized with native semen using standard fish farm technology (control). The removal of mucilage from fertilized roe was performed with tannin, 1 g per 5 liters of water [25]. The roe was incubated in an Osetr device (Russia) for 6 days. During incubation it was processed with organic coloring material (violet K) to avoid saprolegniosis [25]. The hatched sac fries were registered using item-by-item method. Sac fries were maintained in rectangular 250 l basins. The water in the basins was enriched with oxygen by applying a compressor. The young fish that was moved to active nutrition were fed with daphnia.

The morphometric parameters (weight and length) of sac fries (day 1 after hatching), fish larvae (day 8) that were moved to active feed and young fish (day 15) were recorded.

The behavior of fish reared by traditional technologies and of cryo-progeny were evaluated using the "open field" test [26, 27]. The test was conducted individually by placing the analyzed specimen (sac fry, fish larvae, and young fish) in a special device with an applied grid mesh. When an object is placed in the device for the "open sky" test, first, its approximate activity was defined for 3 min (approximate activity, units/min) registering the number of crossings of device coordinate lines by the specimen. The motor activity from minutes 4 to 7 was accepted as baseline activity (baseline activity, units/min). Light was the first irritant (illumination intensity 20 lux), which were turned on

during minute 7 of testing. The illumination intensity was measured with a light meter. During the first 30 s after the exposure the motor activity (the number of crossings of the coordinate grid) was determined as P1, units/min. Nine minutes after the start of the test the second irritant, a low frequency rectangular signal (20 hz), was used (P2, units/min). Bright light was turned on during the minute 11 (100 lux) (P3, units/min), and 13 minutes after the start of testing a high frequency rectangular signal was applied (300 hz) (P4, units/min). A vibroacoustic irritant (P5, units/min) was used at minute 15. Ten individuals were used from the test and control groups during three development phases, sac fry (day 1 after hatching), fish larvae transitioned to active feeding (day 8 after hatching) and young fish (day 15 after hatching).

Mean (M) absolute errors and mean square deviations (σ) were calculated in Microsoft Excel for the parameters. The statistical significance of differences was determined by Student t-criterion [28].

Results. The percentage of mobile cells in the native semen was 90%, in cryopreserved semen was 60%; the time of sperm cell mobility after semen defrosting was 14.2 min vs. 26.4 min in the native semen; the percentage of fertilization in the test group was 50% vs. 80% in the control group. During the rearing period the mortality rate in the test and control groups did not vary significantly and constituted 7 and 5%, respectively.

The dynamics of morphometric parameters of Russian sturgeon progeny (*Acipenser gueldenstaedtii* von Brandt & Ratzeburg 1833) upon fertilization with cryopreserved (test group) **and native** (control group) **semen** ($M\pm\sigma$)

Development stage	Масса, мг		Длина, мм	
	control	test	control	test
Sac fry (day 1)	18.9±0.28	20.5±0.22*	12.3±0.21	14.2±0.2*
Fish larvae transitioned to active feeding (day 8)	28.2±0.55	35.0±0.30*	20.7±0.21	21.4±0.34
Young fish (day 15)	43.8±0.33	47.5±0.33*	24.0±0.15	25.2±0.13*

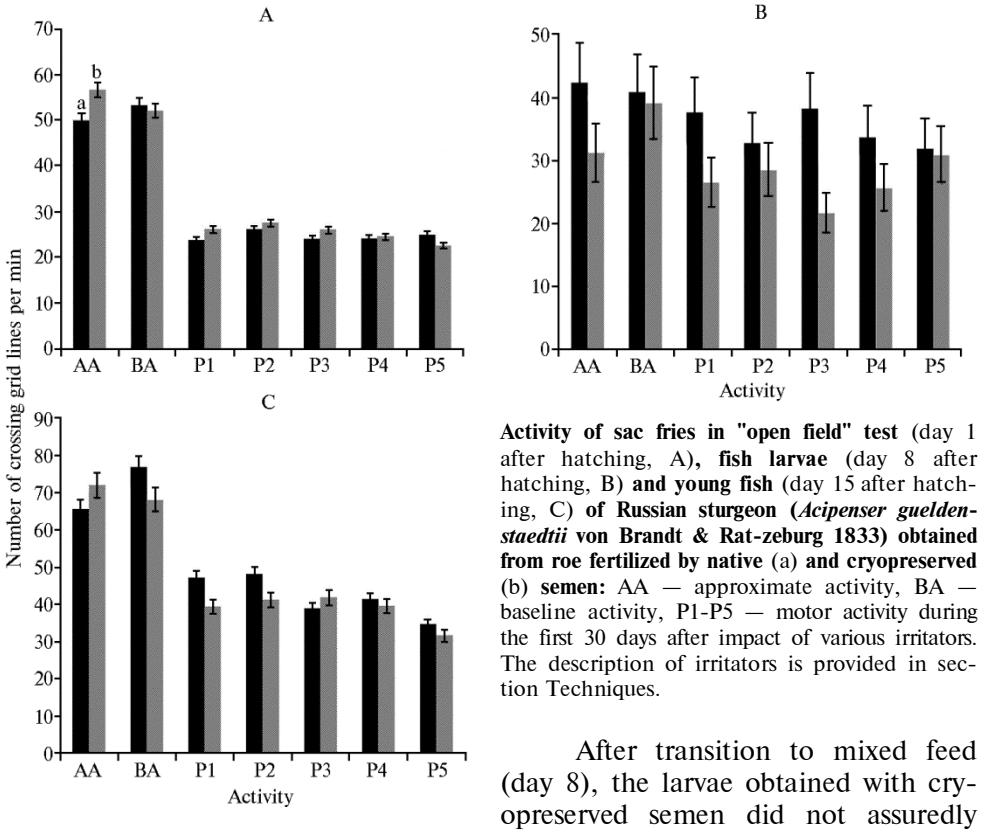
N o t e. The differences with the control group are statistically significant at $p \leq 0.001$.

According to sac fry (day 1) measurement results, the individuals from the test group surpassed the control group (Table) in morphometric parameters. The statistically significant differences were determined ($p \leq 0.001$) both for weight and length of analyzed fish. Comparing fish larvae that transitioned to active feeding (day 8) identified significant differences ($p \leq 0.001$) between test group weight and control group weight; however, length of larvae did not differ. The young fish (day 15) in the test group retained a tendency of surpassing the control group specimens in weight and length.

Any organism finds itself in a new environment begins to display increased motor activity and tries to get oriented. According to the literature, after sac fry hatch they develop a receptor complex; however, without relevant information from the environment, the medulla cannot develop properly [29]. The key system analyzers are concentrated in the medulla, which are responsible for the interconnection of the fry with the environment (lateral line systems, auditory organs, taste receptors), and centers of neuromotor response, feeding and breathing [30]. In this connection we evaluated the quality of fish obtained using cryopreserved semen and native semen by comparing their behavior.

In the "open field" test the sac fries from the control group displayed low orientation activity and concealment behavior changing into slow movements in the test device (see Fig.). The sac fries from the test group were more active and displayed classical response when adapting to unfamiliar environment, which is characteristic of specimen of different evolution groups. No significant difference was observed between orientation and baseline activity either in the control group or in the test group. Response to various irritants by specimens in the

test and control groups did not differ statistically either.



Activity of sac fries in "open field" test (day 1 after hatching, A)**, fish larvae** (day 8 after hatching, B) **and young fish** (day 15 after hatching, C) **of Russian sturgeon (*Acipenser guldenstaedtii* von Brandt & Rat-zeburg 1833) obtained from roe fertilized by native** (a) **and cryopreserved** (b) **semen:** AA — approximate activity, BA — baseline activity, P1-P5 — motor activity during the first 30 days after impact of various irritators. The description of irritators is provided in section Techniques.

After transition to mixed feed (day 8), the larvae obtained with cryopreserved semen did not assuredly differ from those in the control group in terms of central nervous system response with the exception of response to the third irritant, the bright light (100 lux) ($p \leq 0.05$). The specimens in the test group demonstrated concealment behavior, which was indicative of a faster formation of associative bonds in midbrain colliculus as compared to the larvae from the control group, which did not respond to this irritant and maintained activity at baseline level. On day 15 of development the specimens in both test groups responded properly to the irritants by displaying the classical concealment behavior. Significant differences ($p \leq 0.05$) both in the control group and in the test group were observed during analysis of response to irritants. This is indicative of the commencement of connectivity formation in medulla. In general, the groups show no significant differences.

Identical evaluation of behavior of young sturgeons obtained from semen preserved in a cryobank was previously conducted on starlet (*Acipenser ruthenus* Linnaeus, 1758) [31]. Based on the results of the "open field" test, the cryo-progeny did not differ from the young fish in the control group; however, when analyzing the dynamics of motor activity, the test group demonstrated a more active response to the proposed irritants. The authors note that this fact was important for adaptation upon release of young fish in the natural habitat.

Therefore, the young fish of Russian sturgeon species obtained with frozen and defrosted semen in terms of their morphometric parameters have an advantage as compared to the fish obtained by traditional technologies. The cryo-progeny is viable, and in terms of central nervous system reactivity and receptor complex in some cases surpass the control group. The insignificant difference between the development of sac fries, fish larvae and young fish in the test

and control groups can be the result of cryo-resilience of subpopulations of frozen cells. However, when analyzing the responses of fish obtained traditionally and those derived from defrosted semen, no differences have been observed between the control and test groups. Cryopreserved semen can be recommended for government fisheries to be used for fish-rearing of sturgeon species and for private aquaculture firms.

REFERENCES

1. Worm B., Barbier E.B., Beaumont N., Duffy J.E., Folke C., Halpern B.S., Jackson J.B.C., Lotze H.K., Micheli F., Palumbi S.R., Sala E., Selkoe K.A., Stachowicz J.J., Watson R. Impacts of biodiversity loss on ocean ecosystem services. *Science*, 2006, 314: 787-790 (doi: 10.1126/science.1132294)
2. Béné Ch., Arthur R., Norbury H., Allison E.H., Beveridge M., Bush S., Campling L., Leschen W., Little D., Squires D., Thilsted S.H., Troell M., Williams M. Contribution of fisheries and aquaculture to food security and poverty reduction: assessing the current evidence. *World Dev.*, 2016, 79: 177-196 (doi: 10.1016/j.worlddev.2015.11.007).
3. Ottinger M., Clauss K., Kuenzer C. Aquaculture: relevance, distribution, impacts and spatial assessments — a review. *Ocean Coast. Manage.*, 2016, 119: 244-266 (doi: 10.1016/j.ocecoaman.2015.10.015).
4. Bronzi P., Rosenthal H., Gessner J. Global sturgeon aquaculture production: an overview. *J. Appl. Ichthyol.*, 2011, 27: 169-175 (doi: 10.1111/j.1439-0426.2011.01757.x).
5. Safina C., Duckworth A. Fish conservation. In: *Encyclopedia of biodiversity (second edition)*. S.A. Levin (ed.). Princeton University, New Jersey, USA Academic Press, 2013: 443-455 (doi: 10.1016/B978-0-12-384719-5.00315-4).
6. Olesen I., Rosendal G.K., Tvedt M.W., Bryde M., Bentsen H.B. Access to and protection of aquaculture genetic resources — structures and strategies in Norwegian aquaculture. *Aquaculture*, 2007, 1: S47-S61 (doi: 10.1016/j.aquaculture.2007.08.012).
7. Gjedrem T., Robinson N., Rye M. The importance of selective breeding in aquaculture to meet future demands for animal protein: a review. *Aquaculture*, 2012, 350-353: 117-129 (doi: 10.1016/j.aquaculture.2012.04.008).
8. Duncan N.J., Sonesson A.K., Chavanne H. Principles of finfish broodstock management in aquaculture: control of reproduction and genetic improvement. In: *Advances in aquaculture hatchery technology. A volume in Woodhead Publishing Series in Food Science, Technology and Nutrition.* G. Allan, G. Burnell (eds.). Woodhead Publishing, Cambridge, 2013: 23-75 (doi: 10.1533/9780857097460.1.23).
9. Cabrita E., Sarasquete C., Martínez-Páramo S., Robles V., Beirão J., Pérez-Cerezales S., Herráez M.P. Cryopreservation of fish sperm: applications and perspectives (review). *J. Appl. Ichthyol.*, 2010, 26(5): 623-635 (doi: 10.1111/j.1439-0426.2010.01556.x).
10. Herráez P., Cabrita E., Robles V. Fish gamete and embryo cryopreservation: state of the art. In: *Aquaculture biotechnology.* G.L. Fletcher, M.L. Rise (eds.). Wiley-Blackwell, 2011: 303-317 (doi: 10.1002/9780470963159.ch20).
11. Tsvetkova L.I., Pronina N.D., Dokina O.B., Rekubratskii A.V., Parnyshkov V.A. *Voprosy rybolovstva*, 2012, 13(3-51): 538-545 (in Russ.).
12. Labbé C., Robles V., Herraez M.P. Cryopreservation of gametes for aquaculture and alternative cell sources for genome preservation. In: *Advances in aquaculture hatchery technology. A volume in Woodhead Publishing Series in Food Science, Technology and Nutrition.* G. Allan, G. Burnell (eds.). Woodhead Publishing, Cambridge, 2013: 76-116 (doi: 10.1533/9780857097460.1.76).
13. Kopeika J., Thornhill A., Khalaf Y. The effect of cryopreservation on the genome of gametes and embryos: Principles of cryobiology and critical appraisal of the evidence. *Hum. Reprod.*, 2015, 21(2): 209-227 (doi: 10.1093/humupd/dmu063).
14. Robles V., Cabrita E., Kohli V., Herráez M.P. Prospects and development in fish sperm and embryo cryopreservation. In: *Aquaculture research progress.* T.K. Nakamura (ed.). Nova Science Publishers, Inc., NY, 2009: 199-210.
15. Krasil'nikova A.A. *Sovershenstvovanie protsessa kriokonservatsii reproduktivnykh kletok samtsov ryb. Avtoreferat kandidatskoi dissertatsii* [Improvement of cryopreservation of male reproductive cells of fish. PhD Thesis]. Astrakhan', 2015 (in Russ.).
16. Sampath Kumar J.S., Betsy C.J. Cryopreservation of fish gametes and its role in enhancing aquaculture production. In: *Advances in marine and brackishwater aquaculture.* S. Perumal, A.R. Thirunavukkarasu, P. Pachiappan (eds.). Springer, New Delhi, 2015: 241-246 (doi: 10.1007/978-81-322-2271-2_22).
17. Tsai S., Lin C. Advantages and applications of cryopreservation in fisheries science. *Braz. Arch. Biol. Tech.*, 2012, 55(3): 425-434 (doi: 10.1590/S1516-89132012000300014).
18. Ponomareva E.N., Krasil'nikova A.A., Tikhomirov A.M., Firsova A.V. *Yug Rossii: ekologiya,*

razvitie, 2016, 11(1): 59-68 (doi: 10.18470/1992-1098-2016-1-59-68) (in Russ.).
19. Krasil'nikova A.A., Tikhomirov A.M. *Estestvennye nauki*, 2014, 2: 62-69 (in Russ.).
20. Krasilnikova A.A., Tikhomirov A.M. *Vestnik Astrakhanskogo gosudarstvennogo tekhnicheskogo universiteta. Seriya Rybnoe khozyaistvo*, 2014, 2: 72-78 (in Russ.).
21. Krasil'nikova A.A., Tikhomirov A.M. *Estestvennye nauki*, 2015, 3(52): 105-111 (in Russ.).
22. Aramli M.S., Nazari R.M. Motility and fertility of cryopreserved semen in Persian sturgeon, *Acipenser persicus*, stored for 30-60 min after thawing. *Cryobiology*, 2014, 69(3): 500-502 (doi: 10.1016/j.cryobiol.2014.10.006).
23. Dzyuba B., Boryshpolets S., Cosson J., Dzyuba V., Fedorov P., Saito T., Psenicka M., Linhart O., Rodina M. Motility and fertilization ability of sterlet *Acipenser ruthenus* testicular sperm after cryopreservation. *Cryobiology*, 2014, 69(2): 339-341 (doi: 10.1016/j.cryobiol.2014.07.008).
24. Nynca J., Dietrich G.J., Dobosz S., Grudniewska J., Ciereszko A. Effect of cryopreservation on sperm motility parameters and fertilizing ability of brown trout semen. *Aquaculture*, 2014, 433: 62-65 (doi: 10.1016/j.aquaculture.2014.05.037).
25. Chebanov M.S., Galich E.V., Chmyr' Yu.N. *Rukovodstvo po razvedeniyu i vyrashchivaniyu osetrovykh ryb* [Guide to breeding and growing sturgeon]. Moscow, 2004: 148 (in Russ.).
26. Vitvitskaya L.V., Nikonorov S.I., Tikhomirov A.M., Kozlov A.V. V sbornike: *Fundamental'nye nauki — narodnomu khozyaistvu* [In: Fundamental sciences for the national economy]. Moscow, 1990: 123-149 (in Russ.).
27. Nikonorov S.I., Vitvitskaya L.V. *Ekologo-geneticheskie problemy iskusstvennogo vosproizvodstva osetrovykh i lososevykh ryb* [Ecogenetic aspects of artificial reproduction of sturgeons and salmonids]. Moscow, 1993 (in Russ.).
28. Ivanter E.V., Korosov A.V. *Vvedenie v kolichestvennuyu biologiyu* [Introduction to quantitative biology]. Petrozavodsk, 2011 (in Russ.).
29. Shmal'gauzen I.I. *Osnovy sravnitel'noi anatomii* [Basic principles of comparative anatomy]. Moscow, 1947 (in Russ.).
30. Abdurakhmanov G.M., Zaitsev V.F., Lozhnichenko O.V., Fedorova N.N., Tikhonova E.YU., Lepilina I.N. *Razvitie zhiznenno vazhnykh organov osetrovykh v rannem ontogeneze* [The development of vital organs in early ontogeny of sturgeon]. Moscow, 2006 (in Russ.).
31. Ponomareva E.N., Nevalennyi A.N., Belaya M.M., Krasil'nikova A.A. *Vestnik Astrakhanskogo gosudarstvennogo tekhnicheskogo universiteta. Seriya Rybnoe khozyaistvo*, 2017, 4: 118-127 (doi: 10.24143/2073-5529-2017-4-118-127) (in Russ.).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 769-778
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 639.3.03:639.32

doi: 10.15389/agrobiology.2018.4.769eng
doi: 10.15389/agrobiology.2018.4.769rus

DEVELOPING THE BIOTECH METHOD FOR EFFECTIVE REPRODUCTION OF VALUABLE FISH SPECIES POPULATIONS

P.E. GARLOV, N.I. BELIK, N.B. RYBALOVA, B.S. BUGRIMOV

St. Petersburg State Agrarian University, 2, Peterburgskoe sh., St. Petersburg—Pushkin, 196601 Russia, e-mail garlov@mail.ru, biotech@spbgau, nikolaybelik@yandex.ru (✉ corresponding author), wba2009@list.ru
ORCID:
Garlov P.E. orcid.org/0000-0001-6041-3382
Belik N.I. orcid.org/0000-0001-7082-0619
Rybalova N.B. orcid.org/0000-0001-5545-6898
Bugrimov B.S. orcid.org/0000-0001-5955-9587
The authors declare no conflict of interests
Acknowledgements:
The authors would like to thank Yu.N. Bulyakulova (Glavrybvod Northwest branch) and D.A. Yanbukhtin (the Department of Aquatic Bioresources and Aquaculture, SPbSAU) for assistance in the work.
Received July 2, 2017

Abstract

Artificial reproduction of sturgeons and salmonids has shortcomings resulting in a return of commercially raised breeders. These disadvantages primarily are the catch of Atlantic salmon producers in spawning beds, which causes detriment of natural reproduction, and low survival rate of farmed juveniles in nature. In order to increase the efficiency of commercial fish reproduction, we suggest the new methods which are based on fish-specific adaptations during marine feeding period, providing the greatest population productivity and survival. Our objective was the development of a biotechnology for effective farm reproduction of salmon *Salmo salar* (Linne, 1758) and sturgeon *Acipenser stellatus* (Pallas, 1771). First, three main effects have been experimentally established for commercial fish culture in brackish seawater close to critical salinity (4-8 ‰): the highest survival rate, prolongation of high reproductive quality of breeders, and acceleration of juvenile development and growth. Physio-biochemical analysis showed the minimal losses of hemoglobin and serum protein in critical salinity medium, with maximum retention of salts in the blood and in ovarian fluids, apparently by optimizing water-salt balance of the body. The latter is achieved through optimal osmotic gradient between the inner and outer media close to critical salinity limits values. This energy-saving gradient ensures water-salt metabolism and homeostasis of the internal medium and thus the external medium (critical salinity) provides bio-stimulating effect that increases the body resistance. The maintenance of fish brood stocks in the critical range of seawater salinity (3.07-8 ‰) until puberty of producers is proposed by us as an effective method of reproducing populations of Sevruga and Baltic salmon. Then, the breeders naturally matured under specific range of the seawater salinity below a 3.06 ‰ threshold are used to obtain mature sex products. Fertilized eggs are incubated in fish-farm in river water where then larvae and juveniles grow. When recruits sign of readiness to migrate they are placed in seawater salinity 2.5-7 ‰ close to critical range and grow to viable stages. Results of comparative industrial tests of the new biotech reproduction of sturgeon and salmonids in sturgeon and salmon fish farms and in marine cages have shown the effectiveness and advantages of this method which allows preservation of high breeding quality Sevruga producers up to 100 %, and a 5-7-fold growth enhancement of young salmon. This new method can help solving the common problems of rare and endangered populations of commercial fish species restoration, which is in line with a fish farming trend of Conservation Aquaculture aimed at restoring natural environment.

Keywords: fish artificial reproduction, *Acipenser stellatus*, *Salmo salar*, Baltic population, *Rutilus rutilus caspicus*, fish farming, factory sturgeon and salmonids tech breeding, fish farming in brackish sea water

The populations of Atlantic salmon in the northwestern region of Russia and sturgeon species in the southern regions of Russia have lost their commercial value, and are maintained primarily on account of artificial farm reproduction. Unlike sturgeon hatcheries isolated from spawning beds, most salmon hatcheries are located in their aquatic areas, where they remove adult producers at the expense of natural reproduction. The fishing load of salmon hatcheries on pro-

ducers during breeding season and catching of valuable fish species during pres-pawning migration are the reason of progressive decline of their populations [1, 2]. The second reason is the low return of farm-reared producers (up to 2%), especially Atlantic salmon, in relation to the total quantities of released young fish, which is indicative of low survival rates and the need to improve the efficiency of biological technologies of farms. According to calculations, only large two-year old smolts need to be released (40 g and more) in the amounts no less than 150 thousand individuals, which is currently not being performed [2].

The biotechnological basis to solve the current issue of deficit of adult sturgeon and salmon producers [3, 4] is their long-term reservation [2, 5]. The farm technology of breeding Atlantic salmon has become obsolete, it does not envision the stages of formation and maintenance of rearing female stocks, and does not envision release and allocation of young fish in feeding basins [6]. The domestic experience and examples of implemented programs of preserving the populations of Atlantic salmon and sturgeon species in other countries point at the need of a more efficient artificial reproduction (along with restoration of the scope of natural reproduction), and increase of survival rate and heterogeneous variety of farm-reared young fish [2, 7, 8].

In this work we for the first time demonstrate a possibility of maintaining rearing female stocks, wide-scale breeding and progressive multiple acceleration of young Atlantic salmon growth in critical salinity water in case of perennial maritime cage culture fishery. As a result, the producers of starred sturgeon, salmon and roach have been successfully reserved and efficiency of maintenance and use of their rearing female stocks has been proven.

The goal of the research is to develop a complex of biotechnological measures for improvement of artificial reproduction of valuable commercial fish species.

Technoloqies. The experiments (commenced in 1976-1983 and completed in 2010-2013) were conducted at sturgeon fisheries located in Lower Volga regions (Aleksandrovskiy, Bertyulskiy, Ikryaninskiy and Kizyanskiy) and the Don River (Rogozhkinskiy, Vzmorye), and on Nevskiy salmon fishery (salmon hatchery, Leningrad Province, Vsevolozhskoy Region, village of Ostrovki) and at the premises of maritime cage culture fish farm Alkor-Farm LLC (Vyborgskiy Regions, village of Pribylovo, Klyuchevoye fishing ground). In the study starred sturgeon *Acipenser stellatus* (Pallas, 1771), Atlantic salmon *Salmo salar* (Linne, 1758) of Baltic population (Baltic salmon) and roach *Rutilus rutilus caspicus* (Jakowlew, 1870) were involved.

The data of National Research Institute of Lake and River Fish Industry (Saint Petersburg) were used and direct measurements were taken at the cages [2] in order to characterize the primary hydrochemical parameters (salinity, pH, oxygen content, etc.) in the offshore areas of the Vyborg Bay near surface of the water and in the near bottom layer. The findings were identical and conformed to the standards (apart from temperature increase by 3-4 °C during the years of thermal anomalies).

The reservation of a valuable commercial object, the starred sturgeon (69 females in pre-spawning condition) and roach as a large-scale laboratory object (above 350 specimen of both genders in pre-spawning condition) was performed simultaneously and in identical conditions at the aforementioned fish hatcheries in concrete tanks filled with sodium chloride solutions at spawning temperatures during the timeframe required for reproduction, for starred sturgeon it was 5‰ till 1 month, for roach 3, 5 and 12‰ during 55 days. The survivability rate of these species was evaluated in sodium chloride solutions of varying concentration at upper spawning temperatures of 17.4-25.8 °C and oxygen content of

5.2-7.5 mg/l. In the control group the producers were maintained in river water with all other conditions being equal. The fish-rearing qualities of starred sturgeon females were evaluated using conventional methods [9] at fatness coefficient, degree of use in fish-rearing (% of mature female), working fecundity, roe fertilization quality (%), and semen quality (using a 5-point scale).

The levels of hematoglobulin and total protein in blood were determined for evaluation of overall physiological state of fish for 50 roach individuals. For 10 starred sturgeones the osmolarity of blood plasma, chamber (ovarian) fluid and urine was determined using standard procedures [9, 10] with varying water salinity and depending on duration of reservation.

The producers of Baltic salmon were held in river water (control group) in plastic tanks in the Nevskiy salmon fishery (88 females and 75 males in pre-spawning condition) and in sea cages (test group) in the water of the Vyborg Bay with 2.5-4 ‰salinity (44 females and 32 males in pre-spawning condition); the number of mixed-age reared young fish exceeded 1.5 thousand individuals. The average weight, body length and fatness coefficient were determined for producers and young salmon, as well as female fecundity, percentage of mature producers, semen quality and percentage of hatched larvae. The mean morphometrical parameters of two-year-olds and three-year-olds (at least 30 individuals of each age group) were determined for all young fish groups reared in sea cages and were compared with their data from Nevskiy fishery and standard parameters for the Leningrad region.

The novelty of our approach is due to a formalized comparative analysis method generally accepted in patent and invention domain.

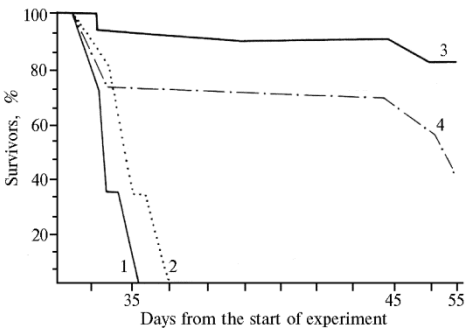


Fig. 1. The survival rate of roach (*Rutilus rutilus caspicus*) producers in sodium chloride solutions of different concentration: 1 — control group (river water), 2 — NaCl 3 ‰, 3 — NaCl 5-7 ‰, 4 — NaCl 12 ‰ (Ikryaninskiy sturgeon fishery, 1979).

The results were processed using variation statistics methods with Microsoft Excel software package. We calculated the arithmetic mean values (M), deviations from mean values (±SEM), mean-square deviations (σ) and variation coefficients (Cv, %).

Results. The deficit of adult producers of sturgeon and salmon species brings the task of creation, maintaining and operating commercial rearing female stocks to the foreground [2-4]. The developed method of lengthy commercial reservation of fish producers in brackish water with critical salinity at 4-8 ‰ [5] increases their use in fish-rearing. The critical salinity, which is the threshold salinity for maturing of gametes of marine and freshwater organisms, allows us to determine the limit of their physiological tolerance and limits of organism interactions with the environment [5, 11, 12].

We have for the first time determined the highest degree of survivability and delayed sexual maturation of roach producers and female starred sturgeons both in sea water and in industrial sodium chloride solution with the same concentration (Fig. 1, 2). In the control group (river water) and NaCl solution (3 ‰) all non-migratory individuals of roach with complete resorption of reproductive products after 35 and 38 days respectively (see Fig. 1) perished. With NaCl 5 and 12 ‰ levels the survival rate of roach producers by the end of the test constituted more than 80 and 40% respectively. The fish-rearing parameters

of starred sturgeon females were analyzed in river water with 5‰ sodium chloride solution levels. The perish rate of females in the control group on the 28th day was about 20% (see Fig. 2) with mass resorption effect of reproductive cells in perished fish. For females of starred sturgeon reserved in the 5‰ solution of NaCl during the period required for reproduction (see Fig. 2) a possibility of obtaining sound progeny was observed. It was determined that critical salinity and even salinity reduced to 2.5 ‰ was optimal for maintenance of producers and reproduction of populations of anadromous species, the Baltic salmon and starred sturgeon.

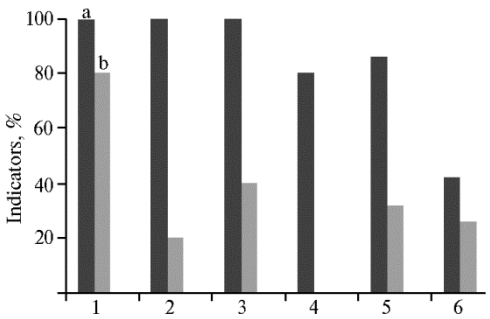


Fig. 2. Fish-rearing indicators of starred sturgeon females (*Acipenser stellatus*) in the test group (a; NaCl 5 ‰) **and in the control group** (b; river water) **28 days after reservation with upper values of spawning temperatures 17.4-25.8°C and oxygen content 5.2-7.5 mg/l** (production test)**:** 1 — survivability, 2 — preserving the physiologically normal state (% of females in this state), 3 —female maturation (% of females in ovulation), 4 — percentage of matured sound females (% of females with fertilized roe of > 50 %), 5 — roe fertilization (one female matured in the control group, 32% of roe fertilization), 6 — sac fry hatching (Ikryaninskiy sturgeon fishery, 1979).

With environment salinity at 5‰, roach producers (50 producers of both genders) and starred sturgeon (10 females) the hematoglobulin and protein levels in blood plasma reduced insignificantly, while salt retention in blood and chamber fluid was maximal. Apparently, this is due to the optimization of the electrolyte balance (Table 1). The electrolyte balance was achieved on account of optimal osmotic gradient between the internal and external environments. This energy-saving gradient ensures preservation of the metabolic and electrolytic homeostasis, i.e. the external environment has biostimulating effect improving body resistance. It is this artificial (modified environment) (NaCl 4-8‰) has the most potential in aquaculture for application in the conditions of close-loop water supply.

1. Physiological state of roach (*Rutilus rutilus caspicus*) and starred sturgeon (*Acipenser stellatus*) in NaCl of varying concentration (Ikryaninskiy sturgeon fishery, 1979)

NaCl, ‰	Reservation timeframe, days	Roach (n = 50 producers, ♀ + ♂)		Starred sturgeon (n = 10 ♀)		
		levels, M±SEM		osmolarity, mosM/l (salinity, ‰)		
		hematoglobulin, g/l (min-max)	total protein, g% (min-max)	blood plasma	cavitary fluid	urine
3	15	6.6±1.1 (5.7-7.95)	9.0±2.95 (7.0-12.9)	–	–	–
5	28	–	6.3±1.5 (4.9-7.9)	164.4 (6.2)	196.0 (7.7)	122.0 (4.5)
	45	9.0±2.95 (7.0-12.9)	6.7±0.7 (5.6-7.0)	–	–	–
12	45	6.3±1.5 (4.9-7.9)	9.0±2.95 (7.0-12.9)	–	–	–
C	34	6.7±0.7 (5.6-7.0)	6.3±1.5 (4.9-7.9)	–	–	–
	28	–	6.7±0.7 (5.6-7.0)	153.0 (5.8)	171.0 (6.6)	155.0 (5.9)

N o t e. C — control group (river water). Gaps mean that the parameter was not defined. The internal environment osmolarity parameters of starred sturgeon were identified and presented by Laboratory of Physiology, Central Research and Development Fishery Institute of the Ministry of Fishery of the USSR.

The fish farm rearing uses the systems of species-specific adaptations only for the river period of life pertaining to the expenditure of financial and power resources and reduction of eurybiontic levels during migration and breeding [2, 13, 14]. The ultimate goal of reproduction (maximum productivity of the population) is achieved in the sea during the feeding period on account of increased survivability and growth. Apparently, fish-rearing needs to use the systems of species-specific phylogenetic adaptations that enable maximum survival rate,

productivity and ecological and physiological plasticity of sexual cycles [15]. These systems are most fully implemented during sea feeding period within very narrow ranges of critical salinity [5, 13]. However, in the currently used fishery technology of breeding Atlantic salmon lack stages of creation and maintenance of rearing female stocks, release and allocation of young fish in the feeding water body [6].

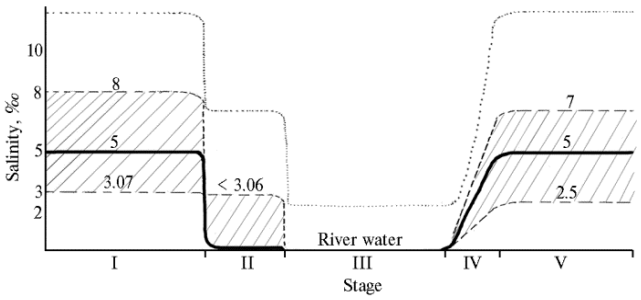


Fig. 3. Salinity patterns at various stages of fishery reproduction of Atlantic salmon (*Salmo salar*) of Baltic population by: I — reservation of female broodstocks in sea cages, II — progeny breeding, III — fishery roe incubation, raising young fish until they are ready to migrate, IV — preadaptation of young fish, V — sea cage breeding of smolts. Unbroken curve means the optimal salinity value, dashed ciurve declares the permitted values, shaded areas are their range; point indicate a curve of anticipated upper values [9].

We have developed a full-cycle salmon reproduction method that includes control of producer reproduction, young fish growth rates and young fish preadaptation to the marine environment [1, 13]. The technology (Fig. 3) includes a wide-scale catching of producers in the sea, maintenance of rearing female stocks in sea cages, breeding and nursing fishery smolts in brackish sea water of critical salinity (AC No. 682197, 965409, patent of the Russian Federation No. 2582347).

2. Comparative fishery and biological parameters of Atlantic salmon (*Salmo salar*) of Baltic population under varying conditions (Leningrad region, 2010-2013)

Parameter	Producers					
	♀+♂		♀		♂	
	sea cages	Nevskiy fishery	sea cages	Nevskiy fishery	sea cages	Nevskiy fishery
Number of individuals	82	163	44	88	32	75
Weight, kg (*M*±SEM)	4.17±0.07	5.00±0.12	3.60±0.05	6.30±0.13	4.40±0.12	2.10±0.14
Mean-square deviation of weight (σ)	0.700	1.616	0.333	1.233	0.700	1.283
Body length (according to Smith), cm (*M*±SEM)	71.60±0.28	74.90±0.71	74.30±0.25	82.00±0.53	63.20±0.04	66.10±0.90
Mean-square deviation of body length (σ)	2.600	9.166	1.683	5.000	0.250	7.833
Fulton's condition factor (Q)	1.02	1.20	1.09	2.60	0.77	1.20
Working fecundity, thousands (*M*±SEM)	–	–	2.40±0.10	4.87±0.03	–	–
Percentage of mature producers (% of maturation)	92.0	84.0	95.0	82.0	97.0	96.0

N o t e. Nevskiy fishery means Nevskiy salmon fishery. The sea cages were located at Alkor-Farm LLC (the Vyborg Bay). Dashes mean that the parameter was not detected.

It is a known fact that during river spawning migration the producers of Atlantic salmon lose their edible qualities and are, therefore, removed from commercial use but continue to be used for artificial reproduction [2, 9]. The presented method allows discontinuing fishing at spawning grounds and growing large viable young fish adapted to the optimal feeding environment, and, ultimately, combine the interests of all reproduction types.

For the first time ripe roe was received and progeny raised to a three-year-old age from producers that maturated naturally in sea cages during the spawning season (October-November) with salinity 2.51-3.06‰ and spawning temperature 3-7°C, with all other conditions being equal with fisheries [2, 13]. The results of working with producers of salmon rearing female stocks using the

new technology and comparing them with the parameters received at Nevskiy salmon fishery enabled us to determine identical high fish-rearing and biological parameters (Tables 2, 3). Furthermore, the producers used by fisheries (from spawning offshore areas) displayed several higher fish-rearing parameters and they were more susceptible to the negative impact of thermal anomalies than those of producers in seas cages. J.M. Elliott at al. [16] reported identical findings.

3. Comparative characterization of Atlantic salmon (*Salmo salar*) producers of Baltic population maintained in sea cages (Alkor-Farm LLC, the Vyborg Bay) at Nevskiy salmon fishery in terms of roe maturation quality (Leningrad region, 2010-2013)

Parameter	Sea cages	Nevskiy fishery
Roe fertilization rate, %	92.0	93.4
Roe put for incubation from 1 batch, thousands of units	90-95	475.8
Sperm quality (mobility), points	5	–
Larvae hatching, % of impregnated roe	81.7	89.7

N o t e. Dashes mean that the parameter was not detected.

The young fish maintained in cages was fed Russian feeds of BimMar company (expenditure 1.3-1.4 kilos). The results of valuation at average water temperature of 3.5 °C, oxygen content of 7-8 mg O_2/l, pH 8-9 (Table 4) indicated that at the ages 1+ and 2+ test young fish maintained in cages increased growth primarily on account of head growth, the length of which increased by 170% while body length increased only by 36%. The body height parameters and fatness coefficient of young fish increased insignificantly, by 35-57%. In the course of analyzing the degree of heterogeneity of individual parameters of young fish we observed that two-year-olds demonstrated the highest variety (1+). Diversity of variation coefficients of two-year-olds reached 23%; for three-year-olds (2+), this parameter was lower and constituted 4.5-17%. This is indicative of declining intensity of development parameters of individuals with age. At the same time, body weight of three-year-olds increased almost by 250%, which points at prevalence of growth processes. This means that upon commencement of smoltification, young fish development is replaced with intensive growth, which corresponds to natural sea feeding [17]. Apparently, the survival rate will increase progressively upon reaching critical salinity 4-8‰ [1, 10, 18]. It is important that this offered method precludes a wide-scale appearance of dwarf males [2, 19]. We believe that the amplified growth and survival rate of young fish if he final fishery cycle is in the sea water are due to the fact that salmon homing is not genetically imprinted [20].

4. Morphometrical parameters of two-year-olds (1+) and three-year-olds (2+) Atlantic salmons (*Salmo salar*) of Baltic population for all batches raised in sea cages (Alkor-Farm LLC, the Vyborg Bay, 2011-2013)

Parameter	*M*±SEM		σ		*Cv*, %	
	1+	2+	1+	2+	1+	2+
Head length (ao), cm	4.60±0.04	7.40±0.04	0.19	0.31	4.20	4.28
Snout length (an), cm	1.80±0.01	2.20±0.02	0.08	0.17	4.77	7.87
Post-orbital head section (po), cm	2.88±0.03	4.20±0.01	0.14	0.09	4.99	2.36
Body length (L), cm	28.70±0.75	39.10±0.22	3.35	1.54	11.66	3.96
Body length (l), cm	26.20±0.43	35.06±0.21	1.93	1.50	7.37	4.29
Maximum body height (gh), cm	6.42±0.06	8.70±0.04	0.31	0.32	4.87	3.76
Minimum body height (ik)	2.19±0.03	3.32±0.03	0.17	0.26	7.89	7.94
Body weight (m), g	280.10±20.08	694.90±14.08	61.34	96.58	17.22	13.91
Fatness coefficient (Q)	1.55	1.61				
Relative growth rate (R)	0.409±0.01	0.490±0.02				

During commercial tests on salmon species, specifically on silver salmon underyearlings *Oncorhynchus kisutsch* (Walbaum, 1792) raised during 1 month in tanks with addition of N-(2-hydroxyethyl)-morpholine or phenethyl alcohol, it was observed that imprinting is formed as late as during the first summer of lar-

vae and young fish raising at fisheries upon transition to active feeding [19, 21]. The release of test young fish and 18-month feeding in the sea resulted in obtaining a bright effect of controllable (obligatory) homing, with 95 and 92%, respectively, of return to the "alien rivers" for each agent. The behavioral response with indications of olfactory imprinting has been observed in sturgeon larvae during transition to active feeding [22]. It is crucial to study behavioral response of sturgeon species for fish-rearing industry and requires a thorough experimental validation based on analysis of species-specific phylogenetic adaptations enabling migration mechanisms [2, 5].

Our findings can be used in a system of measures aimed at preserving rare and endangered fish populations. The idea of their preservation is the underlying idea of a new fish-rearing industry that appeared abroad, i.e. the conservation aquaculture aimed at restoring the natural environment [23]. In Russia, these domains and terms have not been announced or used until now. The conservation aquaculture projects controlled by environment institutions of the USA, Canada and Western Europe include three stages of high-priority action plans: natural reproduction recovery, genetic research and artificial reproduction [23, 24]. These programs are aimed at overcoming a number of negative factors of commercial reproduction of populations, specifically, of domestication of inbred fishery young fish and replacing wild producers with fish-reared with decreased reproductive potential [25-27]. These programs envision ongoing release of fishery-raised young fish when no natural replenishment of the population is available [28]; release of fishery-raised young fish to recover an extinct self-reproducing population [29, 30]; release of fishery-raised young fish to increase the population by supplementation, which is predominant in Russia [8, 31]. This aquaculture domain is based on a combination of effects of natural and fishery reproduction with a focus on recovery of the environmental conditions ensuring natural reproduction of populations [4, 20]. The main objects of these programs in the USA, Canada and Europe are sturgeon and Atlantic salmon populations entered in the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species in the category "endangered species act" [8, 30].

Therefore, industrial sodium chloride solution of critical salinity displays the same biostimulating reservation effects (long-term preservation of fish-rearing quality of producers) as brackish water with the same salt levels. We have successfully reserved the producers of starred sturgeon, Atlantic salmon and roach in critical salinity environment of 4-8‰ with spawning temperature during commercially necessary timeframes. We have determined a possibility to obtain progeny of Atlantic salmon on a wide scale in brackish sea water with salinity up to 3.06 ‰. We have observed the accelerated growth and progressive multiple growth increase of young fish of Baltic salmon population (up to 5-7 times) in brackish sea water of critical salinity in case of perennial sea cage raising. When obtaining progeny from producers in brackish sea water in the areas of feeding and fishing, the load from spawning grounds and commercial dependency of fisheries are released and interests of all types of reproduction and fishing are combined, whereas losses of maintaining broodstocks in optimal reservation environments are minimized. The reduction of processing stages directly at salmon fisheries will allow freeing up additional production capacities in order to improve the efficiency of reproduction. The nursing of young fish in seawater in feeding locations multiplies growth rates, significantly increases young fish survival rate in nature, almost precludes the appearance of "river" dwarf males. Moreover, the smoltification process of young fish shows a wide-scale

synchronous nature because it almost identical to natural, and reduces the costs of fishery products. The survival rate of smolts increases due to their preadaptation for release to natural feeding sites.

REFERENCES

1. Shurukhin A.S., Lukin A.A., Pedchenko A.P., Titov S.F. *Trudy VNIRO*, 2016, 160: 60-69. Available http://atlantniro.ru/images/stories/files/ob.sluwanij/Materiali_ODU_2017.pdf. No date (in Russ.).
2. Garlov P.E., Rybalova N.B., Bugrimov B.S. The necessity for improvement of Atlantic salmon reproduction biotechnology. *Advances in Agricultural and Biological Sciences*, 2016, 2(3): 5-21 (doi: 10.22406/aabs).
3. Petrenko L.A., Malenko A.G., Gorbushina O.A., Shkonda M.V. *Metodicheskie rekomendatsii po vyrashchivaniyu proizvoditelei atlanticheskogo lososya v zavodskikh usloviyakh* [Recommendations for growing Atlantic salmon producers in fish farming]. St. Petersburg, 2004 (in Russ.).
4. Ponomarev S.V. *Lososevodstvo* [Salmon farming]. Moscow, 2012 (in Russ.).
5. Garlov P.E. *Trudy Zoologicheskogo instituta RAN*, 2013, prilozhenie № 3: 75-84 (in Russ.).
6. Yandovskaya N.I., Kazakov R.V., Leizerovich Kh.A. *Instruktsiya po razvedeniyu atlanticheskogo lososya* [Instructions for breeding Atlantic salmon]. Leningrad, 1979 (in Russ.).
7. Trenkler I.V. *Rybovodstvo i rybnoe khozyaistvo*, 2016, 11: 58-70 (in Russ.).
8. Torrissen O., Olsen R.E., Toresen R., Hemre G.I., Tacon A.G.J., Asche F., Hardy R.W., Lall S. Atlantic salmon (*Salmo salar*): the «super-chicken» of the sea? *Rev. Fish. Sci.*, 2011, 19(3): 257-278 (doi: 10.1080/10641262.2011.597890).
9. Pravdin I.F. *Rukovodstvo po izucheniyu ryb* [Guide to the study of fish]. Moscow, 1966 (in Russ.).
10. Golovina N.A., Avdeeva E.V., Evdokimova E.B. *Praktikum po ikhtiopatologii* [Workshop on ichthyopathology]. Moscow, 2012 (in Russ.).
11. Khlebovich V.V. *Kriticheskaya solenost' biologicheskikh protsessov* [Critical salinity of biological processes]. Leningrad, 1974 (in Russ.).
12. Khlebovich V.V. *Trudy zoologicheskogo instituta RAN*, 2013, prilozhenie № 3: 3-6 (in Russ.).
13. Garlov P.E., Bugrimov B.S., Rybalova N.B., Turetskii V.I., Torganov S.V. *Sposob vosproizvodstva populyatsii sevryugi i baltiiskogo lososya. Patent na izobretenie RF № 2582347. FGBOU VO SPbGAU (RU). Opubl. 27.04.2016. Byul. № 12* [Method of reproduction of populations of stellate sturgeon and Baltic salmon. Patent RU № 2582347. Publ. 27.04.2016. Bul. № 12] (in Russ.).
14. Zohar Y., Mucoz-Cueto J.A., Elizur A., Kah O. Neuroendocrinology of reproduction in teleost fish. *Gen. Comp. Endocr.*, 2010, 165(3): 438-455 (doi: 10.1016/j.ygcen.2009.04.017).
15. Garlov P.E. Plasticity of nonapeptidergic neurosecretory cells in fish hypothalamus and neurohypophysis. *Int. Rev. Cytol.*, 2005, 245: 123-170 (doi: 10.1016/S0074-7696(05)45005-6).
16. Elliott J.M., Elliott J.A. Temperature requirements of Atlantic salmon *Salmo salar*, brown trout *Salmo trutta* and Arctic charr *Salvelinus alpinus*: predicting the effects of climate change. *J. Fish Biol.*, 2010, 77(8): 1793-817 (doi: 10.1111/j.1095-8649.2010.02762.x).
17. Boeuf G.J, Payan P. How should salinity influence fish growth? *Comp. Biochem. Physiol.*, 2001, 130(4): 411-423 (doi: 10.1016/S1532-0456(01)00268-X).
18. Deane E.E., Woo N.Y.S. Modulation of fish growth hormone levels by salinity, temperature, pollutants and aquaculture related stress: a review. *Rev. Fish Biol. Fisher.*, 2009, 19(1): 97-120 (doi: 10.1007/s11160-008-9091-0).
19. Stefansson S.O., Björnsson B.Th., Ebbesson L.O.E., McCormic S.D. Smoltification. In: *Fish larval physiology*. R.N. Finn, B.G. Kapor (eds.). Science Publishers, Inc. Enfield (NH) and IBN Publishing Co. Pvt. Ltd, New Delhi, 2008, Chapter 20: 639-681.
20. Hasler A.D., Scholz A.T. *Olfactory imprinting and homing in salmon. Investigations into the mechanism of the imprinting process*. Berlin, Heidelbeg, NY, Tokyo, Springer Verlag, 1983 (doi: 10.1007/978-3-642-82070-0).
21. Stephen D., McCormick S.D., Hansen L.P., Quinn T.P, Saunders R.L. Movement, migration, and smolting of Atlantic salmon (*Salmo salar*). *Can. J. Fish. Aquat. Sci.*, 55(S1), 1998: 77-92 (doi: doi: 10.1139/d98-011).
22. Boiko N.E. Nexachloran and oil contaminations alter memorisation of odors in sturgeon, *Acipenser güldenstädtii* Brandt. *J. Environ. Prot. Ecol.*, 2003, 4(1): 134-140.
23. 65 Dams removed to restore rivers in 2012. In: *Water online*. March, 12, 2013. Available http://www.wateronline.com/doc/dams-removed-to-restore-rivers-0001?atc~c=771+s. No date.
24. Cowx I.G., Nunn A.D., Harvey J.P., Noble R.A.A. *Guidelines for stocking of fish within designated natural heritage sites. Scottish Natural Heritage Commissioned Report No. 513, 2012.* Available http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.738.3232&rep=rep1&type=pdf. No date.
25. Palmé A., Wennerström L., Guban P., Ryman L., Laikre L. *Compromising Baltic salmon genetic diversity: conservation genetic risks associated with compensatory releases of salmon in the Baltic Sea. Havs-*

och vattenmyndigheten rapport 2012. Havs- och vattenmyndigheten, Göteborg, 2012. Available https://www.havochvatten.se/download/18.13780b7613b461ffa9e1a83/1355996777952/rapport-2012-18-compromising-baltic-salmon-english.pdf. No date.
26. Torrissen O., Jones S., Asche F., Guttormsen A., Skilbrei O.T., Nilsen F., Horsberg T.E., Jackson D. Salmon lice — impact on wild salmonids and salmon aquaculture. *J. Fish Dis.*, 2013, 36(3): 171-194 (doi: 10.1111/jfd.12061).
27. Hutchings J.A. Unintentional selection, unanticipated insights: introductions, stocking, and the evolutionary ecology of fishes. *J. Fish Biol.*, 2014, 85(6): 1907-1926 (doi: 10.1111/jfb.12545).
28. Beamesderfer R., Anders P., Young S. Technical basis for the Kootenai Sturgeon Conservation Aquaculture Program. Appendix A. In: *Kootenai River Native Fish Conservation Aquaculture Program Step 2 Submittal*. Kootenai Tribe of Idaho, 2012. Available http://www.restoringthekootenai.org/resources/Step-2-AquaCulture-Doc/Appendix-A.pdf. No date.
29. Culling M., Freamo H., Patterson K., Paul R. Berg P.R., Lien S., Boulding E.G. Signatures of selection on growth, shape, parr marks, and SNPs among seven Canadian Atlantic salmon (*Salmo salar*) populations. *The Open Evolution Journal*, 2013, 7(1): 1-16 (doi: 10.2174/1874404401307010001).
30. Thorstad E.B., Fleming I.A., McGinnity P., Soto D., Wennevik V., Whoriskey F. *Incidence and impacts of escaped farmed Atlantic salmon Salmo salar in nature. NINA Special Report 36*. World Wildlife Fund, Inc., 2008. Available http://www.fao.org/3/a-aj272e.pdf. No date.
31. Lorenzen K. Understanding and managing enhancements: why fisheries scientists should care. *J. Fish Biol.*, 2014, 85(6): 1807-1829 (doi: 10.1111/jfb.12573).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 779-786
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Animal feed and feeding

UDC 639.111.4:636.085.19

doi: 10.15389/agrobiology.2018.4.779eng
doi: 10.15389/agrobiology.2018.4.779rus

MYCOTOXINS DIFFUSION IN FEEDS OF SUMMER PASTURING RATION OF *Rangifer tarandus* IN ARCTIC ZONES OF RUSSIA

E.A. YILDIRIM[1], L.A. ILINA[1], K.A. LAISHEV[1], V.A. FILIPPOVA[1], A.V. DUBROWIN[1], T.P. DUNYASHEV[1], G.Yu. LAPTEV[1], I.N. NIKONOV[2], A.A. YUZHAKOV[3], T.M. ROMANENKO[4], Yu.P. VYLKO[4]

[1]*JSC «Biotrof+»,* 19 korp. 1, Zagrebskii bulv., St. Petersburg, 192284 Russia, e-mail ilina@biotrof.ru (✉ corresponding author), deniz@biotrof.ru, dumova@biotrof.ru, dubrowin.a.v@yandex.ru, timur@biotrof.ru, laptev@biotrof.ru;
[2]*All-Russian Research Veterinary Institute of Poultry Science — Branch of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS,* Federal Agency of Scientific Organizations, 48, ul. Chernikova, St. Petersburg—Lomonosov, 198412 Russia, e-mail ilnikonov@yandex.ru;
[3]*Northwest Center for Interdisciplinary Research of Food Security Problems,* Federal Agency of Scientific Organizations, 7, sh. Podbel'skogo, St. Petersburg—Pushkin, 196608 Russia, e-mail layshev@mail.ru, alyuzhakov@yandex.ru;
[4]*Laverov Federal Center for Integrated Arctic Research (FCIARctic) RAS, Naryan-Mar Agro-Experimental Station,* Federal Agency of Scientific Organizations, 1a, ul. Rybnikov, Naryan-Mar, Nenets AO, 166004 Russia, e-mail nmshos@atnet.ru, vylcko.yury@yandex.ru

ORCID:
Yildirim E.A. orcid.org/0000-0002-5846-5105
Ilina L.A. orcid.org/0000-0003-2789-4844
Laishev K.A. orcid.org/0000-0003-2490-6942
Filippova V.A. orcid.org/0000-0001-8789-9837
Dubrowin A.V. orcid.org/0000-0001-8424-4114
Dunyashev T.P. orcid.org/0000-0002-3918-0948
Laptev G.Yu. orcid.org/0000-0002-8795-6659
Nikonov I.N. orcid.org/0000-0001-9495-0178
Yuzhakov A.A. orcid.org/0000-0002-0633-4074
Romanenko T.M. orcid.org/0000-0003-0034-7453
Vylko Yu.P. orcid.org/0000-0002-6168-8262

The authors declare no conflict of interests

Acknowledgements:
Supported financially by Russian Science Foundation, grant № 17-76-20026 "Rumen microbiocenosis of *Rangifer tarandus* as a basis for developing promising biotechnologies for reindeer herding in Russia's Arctic regions"
Received November 9, 2017

Abstract

In the summer and autumn, the food base of *Rangifer tarandus* consists of up to 300 species of higher plants, of which lichens account for about 15 %. It is shown that in the tissues of some higher plants and in the soil under lichens there are micromycetes capable of producing mycotoxins. In the present work, we are the first to estimate the content of mycotoxins for the components of summer reindeer rations, i.e. *Salix borealis*, *Vaccinium uliginosum, Betula nana*, and *B. pendula.* The aim of the study was to analyze the distribution of mycotoxins in the components of the summer diet of reindeer. Samples of genera *Cladonia* and *Nephroma* lichens, higher plants of the species *Salix borealis*, *Vaccinium uliginosum*, *Betula nana*, *B. pendula*, and mixtures of perennial grasses were collected in early August 2017 in the pastures of the Harp town of the Yamalo-Nenets Autonomous District, the Nelmin-Nos town of Nenets Autonomous District and the Pushnoy town of the Murmansk region. Aflatoxins (AFLA), ochratoxin A (OTA), T-2 toxin (T-2), zearalenone (ZEN), deoxynivalenol (DON) were detected and measured in the samples using ELISA test. During the mycotoxicological evaluation of the summer food ration components of reindeer, we found multiple contaminations with the mycotoxins. The samples of *Embryophyta* representatives revealed a greater number of toxic metabolites compared to samples of the genera *Cladonia* and *Nephroma* lichens. Practically, in all samples of higher plants, the presence of mycotoxins T-2, ZEN and DON produced by *Fusarium* pathogens which affects plants during vegetation, as well as AFLA and OTA metabolites of micromycetes *Aspergillus* sp. and *Penicillium* sp. which previously were considered not adapted for growth and reproduction in plant tissues during the growing season. However, AFLA and OTA were the least represented on virtually all samples. It is of interest that OTA was not detected in any of the lichen samples assayed. In most of the samples, DON Fusarium toxins dominated with accumulation in lichens up to 0.15 mg/kg and in *Embryophyta* samples up to 33.8 mg/kg, as well as ZEN at the amount of up to 0.1227 mg/kg and 2.543 mg/kg, respectively. Mycotoxin contamination of the samples of genus *Cladonia* practically did not have regional differences, whereas in the mixture of perennial grasses and in *V. uliginosum* mycotoxin contamination varied to a large extent depending on the place of plant growth. The mycotoxins are found

in concentrations that may pose a threat to animal health.

Keywords: *Rangifer tarandus*, feed, lichens, mycotoxins, ELISA

Reindeer herding as a source of food, fell, velvet antlers and endocrine-enzyme products plays a leading role for the regions of the Arctic zone of the Russian Federation. The diet of *Rangifer tarandus* consists predominantly of scanty, low-nutritious vegetable fodder. In the winter-spring period, the share of lichens in the diet increases to 70% and only 30% falls on the representatives of *Embryophyta*. In the summer-autumn period, the basis of the animals' food supply is composed of up to 300 species of higher plants (the representatives of the *Salicaceae* and *Poaceae families*, *Bethula nana* species, perennial grasses, etc.), the lichens are not more than 15% [1].

It is shown that the tissues of some higher plants and soil under lichens contain the micromycetes which are able to produce mycotoxins. Thus, T.Yu. Tolpysheva [1] discovered the presence of significant amounts of micromycetes of the *Penicillum* genus in soils under lichens. It is known that some members of this genus can produce ochratoxin A [2)]. A.A. Burkin et al. [3] have found various mycotoxins (alternariol, sterigmatocystin, mycophenolic acid, citrinin, cyclopiazonic acid and others) in the lichens which belong to 20 genera of the *Cladoniaceae, Nephromataceae, Parmeliaceae, Umbilicariaceae, Peltigeraceae* and *Teloschistaceae* families. The presence of micromycetes, which are the producers of mycotoxins, in the tissues of tree and shrub species growing in the Arctic zone of Russia has not been noted before. Nevertheless, the fact of occurrence of toxigenic micromycetes in perennial grasses which also constitute the basis of the reindeer diet is well studied. A.S. Orina et al. [4] have found in the tissues of perennial grasses a significant amount of micromycetes of the *Fusarium* genus. It is known that *F. sporotrichioides* and *F. langsethiae* species are capable of producing T-2 toxin, *F. culmorum* and *F. graminearum* produce zearalenone, *F. culmorum* and *F. graminearum* produce deoxynivalenol [5].

The reindeer physiology (as opposed to cattle) including the state of rumen microflora underwent much less anthropogenic influence. According to a number of researchers [6, 7], the anaerobic microorganisms of reindeer rumen are able to detoxify usnic acid [8] and mycotoxins [1, 9] from lichens. However, the emergence of intoxication in reindeer due to eating lichens has been demonstrated [10].

There are no any publications concerning the containing of toxic metabolites of micromycetes in higher plants being the components of summer pasture diets of reindeers both in domestic and foreign literature. The study of this problem is of considerable scientific interest.

In this work, we for the first time discovered the presence of mycotoxins in such components of summer pasture diets of reindeers as *Salyx boralis, Vaccinium uliginosum, Betula nana* and *B. pendula*.

The objective of these researches was to analyze the prevalence of mycotoxins in the components of the summer diet reindeer of *Rangifer tarandus* using enzyme-linked immunosorbent assay.

Techniques. The samples of reindeer summer diets were collected at the beginning of August 2017 in the territory of tundra and forest-tundra pastures located in the Kharp township (Priuralsky district, Yamalo-Nenets Autonomous Okrug), Nelmin-Nos village (Nenets Autonomous Okrug) and Pushnoy township (Kola district, Murmansk region). The lichens of the *Cladonia* and *Nephroma* genera, higher plants *Salyx boralis* (northern willow), *Salyx Polaris* (polar willow), *Vaccinium uliginosum* (blueberry), *Betula nana* (dwarf birch) and *Betula pendula* (drooping birch) species as well as the mix of perennial grasses have been sampled in 3 replications. The samples have been cleared from the remains of other plants, soil and bark.

The content of aflatoxins (AFLA), ochratoxin A (OTA), T-2 toxin (T-2),

zearalenone (ZEN) and deoxynivalenol (DON) in the samples was investigated using the of enzyme-linked immunosorbent assay (AgraQuant test system, Romer Labs, Inc., Austria) according to manufacturer's recommendations. The mycotoxins, with the exception of DON, were extracted with 70% methanol, DON with distilled water. The solutions of five analyzed mycotoxins of known concentrations were used as the standards. In analysis for ZEN and T-2, 10% hydrochloric acid was used as the stopping solution, for AFLA, OTA and DON the stopping solution was 10% phosphoric acid. The optical density (OD) was measured at λ=450nm (a Stat Fax 303+ microstrip photometer, Awareness Technology, Inc., USA).

The calculation of sample mean (*M*) values) and the error probability, with a rejection of the null hypothesis p, and standard error of means ±SEM was made with Microsoft Excel 2010 software.

Results. The multicomponent samples repeating the composition of the averaged summer pasture diet of reindeers have been formed (Table 1).

1. The composition of the multicomponent samples repeating the average summer pasture diet of reindeer *Rangifer tarandus* (Arctic zone of Russia, 2017)

Component	Percentage of the component in the total diet		
	Kharp township (tundra)	Pushnoy township (forest tundra)	Nelmin-Nos village (tundra)
Cladonia	5	10	10
Nephroma	5	–	–
Salix borealis	5	20	20
Salix polaris	15	–	–
Vaccinium uliginosum	10	–	5
Betula nana	25	20	15
Betula pendula	5	20	20
Mix of perennial herbs	30	30	30

N o t e. Dashes mean that the component has not been represented in the total diet.

Almost in the all investigated samples (both lichens and higher plants) we have detected the presence of mycotoxins (Table 2). To date, the maximum permissible concentration (MPC) of mycotoxins for reindeer feed is not established in Russia. In the Unified Veterinary (Veterinary and Sanitary) Requirements for the products subject to the veterinary control (supervision) (approved by the Decision of the Commission of the Eurasian Economic Community No. 137 dated June 18, 2010) the MPC established for oats, wheat and barley cereals are 0.004 mg/kg for AFLA, 0.005 mg/kg for OTA, 0.06 mg/kg for T-2, 0.1 mg/kg for ZEN, and 1 mg/kg for DON. In most investigated samples we detected the excess of aflatoxins, ochratoxin A, T-2 toxin, zearalenone and DON compared to the above MPC values. The multiple contamination of all samples with mycotoxins (at least with three ones) has been observed that enhanced their toxic impact on animals [4].

The content of mycotoxins largely varied depending on the species. In the samples of higher plants we detected the higher content of toxic metabolites than in *Cladonia* and *Nephroma* lichens. Also, the higher content of mycotoxins in comparison with that for lichens in its pure form has also been found in the multicomponent mixes similar to averaged summer diets.

In the almost all samples of higher plants, we have detected the presence of T-2, ZEN, and DON produced by fusarium pathogens [11] which affects vegetating plants and of AFLA and OTA which are the metabolites of *Aspergillus* sp. and *Penicillium* sp. [12]. Nevertheless, in our experiments, AFLA and OTA were the least represented almost in the all samples. The presence of ochratoxin A was not detected in any of the investigated samples of lichens. Almost in all samples, the DON fusariotoxins produced by *F. culmorum* and *F. graminearum* dominated and accumulated in lichens up to 0.15 mg/kg and in *Embryophyta* up to 33.8 mg/kg, ZEN content was up to 0.1227 mg/kg and 2.543 mg/kg, respectively ($p \leq 0.05$).

The contamination of lichens of *Cladonia* genus by mycotoxins has no geo-

2. The average content (mg/kg) of mycotoxins in the components of the summer pasture diet of the *Rangifer tarandus* reindeers (Arctic zone of Russia, 2017)

Mycotoxin	*Cladonia*	*Nephroma*	*Vaccinium uliginosum*	*Salix borealis*	Mix of perennial herbs	*Betula pendula*	*B. nana*	Mix of the diet components
Kharp township (Priuralsky district, Yamalo-Nenets Autonomous Okrug)								
AFLA	0.005±0.0002*	0.003±0.0006	0.123±0.0190	0.129±0.0100	0.011±0.0047	0.111±0.0054*	–	0.089±0.0041**
OTA	< l. r. d.	< l. r. d.	0.0371±0.00186*	0.0968±0.00800	0.0007±0.00003*	0.0895±0.00640	–	0.041±0.0036
T-2	0.0385±0.00200*	0.0179±0.00083*	1.9690±0.09300*	1.0210±0.10000	0.0004±0.00008	0.4050±0.04700	–	0.1058±0.009
ZEN	0.04±0.002	0.12±0.009	2.54±0.110*	2.44±0.220	0.12±0.008	1.94±0.150	–	0.52±0.021*
DON	0.003±0.0003	0.150±0.0075*	10.300±0.9000	9.810±0.8700	1.550±0.07500*	< l. r. d.	–	1.700±0.0750*
Pushnoy township (Kola district, Murmansk region)								
AFLA	0.003±0.0001*	–	–	0.067±0.0084	0.009±0.0004*	0.111±0.0080	0.107±0.0200	0.057±0.0021*
OTA	< l. r. d.	–	–	0.0307±0.00140*	< l. r. d.	0.0601±0.00690	0.0540±0.00270*	0.0195±0.00310
T-2	0.0098±0.00400	–	–	0.1770±0.03000	< l. r. d.	0.1484±0.00720*	0.1387±0.01200	0.0288±0.00110*
ZEN	0.09±0.004*	–	–	0.56±0.040	0.13±0.008	1483.00±97.600	0.90±0.039*	0.49±0.047
DON	< l. r. d.	–	–	2.790±0.1200*	0.350±0.0370	1.060±0.0890	2.080±0.4800	2.510±0.2400
Nelmin-Nos village (Nenets Autonomous Okrug)								
AFLA	0.0046±0.00058	–	0.005±0.0200	0.129±0.0060*	0.009±0.0003*	–	0.1351±0.0230	0.099±0.0120
OTA	< l. r. d.	–	< l. r. d.	0.1119±0.00970	< l. r. d.	–	0.1017±0.00800	0.0755±0.00310*
T-2	0.0064±0.00100	–	< l. r. d.	0.7615±0.06400	0.0548±0.00210*	–	0.8188±0.03200*	0.4154±0.89000
ZEN	0.09±0.012	–	0.49±0.021*	1.25±0.058*	0.19±0.0094*	–	1.70±0.170	0.79±0.035*
DON	0.020±0.0010*	–	0.130±0.0090	2.600±0.3400	1.020±0.2200	–	33.800±1.7000*	1.530±0.4300

Note. AFLA — aflatoxins; OTA — ochratoxin A; T-2 — T-2 toxin; ZEN — zearalenone; DON — deoxynivalenol. Dashes mean that in this version the analysis for the mycotoxin content was not made; < l. r. d. — below the limit of reliable determination by the ELISA method.

* Statistically significant at $p \leq 0.05$

** Statistically significant at $p \leq 0.01$

graphical differences. The mycotoxins content in the samples of higher plants *V. uliginosum* and perennial grasses varied greatly depending on the place of growing. Thus, this index was the smallest in the samples of perennial grass mix from the forest-tundra pastures of the Pushnoy township compared to those grown in the tundra pastures of the Kharp township and Nelmin-Nos village. In *V. uliginosum* grown in the pasture of the Nelmin-Nos village the presence of OTA and T-2 was not detected, whereas in similar samples from Kharp township their content was 0.0371 and 1.969 mg/kg, respectively ($p \leq 0.05$). The content of AFLA, ZEN and DON in *V. uliginosum* samples from the Nelmin-Nos village was respectively 26.1, 5.2 and 79.2 times more than in the samples from the Kharp township. Summer meteorological conditions in 2017 in the Yamalo-Nenets, Nenets Autonomous Okrug and the Murmansk region were similar, thus the geographical differences in contamination by mycotoxins may be due to the soils dissimilarity in chemical composition and nutrients content. In addition, the described regional features may be related to the specificity of the structural organizations of epiphytic bacterial communities of higher plants. It has been reported that many bacteria including epiphytic perform the biodegradation of the secondary metabolites of micromycetes and have the antibiotic activity against the producers of these metabolites [13-15].

The presence of toxigenic micromycetes (*Fusarium sporotrichioides*, *F. langsethiae*, *F. verticillioides, F. culmorum* and *F. graminearum*), which are able to produce fusariotoxins, in higher plants is widely known [16, 17]. Before, it was assumed [18] that the representatives of the *Aspergillus* and *Penicillium* generam the producers of aflatoxins and ochratoxin A, are not adapted for growing and reproduction on plant during their growing. However in 2014, by quantitative PCR method, the micromycetes of the *Fusarium, Aspergillus* and *Penicillium* genera and their secondary metabolites, the aflatoxins, ochratoxin A, deoxynivalenol, zearalenone and T-2 toxin, have been detected in perennial herb mycobiota [19]). The presence of toxin-producing micromycetes in the mycobiota of trees and shrubs which are the components of the reindeer summer diets have not been detected before.

The issue about the sources of lichens contamination with mycotoxins is also almost unstudied. According to one of the hypotheses put forward by T.Yu. Tolpysheva [20], the contamination of lichens with mycotoxins occurs as the result of contact with the micromycetes *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. from the soil under the lichens [21]. According to T.Yu. Tolpysheva [20], lichens are able to absorb the mycotoxins from soil solution. As the author explains, lichens are the poikilohydric organisms whish passively absorb moisture. Lichens mostly grow on the soil as curtains in which their talli closely contact to keep the moisture longer. This activates the capillary rise of water with the dissolved substances from the soil up the lichens.

Earlier, some Russian [22-24] and foreign [25-27] researchers pointed out the high frequency of the occurrence of mycotoxins and their significant content in the growing fodder herbage for cattle, but the issue about the content of toxic metabolites in the leaves of woody and shrub plants is still unstudied. In foreign literature there are only a few instructions concerning the detection of mycotoxins in the leaves of woody cultivated plants [28].

Thus, in mycotoxicological assessment of the base components of the reindeer summer fodder, the lichens and higher plants growing in tundra and forest-tundra pastures of three regions of the Arctic zone of the Russian Fededi-et, we have found the multiple contamination with aflatoxins, ochratoxin A, zearalenone, T-2 toxin and deoxynivalenol. Our findings indicate that the problem of contamination of higher plants by mycotoxins is much more acute com-

pared to that of lichens. The mycotoxin contamination of *Cladonia* genus almost has no regional differences, whereas the content of mycotoxins in the mix of perennial herbs and in *V. uliginosum* varies greatly depending on the place of growing. The detected mycotoxins are present in the concentrations which may pose a threat to animals.

REFERENCES

1. Chikalev A.I., Yuldashbaev Yu.A., Rodionov G.V. *Olenevodstvo* [Reindeer herding]. Moscow, 2015 (in Russ.).
2. Bennett J.W., Klich M. Mycotoxins. *Clin. Microbiol. Rev.*, 2003, 16(3): 497-516 (doi: 10.1128/CMR.16.3.497-516.2003).
3. Burkin A.A., Kononenko G.P. *Prikladnaya biokhimiya i mikrobiologiya*, 2013, 5(49): 522-530 (doi: 10.7868/S0555109913050036) (in Russ.).
4. Orina A.S., Gavrilova O.P., Gagkaeva T.Yu. *Zashchita i karantin rastenii*, 2017, 6: 25-27 (in Russ.).
5. Diaz D. *Mikotoksiny i mikotoksikozy* [Mycotoxins and mycotoxicoses]. Moscow, 2006 (in Russ.).
6. Palo R.T. Usnic acid, a secondary metabolite of lichens and its effect on in vitro digestibility in reindeer. *Rangifer*, 1993, 13: 39-43.
7. Sundset M.A., Edwards J.E., Cheng Y.F., Senosiain R.S., Fraile M.N., Northwood K.S., Praesteng K.E., Glad T., Mathiesen S.D., Wright A.D. Rumen microbial diversity in Svalbard reindeer, with particular emphasis on methanogenic archaea. *FEMS Microbiol. Ecol.*, 2009, 70(3): 553-562 (doi: 10.1111/j.1574-6941.2009.00750.x).
8. Luzina O.A., Salakhutdinov N.F. *Bioorganicheskaya khimiya*, 2016, 3(42), 2016: 276 (doi: 10.7868/S0132342316030106) (in Russ.).
9. Burkin A.A., Kononenko G.P. *Izvestiya Rossiiskoi akademii nauk. Seriya Biologicheskaya*, 2014, 3: 228 (doi: 10.7868/S0002332914030047) (in Russ.).
10. Kingsbury J.M. *Poisonous plants of the United States and Canada*. NY, 1964.
11. Kononenko G.P., Burkin A.A. V sbornike: *Uspekhi meditsinskoi mikologii* [Advances in medical mycology]. Moscow, 2003: 141-144 (in Russ.).
12. Burkin A.A. V sbornike: *Uspekhi meditsinskoi mikologii* [Advances in medical mycology]. Moscow, 2003: 122-124 (in Russ.).
13. Hernandez-Mendoza A., Garcia H.S., Steele J.L. Screening of *Lactobacillus casei* strains for their ability to bind aflatoxin B1. *Food Chem. Toxicol.*, 2009, 47(6): 1064-1068 (doi: 10.1016/j.fct.2009.01.042).
14. Čvek D., Markov K., Frece J., Friganović M., Duraković L., Delaš F. Adhesion of zearalenone to the surface of lactic acid bacteria cells. *Croatian Journal for Food Technology, Biotechnology & Nutrition*, 2012, 7(Special Issue): 49-52.
15. Shi L., Liang Z., Li J., Hao J., Xu Y., Huang K., Tian J., He X., Xu W. Ochratoxin A biocontrol and biodegradation by *Bacillus subtilis* CW 14. *J. Sci. Food Agr.*, 2014, 94(9): 1879-1885 (doi: 10.1002/jsfa.6507).
16. Levitin M.M., Novozhilov K.V., Afanasenko O.S., Mikhailov L.A., Mironenko N.V., Gagkaeva T.Yu., Gannibal F.B. V knige: *Mikologiya segodnya. Tom 2* [In: Mycology today. V. 2]. Moscow, 2011: 261-274 (in Russ.).
17. Gagkaeva T.Yu., Gavrilova O.P. *Materialy Mezhdunarodnoi nauchnoi konferentsii, posvyashchennoi 150-letiyu so dnya rozhdeniya chl.-korr. AN SSSR, prof. A.A. Yachevskogo «Problemy mikologii i fitopatologii v XXI veke»* [Proc. Int. Conf. dedicated to 150 anniversary of Prof. A.A. Yachevskii «Challenges of mycology and phytopathology in the 21st century»]. St. Petersburg, 2013: 400 (in Russ.).
18. Christensen C.M., Kaufmann H.H. *Storage of cereal grains and their products. 2nd ed.* American Association of Cereal Chemists. St. Paul, Minnesota, 1974: 158-192.
19. Laptev G.Yu., Novikova N.I., Il'ina L.A., Iyldyrym E.A., Soldatova V.V., Nikonov I.N., Filippova V.A., Brazhnik E.A., Sokolova O.N. Dynamics of mycotoxin accumulation in silage during storage. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2014, 6: 123-130 (doi: 10.15389/agrobiology.2014.6.123eng).
20. Tolpysheva T.Yu. *Vestnik Moskovskogo Universiteta. Seriya 16: Biologiya*, 2014, 3: 37-41 (in Russ.).
21. Girlanda M., Isocrono D., Bianco C., Luppi-Mosca A.M. Two foliose lichens as microfungal ecological niches. *Mycologia*, 1997, 89(4): 531-536 (doi: 10.2307/3760987).
22. Burdov L.G. *Monitoring mikotoksinov, profilaktika i lechenie mikotoksikozov v Udmurtskoi Respublike. Kandidatskaya dissertatsiya* [Monitoring of mycotoxins, mycotoxicosis prevention and treatment in the Udmurt Republic. PhD Thesis]. Kazan', 2013 (in Russ.).
23. Burkin A.A., Kononenko G.P. Mycotoxin contamination of meadow grasses in European Rus-

sia. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2015, 50(4): 503-512 (doi: 10.15389/agrobiology.2015.4.503eng).
24. Laptev G.Yu., Novikova N.I., Dubrovina E.G., Il'ina L.A., Iyldyrym E.A., Filippova V.A., Nikonov I.N., Brazhnik E.A. *Kormoproizvodstvo*, 2014, 10: 35-39 (in Russ.).
25. Boudra H., Morgavi D.P. Reduction in fusarium toxin levels in corn silage with low dry matter and storage time. *J. Agr. Food Chem.*, 2008, 56(12): 4523-4528 (doi: 10.1021/jf800267k).
26. Mansfield M.A., Jones A.D., Kuldau G.A. Contamination of fresh and ensiled maize by multiple *Penicillium* mycotoxins. *Phytopathology*, 2008, 98(3): 330-336 (doi: 10.1094/PHYTO-98-3-0330).
27. Teller R.S., Schmidt R.J., Whitlow L.W., Kung L. Jr. Effect of physical damage to ears of corn before harvest and treatment with various additives on the concentration of mycotoxins, silage fermentation, and aerobic stability of corn silage. *J. Dairy Sci.*, 2012, 95(3): 1428-1436 (doi: 10.3168/jds.2011-4610).
28. Watanabe I., Kakishima M., Adachi Y., Nakajima H. Potential mycotoxin productivity of *Alternaria alternata* isolated from garden trees. *Mycotoxins*, 2007, 57(1): 3-9 (doi: 10.2520/myco.57.3).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 787-798
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.5:636.084.524

doi: 10.15389/agrobiology.2018.4.787eng
doi: 10.15389/agrobiology.2018.4.787rus

THE RATION RECIPES DEVELOPED TO IMPROVE EFFECTIVE AND SAFE BIOFORTIFICATION OF HEN (*Gallus gallus* L.) EGGS

A.Sh. KAVTARASHVILI, I.L. STEFANOVA, V.S. SVITKIN, E.N. NOVOTOROV

Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Federal Agency of Scientific Organizations, 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail alexk@vnitip.ru (✉ corresponding author), dp.vniipp@mail.ru, 89267796966@yandex.ru, en-5506040@mail.ru
ORCID:
Kavtarashvili A.Sh. orcid.org/0000-0001-9108-1632
Stefanova I.L. orcid.org/0000-0002-4394-5149
Svitkin V.S. orcid.org/0000-0002-4161-0986
Novotorov E.N. orcid.org/0000-0003-4478-3206
The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation, grant No 16-16-04047
Received March 1, 2018

Abstract

Enrichment of chicken eggs with ω-3 polyunsaturated fatty acids (PUFAs) is relevant worldwide, but scientists and practitioners face certain challenges. Effective biofortification requires dietary source of ω-3 PUFAs bioavailable for laying hens that will not compromise livability, health, and productivity of layers. Since any increase in ω-3 PUFA level in dietary lipids can deteriorate the oxidative stability of egg lipids causing faster quality loss and emergence of fishy odor and taste in stored and/or cooked eggs, dietary antioxidants should be used of which vitamin E and selenium (Se) are the most effective. High costs of the ω-3 PUFA-enriched diets for layers should also be diminished. The most popular source of dietary ω-3 PUFAs for layers is flax (seed, oil, cake); however, its dietary level should not exceed 15 % even when used with the appropriate multi-enzyme preparations. This paper is the first to report on comparative study of low-cost feed recipes that we suggest for effective concurrent enrichment of eggs with ω-3 PUFAs, vitamin E, and Se with no negative impacts on layers' livability and productivity. The trials were performed in 2016-2017 at the Zagorskoye Center for Genetics & Selection (Moscow Province) on SP 789 cross layers from 140 to 200 days of age. In the Trial 1 the diets for layers were supplemented with flaxseed oil (3 %) and cake (5 or 10 %), synthetic vitamin E (DL-α-tocopherol, 100 or 150 ppm), and Se preparations Sel-Plex® (Alltech, USA), DAFS-25 (Russia), and sodium selenite (0.5 ppm of Se). These doses of the additives led to a 4.5-4.7-fold increase in ω-3 PUFA level, a 1.9-3.6-fold increase in vitamin E and a 1.5-2.2-fold increase in Se content in the edible parts of eggs. Additionally, egg production was 0.6-4.0 % higher, total egg weight was 0.3-8.4 % higher, and feed conversion ratio was improved by 4.1-9.4 %. In Trial 2 layers were fed with optimized doses of the additives determined in the previous trial (flaxseed oil 3 %, flaxseed cake 5 %, vitamin E 150 ppm, Se 0.5 ppm); the comparative efficiency of different Se sources (Sel-Plex®, Sel-Plex® + DAFS-25 at 1:1, Sel-Plex® + sodium selenite at 1:1) and organic vitamin E vs. synthetic vitamin E preparation was studied. The combinations of Sel-Plex® with other Se sources and the substitution of organic vitamin E for the synthetic source improved egg production, egg weight, feed conversion ratio, and decreased diet cost. The best results were found for the mixture of Sel-Plex® and selenite and an organic source of vitamin E. In this, the content of total ω-3 PUFAs in eggs was 4.9 times higher compared to control (with ω-6/ω-3 PUFAs ratio 2.3:1 vs. 14.2:1 in control), the contents of individual ω-3 PUFAs were also significantly higher, i.e. 7.1-fold for α-linolenic acid, 1.8-fold for eicosapentaenoic acid, 3.2-fold for docosapentaenoic acid, and 3.8-fold for docosahexaenoic acid. The content of vitamin E in the eggs was 2.8 times higher, Se level was 2.2 times higher. In this trial, the egg production improved by 10.1 % compared to control, egg weigh output per layer was 13.2 % higher. Feed expenses per 10 eggs lowered by 7.6 %, and per 1 kg of egg weight by 9.9 %. Total diet costs were 1.2 % lower.

Keywords: chicken, functional eggs, ω-3 polyunsaturated fatty acids, selenium, vitamin E, diet cost

Market of functional products increasing the supply of all necessary nutrients to the consumers and promoting the disease prevention has been growing strongly over the last few years [1-3]. Products rich in essential fatty acids, pri-

marily ω-3 polyunsaturated fatty acids (PUFAs), i.e. α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which cannot be synthesized in sufficient amounts by animals and birds, are of particular interest [4]. Humans need these substances for the development of brain, visual function and for prevention of cardiovascular diseases, etc. [5]. The optimum ratio of ω-6/ω-3 PUFA in human diet is still debatable. Opinions vary from 1:1, pleading the fact that the two PUFA groups compete with each other in metabolic processes [6], and to 2-3:1 [7]. In developed countries, this ratio is remarkably high. Estimates vary from 10:1 to 25:1 [8].

Current diets of layers based on corn and other grains are leaning heavily toward ω-6 PUFA (with small contents of ALA and virtually non-existent DHA and EPA) [9]. Vegetable oils (corn, soy, sunflower, rape) used in the diets also contain considerable amounts of ω-6 PUFA (mostly linolenic acid, LA) and very little ω-3 PUFA [10], excluding however certain species of rape [11]. With this kind of diet, ω-3 PUFA content in the egg is small and the ω-6/ω-3 PUFA ratio is way above the optimum value [12].

As the most available source of complete protein and fatty acids, chicken eggs represent a food product that is most suitable for added functional properties [13]. Egg white covers approx. 12% of a daily demand of a human body and, therefore, is considered to be a model complete protein. Egg yolk contains saturated, monounsaturated and polyunsaturated fatty acids (approx. 7 % of a daily demand of a human body, including approx. 11% of essential fatty acids) [7]. Amino acid composition of a chicken egg virtually does not depend on the diet of the layer [13], whereas lipid composition largely depends on the lipid profile of the diet [4, 14]. Thanks to high-speed and adaptable metabolism, biologically active substances take 2-4 weeks to transit from the layer's diet to eggs, so one can vary the composition of the egg yolk by modifying the diet of the layer [4, 15, 16]. However, enriched diets may have negative impact on physiological condition of the birds and boost the cost of products (by 15-20 % for functional poultry products on retail market) [1, 17].

Enrichment of eggs with ω-3 PUFA requires a proper source where high biological availability of this fatty acid would have little negative impact on the egg production, layers' health and quality of eggs, including organoleptic parameters. One should control the ω-6/ω-3 PUFA ratio both in the diet and in the obtained eggs. Increase in the share and extent of PUFA (including ω-3 variety) undersaturation in the diet will reduce oxidation stability [18, 19] and cause toxic oxidation products to occur in the body of the layer and to carry over into the eggs thereby compromising their quality during storage and/or cooking [20]. In addition, enrichment of eggs with ω-3 PUFA often results in unpleasant fishy odor [21].

ω-3 PUFA in the layers' diet mostly comes from flax products (seed, oil, cake) wherein ALA contents is higher than in any other oil crops: ALA comprises over 50% of lipids in flax seed [22]. Flax oil is less frequent in the diet of the poultry than seed or cake due to insufficient availability and relatively high cost of this product [23]. Flax products diet, however, causes the odor of spoiled fishy in the eggs (probably, due to oxidation of PUFA-enriched lipids). Whole flax seed contains approx. 40% of fat, 20-25% protein and 3-10% adhesive substances (held mostly in seed coats), which, along with lignan phytoestrogens , linatine (pyridoxine antagonist) and linamarin (cyanogenetic glucoside), are deemed to be major anti-nutritional factors of flax [24]. Generally recommended diet of a grown bird should contain up to 10% of flax seed [23] which should be ground, autoclaved, granulated, extruded, subjected to microwave thermal treatment, etc., to enhance availability of protein and ω-3 PUFA [25, 26]. Phytoestrogens in flax

seed negatively impact egg and yolk weight, and adhesive substances reduce the egg-laying capacity. Arabinoxylanic fraction of the adhesive substance in the seed vastly increases chymus viscosity, thereby reducing the effect of dietary nutrients [27]. The diets with rape should be enriched with ferments capable of breaking the non-starch polysaccharides [28, 29]. There are few similar studies known in the context of flax. There were reports confirming the improved egg-laying capacity and feed conversion rate if the layers were fed 15% of flax seed with multienzyme complex Superzyme®-OM (Canadian Bio-Systems, Inc., Canada) that helps break the non-starch polysaccharides (at the same dosage of flax seed without enzyme additives, egg-laying capacity and shell mass went down compared to control without flax seed and enzyme additives). Enzyme application would increase total ω-3-PUFA (from 546 mg to 578 mg per 1 egg of 60 g) and DHA (from 91.8 mg to 101.9 mg) (30).

Oxidation stability of PUFA-enriched lipids is increased by antioxidants added to the feed, e.g. vitamins E, A and C, carotenoids, Se, I, etc., which also are valuable bioactive agents. It would partially solve the problem of fishy odor in eggs. One should also use fresh feed with minimum-oxidation lipids [20, 31, 32]. At current prices on quality feed sources of ω-3-fatty acids, Western producers of FA-enriched eggs spend almost twice as much as on non-enriched eggs [7], so antioxidants without ω-3-fatty acids appear to be a feasible alternative.

We are the first to study the potential of biofortification of edible eggs by vitamin E, selenium and ω-3 PUFA in Russian cross breed chickens. This paper gives the dosages we established for flax cake, oil, fatty acids, organic source of vitamin E, Sel-Plex® and sodium selenite, which increase egg-laying capacity and egg weigh output per layer without negative physiological effect, while cutting the feed consumption per unit of product and the costs for compound feed.

This paper is dedicated to the study of efficiency of simultaneous enrichment of edible chicken eggs with ω-3 polyunsaturated fatty acids, vitamin E and selenium with the help of feed additives, and to assessment of impact of the latter on health and productivity of poultry.

Methods. Trials were held in the animal facility Zagorskoye Center for Genetics and Selection (Sergiev Posad, Moscow Region) in 2016-2017 on SP 789 cross layers (*Gallus gallus* L.) aged 140 to 200 days and held in poultry cages (5 layers in each cage) at intermittent lighting 2C:5T:3C:2T:3C:9T.

Six gropus, each comprising of 30 layers, were formed for determination of necessary dosage of ω-3 PUFA, vitamin E and selenium by the analogue method. Group I (control) was given standard diet (basic diet, BD) comprising of wheat (56.1%), bran (11.1 %), soy bean meal (9.3 %), sunflower cake (9.3 %), sunflower oil (3.0 %); ω-6 and ω-3 PUFA content is 3.62% and 0.14% respectively, their ratio is 25.9:1; synthetic vitamin E (DL-α-tocopherol) content is 10 g, pure selenium (sodium selenite as a source) makes 0.2 g/t of compound feed. In test groups II and III, sunflower oil in BD was totally replaced by flaxseed oil (3 %), cutting the share of wheat, soy bean meal, sunflower cake and wheat bran down to 55.7; 7.0; 8.2 and 9.9 respectively, with adding 5% of flaxseed cake. In test group II, selenium came from preparation Sel-Plex® (Alltech, USA), in test group III the Se source was DAFS-25 (LLC Sulfat, Russia). In the diet of test groups II and III, the amount of ω-6 and ω-3 PUFA was 2.09% and 1.97% respectively, at 1.06:1; vitamin E (DL-α-tocopherol) was 100 g/t, pure selenium was 0.5 g/t of compound feed. In test groups IV-VI, sunflower oil was also replaced with flaxseed oil, with reducing the content of soy bean meal, sunflower cake and wheat bran to 4.7%. 7.1% and 8.9% respectively, and adding 10% flaxseed cake to the diet. Test groups IV, V and VI were was fed Sel-Plex®, DAFS-25, and sodium selenite, respectively. In these diets, ω-6 and ω-3 PUFA content

was 1.82% and 2.35% respectively (at 0.77:1) with 150 g vitamin E (DL-α-tocopherol) and 0.5 g pure selenium per ton of compound feed. Diets of all groups were supplemented with 0.01% enzyme preparations Feedbest W (xylanase and β-glucanase) and Feedbest P (3-phytase) (LLC Sibbiofarm, Russia).

Seven groups, each comprising of 30 layers, were formed for determination of complex enrichment of edible chicken eggs with ω-3 PUFA, non-organic and organic forms of vitamin E and selenium by analogue method. Group I (control, BD) was fed wheat (57.2%), bran (5.5%), soy bean meal (10.4%), sunflower cake (8.6%), corn gluten (3.0%), sunflower oil (4.0%). Content of ω-6 and ω-3 PUFA was 3.69% and 0.12% respectively (at 30.8:1), vitamin E (DL-α-tocopherol) is 10 g/t, pure selenium (source: sodium selenite) is 0.2 g/t compound feed. In test groups II-IV, 3% sunflower oil was replaced with flaxseed oil, content of wheat, bran and soy bean meal was reduced to 56.8%; 4.3% and 6.5% respectively; sunflower cake increased to 9.1% and 5% of flaxseed cake added. In test groups II, III and IV selenium came from Sel-Plex®, Sel-Plex® and DAFS-25 (1:1), and Sel-Plex® and sodium selenite (1:1). Content of ω-6 and ω-3 PUFA in the diets is 2.49% and 2.16% respectively (at 1.15:1), with 150 g/t vitamin E (DL-α-tocopherol) and 0.5 g/t pure selenium. In test groups V-VII, sunflower oil (4%) in BD was replaced with flaxseed oil (3%) in combination with fat production waste (a substrate containing over 90% fats, 280 μg/g natural carotenoids and over 11300 μg/g α-tocopherola, the organic form of vitamin E) (1.5%), with adding flaxseed cake (5%), reducing bran and soy bean meal (down to 2.64% and 6.53% respectively) and increasing sunflower cake contents (up to 9.63%). Se-source in test groups V, VI and VII was Sel-Plex®; Sel-Plex® and DAFS-25 (1:1); and Sel-Plex® and sodium selenite (1:1). Content of ω-6 и ω-3 PUFA was 2.50% and 2.23% (at 1.12:1), with 150 g/t organic vitamin E (D-α-tocopherol) and 0.5 g/t pure selenium. Diets of all groups were supplemented with enzyme preparation Record (100 g/t feed; CJSC Ferment, Belarus).

Livability of population was controlled on a daily basis, live weight control involved individual weighing of the entire population aged 140 and 200 days. Egg production per laying hen was estimated by the number of eggs laid by each group; egg weight by individual weighing of each egg the hens laid within 3 consecutive days in the middle of each month; output per hen (weight) — by the number of eggs laid and average weight of the eggs for each group; output per hen for category — by weighing and visual inspection of the eggs laid by the hens within 3 consecutive days in the middle of each month (national standard of the Russian Federation GOST 31654-2012 “Edible Chicken Eggs. Specifications”). Feed consumption was estimated by daily control of the feed served and feed stock available at the end of each week; feed consumption per 10 eggs and per 1 kg of egg weight was calculated on the basis of data on feed consumption, egg production and egg output (by weight). Weight of the egg white, egg yolk and egg shell was determined by generally accepted methods in the middle of each month; contents of ω-3 PUFA, vitamin E and selenium in the yolk and in the egg white were quantified on days 30 and 60 days of examination.

Se weight fraction was measured by electrothermal atomic absorption spectrometry (spectrometer Duo 240 FS/240Z, Varian, USA). Samples were decomposed by microwave sample preparation system Milestone START D (Milestone Systems, Italy). Weight fraction of vitamin E was measured by high-performance liquid chromatography (chromatographic system Knauer, Knauer Engineering GmbH Industrieanlagen & Co., Germany). Samples were prepared by the commonly accepted alkaline saponification method followed by extraction with diethyl ether. Weight fraction of crude fat was measured using the Randall method and extractor VELP Ser148 (VELP, Italy); fatty acids composition was

studied by capillary gas-liquid chromatography using gas chromatograph Kristall-2000M (Chromatech, Russia). Methyl ethers of fatty acids were separated in capillary column Stabilwax®-DE (Restek, USA) (l = 60 m, internal Ø 0.32 mm, film thickness 0.5 mm) and registered by flame-ionization detector Kristall-2000M (CJSC SBC Chromatech, Russia).

Data processing proceeded by variation statistics in Microsoft Excel. Mean values (*M*) and standard error of mean (±SEM) are set forth in the tables. Reliability was estimated by the Student *t*-test. Variations were considered statistically significant at $p < 0.05$.

Results. The purpose of Test 1 was to estimate the dose of sources of ω-3 PUFA, vitamin E and selenium in the diet of layers. Over the 60-day period, livability of the flock in every trial group was 100% (Table 1). Live weight of layers aged 140 days was virtually the same in groups I-VI. However, the biggest live weight among the layers aged 200 days was in group II (1.4%-3.6% higher than in the remaining groups). Group III yielded the worst results, 0.9% below the control group. In terms of egg-laying capacity, groups II, III, V and VI were way ahead of the others with the negligible difference between them. Group I demonstrated the lowest egg-laying capacity, 0.6%-3.9% behind the others. Group II had the highest mean weight of the egg, mean egg weight output per layer, as well as the output of super and grade 1 eggs (respectively 4.5%-6.7%; 5.0%-8.4%; 3.7%-9.7% and 11.0%-23.8% higher than in the remaining groups). Group V yielded the smallest egg weight, while the control group yielded the smallest egg weight output. Tendency toward the drop in the egg weight was in the poultry receiving diet with 10% of flaxseed cake and pure selenium (0.5 g/t) in DAFS-25. The difference in weight of eggs was significant between group II and groups I, III-VI ($p < 0.001$). In recent studies [33], addition of 10% flaxseed cake to the diet increased egg production by 4% ($p < 0.05$), with the egg weight considerably lower than in control group. According to another report [34], increase in vitamin E content from 100 to 200 g/t boosted egg laying ($p < 0.01$) while the egg weight dropped ($p < 0.01$).

1. Body weight, egg production, egg quality and feed consumption in SP 789 cross layers fed with varying diets including ω-3 polyunsaturated fatty acids, vitamin E and selenium (*M*±SEM, Zagorskoye CGS, Sergiev Posad, Moscow Region, 2016-2017)

Indicator	Group (n = 30)					
	I (control)	II	III	IV	V	VI
Live weight, g:						
age 140 days	1307±16.42	1308±17.93	1312±11.31	1309±15.31	1308±13.60	1318±16.01
age 200 days	1616±26.29	1658±26.28	1601±27.72	1620±38.28	1613±22.16	1635±24.95
Egg production per layer, pcs.	47.5	49.2	49.0	47.8	49.3	49.4
Egg laying intensity, %	79.2	82.0	81.7	79.7	82.2	82.3
Mean weight of an egg, g	56.2±0.50	58.7±0.43*	55.4±0.49*	55.8±0.48	55.0±0.46	55.9±0.48*
Egg output by grade, %:						
Premium	2.04	1.94	1.97	1.32	1.35	1.97
Super (0)	3.40	10.97	5.92	7.24	1.35	1.32
1	50.34	61.29	37.50	43.42	45.95	48.03
2	40.14	21.94	49.34	44.08	48.97	45.39
3	0.68	0.64	1.32	1.31	0.67	0.66
breakage and cracks	3.40	3.22	3.95	2.63	2.70	2.63
Egg weight output per layer, kg	2.675	2.900	2.722	2.683	2.717	2.761
Feed consumption:						
per 1 hen/day, g	109.2	107.0	106.7	103.1	104.5	106.5
per 10 eggs, kg	1.38	1.31	1.31	1.29	1.27	1.29
per 1 kg of egg weight, kg	2.45	2.22	2.35	2.31	2.31	2.31
Cost per 1 t compound feed, RUB	14098	14504	14282	14374	14151	14104

N o t e. Groups and accounting methods (Test 1) are described in section Techniques.
* Difference with control group is statistically significant at $p < 0.001$.

The lowest feed consumption per hen for day was in group IV (1.3%-5.6% below the other groups), while group I (control) kept the maximum feed

consumption. The cost of feed per 10 eggs was the lowest in group V, and in terms of 1 kg of egg weight in group II (respectively 1.6%-8.0% and 3.9%-9.4% smaller than in the remaining groups). These indicators were the highest in control group where minimum egg production and maximum feed consumption per hen for day were recorded.

2. Morphological and chemical characterization of eggs laid by SP 789 cross layers fed various diets including ω-3 polyunsaturated fatty acids (PUFA)**, vitamin E and selenium** (*M*±SEM, Zagorskoye CGS, Sergiev Posad, Moscow Region, 2016-2017)

Indicator	Group (n = 30)					
	I (control)	II	III	IV	V	VI
Egg yolk weight:						
absolute, g	14.27±0.44	14.23±0.32	13.99±0.31	13.69±0.34	14.20±0.30	14.49±0.34
relative, %	23.95	23.63	24.44	23.38	24.70	24.18
Egg while weight:						
absolute, g	39.00±0.56	39.62±0.66	37.1±0.57*	38.54±0.46	37.08±0.49*	38.94±0.60
relative, %	65.45	65.79	64.80	65.80	64.51	65.00
Egg shell weight:						
absolute, g	6.32±0.10	6.37±0.13	6.16±0.08	6.34±0.10	6.20±0.09	6.48±0.08
relative, %	10.60	10.58	10.76	10.82	10.79	10.82
Contents per 100 g of the edible part of egg (bulk samples):						
Se, μg	28.3	61.6	58.2	61.8	58.9	43.1
vitamin E, mg	2.31	4.47	5.04	6.40	8.35	5.50
ω-6 PUFA, mg	2181	1630	1504	1535	1321	1410
ω-3 PUFA, mg	172	767	767	807	767	796
including						
α-linolenic, mg	69	539	537	547	577	515
ω-3-eicosapentaenoic, mg	12	22	26	25	28	29
ω-3-docosapentaenoic, mg	8.5	29	30	31	31	29
ω-3-docosahexaenoic, mg	64	148	146	174	152	144
ω-6/ω-3 PUFA ratio	12.7:1	2.1:1	2.0:1	1.9:1	1.7:1	1.8:1

N o t e. Groups and accounting methods (Test 1) are described in section Techniques.
* Difference with control group is statistically significant at $p < 0.05$.

According to morphological and chemical study of the eggs (Table 2), the groups did not differ too much in terms of the absolute and relative weight of the egg yolk over the trial period (60 days). Maximum absolute weight of the egg white was in group II (1.5%-6.9% above the other groups). The difference in this value proved to be significant between groups I and III, V ($p < 0.05$); II and III, V ($p < 0.01$); VI and III, V ($p < 0.05$); IV and V ($p < 0.05$). In terms of the relative weight of egg white, the leading groups were group II and IV where Se source was Sel-Plex®, i.e. the weight of eggs in these groups grew due to egg white. There were no considerable differences between the groups for absolute and relative weight of the egg shell.

In groups II-VI, vitamin E content in 100 g of the edible part of egg was 1.9-3.6 times higher than in the control. Increasing the dose of vitamin from 100 g/t to 150 g/t compound feed naturally increased its content in the egg itself. Similar correlation was observed in other studies [34, 35], i.e. vitamin E content in the egg directly depended on the dose thereof in the diet.

Selene content in 100 g of the edible part of egg in the test groups was 1.5-2.2 higher than in the control. Of all test groups, this value was the lowest in group VI where sodium selenite was used. Our data correlate with the findings of other authors [36] who report that selenium content in egg white and egg yolk depends on the dosage and the form of selenium in the layers' diet.

Deposition of ω-3 PUFA per 100 g of the edible part of egg in test groups was 4.5-4.7 times higher compared to control, with 7.5-8.4-fold α-linolenic acid, 1.8-2.4-fold eicosapentaenoic acid, 3.2-3.4-fold docosapentaenoic acid, and 2.3-2.7-fold docosahexaenoic acid. The ω-6/ω-3 PUFA ratio in groups II and VI was 1.7-2.1:1 vs. 12.7:1 in control. It should be noted that add-

ing 5% flaxseed cake (groups II and III) and 10% flaxseed cake (groups IV-VI) in combination with 3% flaxseed oil have very little impact on ω-3 PUFA in eggs, whereas decrease in ω-6 to ω-3 PUFA ratio occurs due to decreasing accumulation of ω-6 PUFA. The test groups where DAFS-25 was the Se source (III and V) and sodium selenite (VI) show a tendency toward decrease of ω-6/ω-3 PUFA ratio. These data correlate with the findings of N. Gjorgovska et al. [34] who reported an increase in the content of these fatty acids in the eggs of chickens fed with a higher dose of ω-3 PUFA (DHA and EPA). Fresh eggs and the eggs from control hens and groups II-III stored for 25 days at room temperature had no foreign odor or flavor. Fresh eggs from groups IV-VI had no foreign odor or flavor, too, but when these eggs were stored, a slight fishy odor and flavor emerged before and after cooking.

Complex enrichment of chicken eggs with ω-3 PUFA, selenium and vitamin E during the first test increased the cost of feed in groups II-VI by 0.04%-2.88% compared to the control group. That is why in the next experiment we endeavored to obtain similar results for biofortification of edible eggs without causing the increase in cost of the compound feeds.

In Test 2 (Table 3), livability in each group was also 100%. No significant difference in live weight of hens aged 200 days was detected. The highest productivity and egg weight output per layer was in group VII (2.4%-10.1% and 3.0%-13.2% higher than in other groups). Control group yielded the lowest values. Following the replacement of synthetic source of vitamin E with organic (fatty acids), mean egg weight increased in groups V-VII by 0.6-0.8 g or by 1.1%-1.4%, with group VII dominance. The lowest egg weight was in the control group, 0.6-1.6 g (or 1.1%-2.8%) lower than in other groups. Difference in these values between groups V-VII and group I was significant ($p < 0.05$). Available literature provides no data regarding the impact of any such fatty acid preparations on the effective accumulation of vitamin E in eggs.

3. Body weight, egg productivity, egg quality and feed consumption with SP 789 cross layers following optimization of diet by inclusion of ω-3 polyunsaturated fatty acids, non-organic and organic forms of vitamin E and selenium (*M*±SEM, Zagorskoye CGS, Sergiev Posad, Moscow Region, 2016-2017)

Indicator	Group (n = 30)						
	I (control)	II	III	IV	V	VI	VII
Live weight, g:							
age 140 days	1372±19.87	1370±22.68	1392±21.24	1396±23.51	1379±20.08	1366±22.37	1390±34.89
age 200 days	1543±22.87	1592±27.18	1615±32.28	1597±34.90	1602±30.08	1577±27.47	1566±29.76
Egg production per layer, pcs.	46.5	48.6	49.0	49.2	49.6	50.0	51.2
Egg laying intensity, %	77.6	81.0	81.7	82.1	82.7	83.3	85.4
Mean weight of an egg, g	54.9±0.48	55.5±0.44	55.6±0.40	55.7±0.36	56.1±0.34*	56.2±0.40	56.5±0.43*
Egg weight output per layer, kg	2.555	2.691	2.725	2.757	2.784	2.808	2.893
Feed consumption:							
per 1 hen/day, g	111.7	111.9	111.7	112.5	114.8	114.1	113.6
per 10 eggs, kg	1.44	1.38	1.37	1.37	1.39	1.37	1.33
per 1 kg of egg weight, kg	2.62	2.50	2.46	2.45	2.47	2.44	2.36
Cost per 1 t compound feed, RUB	14863	14965	14878	14855	14827	14711	14688

N o t e. Groups and accounting methods (Test 1) are described in section Techniques.
* Difference with control group is statistically significant at $p < 0.05$.

The lowest feed consumption rates (per 1vhen/day) were in groups I, II and III. Group V had the highest consumption (2.8% higher than control group). The lowest feed consumption per 10 eggs and 1 kg of egg weight (respectively 2.9-7.6 and 3.3%-9.9% smaller than in other groups) was in group VII where the maximum productivity and egg weight output per layer were observed.

Cutting the content of Sel-Plex® by half and balancing the diet with DAFS-25 in groups III and VI reduced the costs per 1 t compound feed by RUB 87 and RUB 116 (or 0.58% and 0.78%) respectively; and replacement of

50% of Sel-Plex® with sodium selenite in groups IV and VII reduced the costs per 1 t feed by RUB 110 and RUB 139 (or by 0.74% and 0.94%), respectively, as compared to groups and V. Replacement of the synthetic source of vitamin E with organic one (fatty acids from oil and fat production waste) in groups V-VII reduced the costs per 1 t compound feed by RUB 138-167 (or by 0.92%-1.12%) vs. groups II-IV. Group VII showed the lowest results, by RUB 175 (or 1.18%) lower than in control group and by RUB 23-277 (0.16%-1.85%) below groups II-VI.

The groups do not differ significantly in absolute and relative weight of the egg yolk, white and shell (Table 4). Se concentration in 100 g of the edible part of the egg in groups II-VII was 2.2-2.3 times, and vitamin E — 2.0-2.8 times higher than in the control group. Test groups show virtually similar Se content. A higher vitamin E concentration per 100 g of the edible part of egg was in group IV with its synthetic source and in group VII with its organic source. Accumulation of ω-3 PUFA per 100 g of the edible part of egg in groups II-IV was 3.4-5.0 times higher than in the control group, with 5.2-8.0-fold α-linolenic acid, 1.4-2.4-fold eicosapentaenoic acid, 1.5-3.2-fold docosapentaenoic acid, and 2.3-3.8-fold docosahexaenoic acid levels. Ratio ω-6 ω-3 PUFA in groups II-IV was 2.3-2.9:1 against 14.2:1 in control group. It should be noted that introduction of either synthetic or organic vitamin E in the feed caused the highest content of ω-3 and ω-6 PUFA per 100 g of the edible part of egg in groups where selenium sources were Sel-Plex® with sodium selenite in proportion 1:1 (groups IV and VII).

4. Morphological and chemical characterization of eggs laid by SP 789 cross layers fed optimized diets including ω-3 polyunsaturated fatty acids, non-organic and organic forms of vitamin E and selenium (*M*±SEM, Zagorskoye CGS, Sergiev Posad, Moscow Region, 2016-2017)

Indicator	Group (n = 30)						
	I (control)	II	III	IV	V	VI	VII
Egg yolk weight:							
absolute, g	12.38±0.31	12,58±0,32	12,56±0,28	12,64±0,34	12,72±0,39	12,80±0,32	12,89±0,43
relative, %	22.55	22,67	22,59	22,69	22,68	22,78	22,81
Egg while weight:							
absolute, g	36.32±1.15	36,77±1,18	36,81±1,04	36,89±0,97	37,16±0,97	37,15±0,95	37,34±1,02
relative, %	66.16	66,26	66,20	66,24	66,23	66,10	66,09
Egg shell weight:							
absolute, g	6.20±0.21	6,14±0,17	6,23±0,21	6,17±0,17	6,22±0,20	6,25±0,22	6,27±0,16
relative, %	11.29	11,07	11,21	11,07	11,09	11,12	11,10
Contents per 100 g of the edible part of egg (bulk samples):							
Se, μg	27.1	61,1	61,2	60,3	61,5	59,3	60,9
vitamin E, mg	3.10	6,16	7,04	7,95	8,19	8,59	8,82
ω-6 PUFA, mg	2717	2385	2312	2445	1915	2035	2148
ω-3 PUFA, mg	192	881	790	960	655	778	949
including							
α-linolenic, mg	77	618	502	581	400	490	544
ω-3-eicosapentaenoic, mg	11	25	19	26	15	19	20
ω-3-docosapentaenoic, mg	17	36	26	45	34	33	55
ω-3-docosahexaenoic, mg	87	202	243	308	206	236	330
ω-6/ω-3 PUFA ratio	14.2:1	2,7:1	2,9:1	2,5:1	2,9:1	2,6:1	2,3:1

N o t e. Groups and accounting methods (Test 1) are described in section Techniques.

According to P. Weill et al. [37], the use of 5% dietary extruded flaxseed, as compared to standard feed, results in 3.8 times higher content of n-3 PUFA in edible eggs, including 2.4 times higher DHA. Findings of I.D. Bean et al. [38] also indicate a significantly (at $p < 0.001$) increased content of linolenic acid, DHA and total ω-3 fatty acids in the eggs of layers fed with flaxseed.

Therefore, simultaneous use of dietary ω-3 polyunsaturated fatty acids (PUFA), selenium and vitamin E in tested doses will boost content of these substances in edible eggs without negative impact on livability and productivity of hens. For complex enrichment of edible eggs with ω-3 PUFA, vitamin E and

selenium, one needs to use dietary flaxseed oil and cake (3% and 5% respectively, with adding enzyme preparation), 150 g/t vitamin E and 0,5 g/t selenium. Replacement of half-dose of Sel-Plex® with DAFS-25 and sodium selenite and synthetic vitamin E (DL-α-tocopherol) with its organic source (D-α-tocopherol) enhances productivity and total egg weight per layer, while cutting the feed consumption per unit of product and the costs. Best results are achieved when Se sources are Sel-Plex® and sodium selenite (1:1) and the organic source of vitamin E are fatty acids of fat production waste. This group 4.9 times exceeded the control group for ω-3 PUFA content per 100 g of the edible part of egg (with ω-6/ω-3 PUFA ratio 2.3:1 against 14.2:1 in control group), including 7.1-fold increase in α-linolenic acid, 1.8-fold increase in eicosapentaenoic acid, 3.2-fold increase in docosapentaenoic acid, 3.8-fold increase in docosahexaenoic acid, 2.8-fold increase in vitamin E, and 2.2-fold increase in selenium. Layers' productivity is 10.1% higher, egg weight output per layer is 13.2% higher, whereas feed consumption per 10 eggs and 1 kg of egg weight reduces by 7.6% and 9.9% respectively, and feed consumption reduces by 1.2%.

REFERENCES

1. Siru I., Kápolna E., Kápolna B., Lugasi A. Functional food. Product development, marketing and consumer acceptance — A review. *Appetite*, 2008, 51: 456-467 (doi: 10.1016/j.appet.2008.05.060).
2. Kavtarashvili A.Sh., Stefanova I.L., Svitkin V.S., Novotorov E.N. Functional egg production. I. The role of -3 polyunsaturated fatty acids (review). *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2017, 52(2): 349-366 (doi: 10.15389/agrobiology.2017.2.349eng).
3. Perić L., Rodić V., Milošević N. Production of poultry met and eggs as functional food: challenges and opportunities. *Biotechnology in Animal Husbandry*, 2011, 27(3): 511-520 (doi: 10.2298/BAH1103511P).
4. Leskanich C.O., Noble R.C. Manipulation of the n-3 polyunsaturated fatty acid composition of avian eggs and meat. *World Poultry Sci. J.*, 1997, 53(2): 155-183 (doi: 10.1079/WPS19970015).
5. Simopoulos A. Evolutionary aspects of diet and essential fatty acids. *World Rev. Nutr. Diet*, 2001, 88: 18-27 (doi: 10.1159/000059742).
6. Stoll A. *The omega-3 connection*. Simon and Schuster, NY, 2001: 40.
7. Surai P. *Kormi i fakti*, 2016, 4(68): 4-8 (in Russ.).
8. Simopoulos A.*P. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed. Pharmacother., 2002, 56(8): 365-379* (doi: 10.1016/S0753-3322(02)00253-6).
9. Sparks N.H.C. The hen's egg — is its role in human nutrition changing? *World Poultry Sci. J.*, 2006, 62(2): 308-313 (doi: 10.1079/WPS200599).
10. Okolelova T.M. *Ptitsevodstvo*, 2013, 5: 15-19 (in Russ.).
11. Antongiovanni M., Minieri S., Buccioni A., Galligani I., Rapaccini S. Transfer of dietary fatty acid from butyric acid fortified canola oil into the meat of broiler. *Ital. J. Anim. Sci.*, 2009, 8(Suppl. 2): 754-756 (doi: 10.4081/ijas.2009.s2.754).
12. Shapira N. Every egg may have a targeted purpose: toward a differential approach to egg according to composition and functional effect. *World Poultry Sci. J.*, 2010, 66(2): 271-284 (doi: 10.1017/S0043933910000322).
13. Surai P. *Kombikorma*, 2010, 6: 95-96 (in Russ.).
14. Arkhipov A.V. *Lipidnoe pitanie, produktivnost' ptitsy i kachestvo produktov ptitsevodstva* [Lipid nutrition, poultry performance and quality of poultry products]. Moscow, 2007 (in Russ.).
15. Griminger P. Lipid metabolism. In: *Avian physiology*. P.D. Sturkey (ed.). Springer, NY, 1986: 345-358.
16. Hargis P.S., Van Elswyk M.E., Hargis B.M. Dietary modification of yolk lipid with menhaden oil. *Poultry Sci.*, 1991, 70(4): 874-883 (doi: 10.3382/ps.0700874).
17. Guyonnet V. Eggs and egg products: consumers' attitudes, perceptions and behaviors. *Proc. XXIV World's Poultry Congress (5-9 August 2012 Salvador, Bahia-Brazil)*. Salvador, 2012: 1-10.
18. Komprada T. Eicosapentaenoic and docosahexaenoic acids as inflammation-modulating and lipid homeostasis influencing nutraceuticals: A review. *J. Funct. Food.*, 2012, 4(1): 25-38 (doi: 10.1016/j.jff.2011.10.008).
19. Plourde M., Cunnane S.C. Extremely limited synthesis of long chain polyunsaturates in adults: implications for their dietary essentiality and use as supplements. *Appl. Physiol. Nutr. Me.*, 2007, 32(4): 619-634 (doi: 10.1139/H07-034).
20. Scheideler S.E., Froning G.W. The combined influence of dietary flaxseed variety, level, form and storage conditions on egg production and composition among vitamin E-supplemented hens. *Poultry Sci.*, 1996, 75(10): 1221-1226 (doi: 10.3382/ps.0751221).

21. Bubel F., Dobrzański Z., Bykowski P., Patkowska-Sokoła B., Trziszka T. Enrichment of hen eggs with omega-3 polyunsaturated fatty acids — physiological and nutritional aspects. *Acta Sci. Pol., Medicina Veterinaria*, 2011, 10(3): 5-18.
22. Caston L.J., Leeson S. Dietary flax and egg composition. *Poultry Sci.*, 1990, 69(9): 1617-1620 (doi: 10.3382/ps.0691617).
23. Newkirk R. *Flax: feed industry guide*. Flax Canada 2015 Inc., Winnipeg, MB, 2008.
24. Morris D.H. *Flax: A health and nutrition primer*. Flax Council of Canada, Winnipeg, MB, 2007.
25. Shen Y., Feng D., Oresanya T.F., Chavez E.R. Fatty acid and nitrogen utilization of processed flaxseed by adult chickens. *J. Sci. Food Argic.*, 2005, 85(7): 1137-1142 (doi: 10.1002/jsfa.2073).
26. Feng D., Shen Y., Chavez E.R. Effectiveness of different processing methods in reducing hydrogen cyanide content of flaxseed. *J. Sci. Food Agric.*, 2003, 83(8): 836-841 (doi: 10.1002/jsfa.1412).
27. Alzueta C., Rodriguez M.L., Cutuli M.T., Rebole A., Ortiz L.T., Centeno C., Trevino J. Effect of whole and demucilaged linseed in broiler chicken diets on digesta viscosity, nutrient utilization and intestinal microflora. *Brit. Poultry Sci.*, 2003, 44(1): 67-74 (doi: 10.1080/0007166031000085337).
28. Slominski B.A., Meng X., Campbell L.D., Guenter W., Jones O. The use of enzyme technology for improved energy utilization from full-fat oilseeds. Part II: Flaxseed. *Poultry Sci.*, 2006, 85(6): 1031-1037 (doi: 10.1093/ps/85.6.1031).
29. Egorova T.A., Lenkova T.N. Rapeseed (*Brassica napus* L.) and its prospective usage in poultry diet (review). *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2015, 50(2): 172-182 (doi: 10.15389/agrobiology.2015.2.172eng).
30. Jia W., Slominski B.A., Guenter W., Humphries A., Jones O. The effect of enzyme supplementation on egg production parameters and omega-3 fatty acid deposition in laying hens fed flaxseed and canola seed. *Poultry Sci.*, 2008, 87(10): 2005-2014 (doi: 10.3382/ps.2007-00474).
31. Van Elswyk M.E. Nutritional and physiological effects of flaxseed in diets for laying fowl. *World Poultry Sci. J.*, 1997, 53(3): 253-264 (doi: 10.1079/WPS19970020).
32. Kavtarashvili A.Sh., Stefanova I.L., Svitkin V.S., Novotorov E.N. Functional egg production. II. The roles of selenium, zinc, and iodine (review). *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2017, 52(4): 700-715 (doi: 10.15389/agrobiology.2017.4.700eng).
33. Cherian G., Quezada N. Egg quality, fatty acid composition and immunoglobulin Y content in eggs from laying hens fed full fat camelina or flax seed. *J. Anim. Sci. Biotechno.*, 2016, 7: 15 (doi: 10.1186/s40104-016-0075-y).
34. Gjorgovska N., FIlev K. Multi-enriched eggs with omega 3 fatty acids, vitamin E and selenium. *Archiva Zootechnica*, 2011, 14(2): 28-35.
35. Maziar M.A, Shariatmadari F., Loftollahian H., Mazuji M.T. Effects of supplementing layer hen diets with selenium and vitamin E on egg quality, lipid oxidation and fatty acid composition during storage. *Can. J. Anim. Sci.*, 2008, 88: 475-483.
36. Pappas A.C., Karadas F., Surai P.F., Speake B.K. The selenium intake of the female chicken influences the selenium status of her progeny. *Comp. Biochem. Phys. B*, 2005, 142(4): 465-474 (doi: 10.1016/j.cbpb.2005.09.013).
37. Weill P., Schmitt B., Chesneau G., Daniel N., Safraou F., Legrand P. Effects of introducing linseed in livestock diet on blood fatty acid composition of consumers of animal products. *Ann. Nutr. Metab.*, 2002, 46(5): 182-191 (doi: 10.1159/000065405).
38. Bean I.D., Leeson S. Long-term effects of feeding flaxseed on performance and egg fatty acid composition of brown and white hens. *Poultry Sci.*, 2003, 82(3): 388-394 (doi: 10.1093/ps/82.3.388).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 799-810
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.5:636.086.78

doi: 10.15389/agrobiology.2018.4.799eng
doi: 10.15389/agrobiology.2018.4.799rus

ADDITION OF Quercus cortex EXTRACT TO BROILER DIET CHANGES SLAUGHTER INDICATORS AND BIOCHEMICAL COMPOSITION OF MUSCLE TISSUE

V.A. BAGIROV[1], G.K. DUSKAEV[2], N.M. KAZACHKOVA[2], Sh.G. RAKHMATULLIN[2], E.V. YAUSHEVA[2], D.B. KOSYAN[2], SH.A. MAKAEV[2], Kh.B. DUSAEVA[3]

[1]*Ernst Federal Science Center for Animal Husbandry,* Federal Agency of Scientific Organizations, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail vugarbagirov@mail.ru;
[2]*Federal Research Centre of Biological Systems and Agrotechnologies RAS,* Federal Agency of Scientific Organizations, 29, ul. 9 Yanvarya, Orenburg, 460000 Russia, e-mail gduskaev@mail.ru (✉ corresponding author), vniims.or@mail.ru, vasilena56@mail.ru, shahm2005@rambler.ru, kosyan.diana@mail.ru;
[3]*Orenburg State University*, 13, prosp. Pobedy, Orenburg, 460018 Russia, e-mail albordusaeva@mail.ru
ORCID:
Bagirov V.A. orcid.org/0000-0001-5398-8815
Duskaev G.K. orcid.org/0000-0002-9015-8367
Kazachkova N.M. orcid.org/0000-0002-0871-736X
Rakhmatullin Sh.G. orcid.org/0000-0003-0143-9499
Yausheva E.V. orcid.org/0000-0002-1589-2211
Kosyan D.B. orcid.org/0000-0002-2621-108X
Makaev Sh.A. orcid.org/0000-0001-5836-0330
Dusaeva Kh.B. orcid.org/0000-0001-9176-0694

Acknowledgements:
Samples were analyzed in the Shared Use Center FRC BSAT RAS (Ros.RU № 000121 PF59 of 12.10.15) and in testing laboratory of the Center for Biotic Medicine NPO (Registration Certificate of ISO 9001: 2000, Number 4017-5.04.06).
Supported financially by Russian Science Foundation (project № 16-16-10048)
Received February 17, 2018

Abstract

Following world trends in feeding farm animals, it should be noted an increasing interest in polyphenolic substances derived from plant extracts. Recent studies indicate the ability of these substances to stimulate growth of animals and poultry, to reduce the risk of diseases and to improve consumer properties of products. This paper is the first our report to show that dietary Quercus cortex extract, when used separately or in combination with amylolytic and cellulolytic enzymes, promotes slaughter indices of Smena 8 cross broilers and can change meat composition on fatty acids and some microelements. For the experiment, 7-day chicken broilers were divided by analogs' method into 4 groups (n = 30, a total of 120 birds). Control group received main diet, Quercus cortex extract was added to the diets of groups I and II (2.5 ml/kg LW), group II and group III were also fed with an enzymatic preparation containing glucoamylase and concomitant cellulolytic enzymes (5 g/10 kg of feed). Dietary Quercus cortex extract led to an increase in pre-slaughter weight by 4.4-16.6 % over the entire experiment as compared to the control broilers, and caused changes in small intestine microbiota. The counts of *Bacteroidetes* and *Firmicutes* phyla were 5.1 % and 4.0 % higher, respectively, while *Proteobacteria* counts decreased by 3.2 %. Enzyme supplement increased the abundance of *Bacteroidetes*, *Firmicutes* and *Proteobacteria* phyla by 7.3 %, 6.5 % and 5.8 %, and combination of the enzyme preparation with the extract of oak bark increased the counts of *Actinobacteria* phyla by 9.0 %. In group II, the dry matter content in pectoral muscle was 1.29 % higher ($p \leq 0.05$) and crude fat was 1.35 % higher ($p \leq 0.05$), in group I and group III these values increased by 0.76 and 0.87 %, and by 0.08 and 0.33 %, respectively. The total concentration of unsaturated fatty acids was almost at the same level, and some peculiarities have been identified for only certain acids. The most obvious one was a decline in monounsaturated myristoleinic acid level in the experimental groups ($p \leq 0.05$) while the level of palmitoleic acid was rising ($p \leq 0.05$). In group I, the concentration of linolenic acid was higher ($p \leq 0.05$) compared to that in all test groups and the control group. The total concentration of saturated fatty acids in muscle samples of control and test groups also remained practically unchanged and leveled due to long-chain fatty acids which content in the test groups increased vs. a decrease in myristic acid level ($p \leq 0.05$). As a result of Quercus cortex extract supplement, a significant increase in magnesium level ($p \leq 0.05$) was found in group I. In all test groups, we observed a decrease in the level of calcium ($p \leq 0.05$) and iron, zinc, copper, cobalt and iodine, the trace elements ($p \leq 0.05$), in the broilers' muscle tissue as compared to the control group. Thus, the dietary Quercus cortex extract contributes to the increase in slaughter indices of broilers, can change fatty acid and elemental profile of muscle tissue and influences the microbiome of small intestine.

Keywords: Quercus cortex extract, broilers, muscle tissue, chemical composition, fatty acids, bioelements

The development of resistance to antimicrobial drugs and discussed possibility of transferring the resistance genes from animals to humans causes the growing concern that leads to the refusal of using feed antibiotics. However, the exclusion of them from diets decreases the efficiency of technologies in livestock and poultry farming [1].

One of the global trends in the practice of feeding farm animals and poultry is the growing interest to the perspectives of using plant extracts, including those containing tannins. Thus, the performed studies point out to the growth-promoting effect of these compounds to various animals and birds species [2, 3], while medicinal plants and tannins can reduce the risk of emergence of animal diseases [4-6] and change the consumer properties of a product [7-9]. The positive effect of the tannin derivatives of *Emblica officinalis* (Emblica, Indian Gooseberry) on the baby chickens' humoral immune responses against the coccidiosis infection [10] has been noted. The ability of garlic and pennywort to improve the growth of broiler chickens and to cause the positive changes the intestinal microbial communities and the fatty acid composition of the pectoral muscles has been studied; also these plants exhibit the immunomodulating effect and control the intestinal enteropathogens that influence on the results of feed conversion [11]. It is known that some medicinal and edible plants have a positive effect on the growth and economic effectiveness when growing poultry, for example, the positive effect of certain medicinal herbal mixtures on the lipid metabolism in the liver and on the organism's antioxidant status has been reported [12-15].

Poultry meat has a lot of beneficial nutritional properties, the important one of which is low content of lipids and relatively high content of polyunsaturated fatty acids [16-18].

The application of multienzyme complexes causes the active growth of intestinal microflora and the loss of a part of the substances reduced by enzymes. The suppression of microflora enables an increase in productive effect of enzymes significantly (by 30-35%). The combined use of an antibiotic and an enzyme preparation was discussed as a promising approach [19, 20]. It was reported that nitrogen digestibility increased by 37.0% and the pronounced synergism of these substances has been noted [19].

In the present paper, it was shown for the first time that the inclusion of the extract of Quercus cortex in the enzyme-containing diet of broilers contributes to the improvement of the slaughter qualities of poultry, changes in the fatty acid and elemental profile of muscle tissue and in the structure of the intestinal microbiome.

Our objective was to study the effect of Quercus cortex extract on the productive and quality parameters of poultry with enzyme containing diet.

Techniques. The researches were conducted in vivarium conditions (Orenburg region, 2017) on the chickens of the Smena 8 cross (n = 120). The 7-day-old broiler chickens selected for the experiment were divided into 4 groups using the analog method (n = 30). During the experiment, all the birds were in the same living conditions. The basal diets (BD) have been composed with taking into account the recommendations of the All-Russian Research and Technological Institute of Poultry (V.I. Fisinin, I.A. Egorov, T.N. Lenkova and others Guidelines for Optimizing the Recipes of Combined Feeds for Poultry. Moscow, 2009).

According to the scheme of experiment, the control group had been receiving the BD, the experimental group I — BD + extract of Quercus cortex (2.5 ml per 1 kg of body weight), experimental group II — BD + extract of

Quercus cortex (2.5 ml/kg of body weight) + enzyme preparation (5 g per 10 kg of feed), experimental group III — BD + enzyme preparation (5 g per 10 kg of feed). The enzyme preparation (GlucoLux-F, Sibbiopharm, Russia) containing glucoamylase and accompanying cellulolytic enzymes (xylanase, β-glucanase, cellulase) has been applied according to the manufacturer's standards. The birds were fed twice a day; the feed intake was accounted daily. The birds' living conditions and the procedures when conducting the experiments met the requirements of the instructions and recommendations of the Russian Specifications (Order of the Ministry of Health of the USSR No. 755 dated August 12, 1977) and of The Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996). Every effort to minimize the birds' suffering and to reduce the number of used samples has been made. The birds' decapitation under the Nembutal ether has been made on the 42nd day.

To prepare extract of Quercus cortex, 50 g of crushed bark (dosage form) were poured with 500 ml of hot (70 °C) distilled water, heated in a boiling water bath for 30 min, percolated and filtered (White Ribbon ashless filters, d 70 mm, ApexLab, Russia). The filtered extract was analyzed by the chromatography-mass spectrometry using a GQCMS 2010 Plus gas chromatograph with the mass-selective detector (Shimadzu, Japan) on the HP-5MS column. For the results interpreting the GCMS Solutions and GCMS PostRun Analysis (Shimadzu, Japan) software was used; for the compound identification the set of CAS spectra libraries (https://www.cas.org), NIST08 (https://www.nist.gov), Mainlib (http://catalog.mainlib.org), Wiley9 (http://www.sisweb.com) has been used. The number of identified components was evaluated by the relative value (%) correlating the peak area of a component with the total peak area of the extract.

The chemical composition of the broiler tissues after the slaughter was determined by standard methods (State Standards GOST 13496.15-97, GOST 51479-99, GOST 23042-86, GOST 25011-81, GOST R 53642-2009), for analysis of fatty-acid composition of the muscle tissue a Crystal-4000 Lux gas chromatograph (NPF Meta-Chrom LLC, Russia) and the Lyumakhrom liquid chromatograph (Lyumeks, Russia) (GOST 51486-99) were used.

In analyzing the elemental composition of the tissues, the biosubstrates were ashed in the MD-2000 microwave decomposition system (PerkinElmer, Inc., USA). The element content in ash was determined (Elan 9000 mass-spectrometer, Optima 2000 V atomic emission spectrometer, PerkinElmer, Inc., USA).

The samples content of the broiler small intestine were placed into the sterile Eppendorf microtubes with the snap-on lid (Nuova Aptaca S.R.L., Italy); DNA was extracted and purified according to the modified method [21]. DNA purity (according to OD_{260}/OD_{280}) was assessed with a NanoDrop spectrophotometer (Thermo Scientific, USA), for measuring concentration (ng/μl) a Qubit 2.0 fluorimeter (Invitrogen/Life Technologies, USA) was used. DNA concentration was measured 3 times: after extraction, after the first polymerase chain reaction (PCR) with the specific 16S prokaryotic primers, and after the second PCR with the adapters and indices of Nextera XT protocols. The analysis of microflora was made by metagenomic sequencing (Illumina MiSeq, Illumina, USA) with the MiSeq® Reagent Kit v3 (600 cycles). The bioinformatic processing of the results was performed with PEAR software (Pair-End AssembeR, PEAR v0.9.8) [22].

Filtering, dereplicating, removing chimeric sequences, clustering, sorting (single-tons cutoff) and removing the contamination were performed in the USEARCH program (usearch v8.0.1623_i86linux32, http://drive5.com/usearch).

We used -fastq_filter algorithm for filtering, -derep_prefix algorithm for replication, and -cluster_otus algorithm for clustering and deleting chimeric sequences [23-26]. The VAMPS resource (Visualization and Analysis of Microbial

Population Structures, https://vamps.mbl.edu/) [27] was used for visualization. The sequencing results were processed with Microsoft Excel software package.

The experiment was completed with the balance experiments for determining the feed digestibility and nutrients utilization by the birds [28]. Ammonia in the average sample of the bird manure was fixed with the 0.1 N oxalic acid (4 ml per 100 g of manure). Upon the completion of a balance experiment, the samples were dried at 60-70 °C and kept in airtight container. The loss of substances and the amount of assimilated feed were calculated from daily accounting of the manure weight and composition.

The statistical processing was performed using SPSS Statistics 20 software (IBM, USA). The mean (M) and root-mean-square deviations ($\pm\sigma$) as well as the standard deviation errors ($\pm$SEM) were calculated. Nonparametric Wilcoxon test was used for comparison of the variants. The differences were considered statistically significant at $p < 0.05$.

Results. In the prepared oak bark extract, we have identified 35 compounds (Table 1) among which we detected the substances (10%) exhibiting the activity against the first-type Quorum Sensing system. The data on the investigation of the biological properties of these substances are partly presented by us earlier [29, 30].

1. Chemical compounds identified in the prepared extract of the Quercus cortex oak's bark

Name of the identified compound (under IUPAC))	Peak area, %
Propantriol-1,2,3*	3.56
Decane*	0.30
2-furancarboxylic acid*	0.30
1,3,5-triazin-2,4,6-triamine*	0.59
Pentadecane*	0.25
2,3-dihydroxypropanal*	0.35
Butanedioic acid*	0.30
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one*	1.19
2-amino-9-[3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-3H-purin-6-one*	0.59
Cyclopentane-1,2-diol**	0.30
1,2:5,6-dinghydrogalaccitol**	0.89
5-hydroxymethylfurfurol*	1.98
Acetylcysteine, (R)-2-acetamido-3-mercaptopropanoic acid*	0.89
1-methylundecyl ester of 2-propenoic acid**	1.39
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one**	0.79
1-(2-hydroxyethyl)-4-methylpiperazine**	1.33
6-(4-hydroxy-6-methoxy-2-methyl-tetrahydro-pyran-3-yloxy)-2-methyl-dihydro-pyran-3-one**	0.79
1,2,3-trihydroxybenzene*	0.99
2-methyl-5-nitropyrimidine-4,6-diol**	0.99
4-hydroxy-3-methoxybenzaldehyde*	0.53
2-amino-9-[3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-3H-purin-6-one*	25.6
1,6-anhydro-β-D-glucopyranose*	6.14
1-(β-D-arabinofuranosyl)-4-O-trifluoromethyluracil**	0.99
4-hydroxy-3-methoxybenzoic acid**	0.69
1,6-anhydro-β-D-glucofuranose*	0.89
4-propyl-1,3-benzenediol*	1.38
1,2,3,4,5-cyclohexanpentol*	36.38
4-(hydroxymethyl)-2,6-dimethoxyphenol*	0.37
4-(3-hydroxy-1-propenyl)-2-methoxyphenol*	4.45
9 -[(2R, 3R, 4S, 5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-3H-purin-2,6-dione*	0.30
7-hydroxy-6-methoxy-2H-1-benzopyran-2-one*	0.48
methyl-α-D-glucopyranoside*	1.19
2H-1-benzopyranone-2*	0.30
2-ethoxy-6-(methoxymethyl)-phenol**	0.75
3,4,5-trimethoxyphenol**	1.79

* The components identified with the probability > 90%
** The components identified with the probability < 90%

The dietary Quercus cortex extract in group I led to the increase in preslaughter weight by 4.4% compared to the control, and in the weight of the semi-eviscerated carcass by 4.7% (by the end of the experiment). In the experi-

mental group II, with enzyme containing diet, the pre-slaughter weight increased by 16.6% ($p \leq 0.05$) and the weight of the semi-eviscerated carcass by 21.5%. In the groups, the share of muscle tissue in the weight of the semi-eviscerated carcass amounted to 55.5-57.3%. The positive effect of tannin containing preparations on the slaughter parameters of broiler chickens is known [31]; in particular, it has been noted upon the inclusion in the diet of the chestnut-based additives with 77%tannin content. These data have been confirmed in our research, and when comparing the obtained results, it is necessary to take into account that the concentration of tannins in the Quercus cortex extract is less than 20%.

The investigation of the muscle tissue chemical composition (Table 2) revealed the increase in the content of dry matter by 1.29% ($p \leq 0.05$) and of crude fat by 1.35% ($p \leq 0.05$) compared to the control in test group II, and by 0.76 and 0.87%, 0.08 and 0.33%, respectively, in test groups I and III. In tes groups there was a tendency to increase the protein content in pectoral muscles. This is consistent with the data of the previously performed assessment of the influence of polyphenolic substances on the chemical composition of meat and the results of feeding broilers with exogenous amylase in combination with xylanase [32, 33].

2. Chemical composition (%) of pectoral muscle tissue of 42-day-old broiler chickens of Smena 8 cross fed with dietary oak bark extract and biopreparation with amylolitic and cellulolytic activity ($M \pm$SEM, $n = 11$, the experiment in vivarium conditions)

Parameter	Group			
	control	I	II	III
Mass fraction of dry matter	25.19±0.17	25.61±0.27	26.48±0.42*	25.72±0.89
Mass fraction of crude fat	4.54±0.64	5.56±1.02	5.89±0.40*	5.81±0.85
Mass fraction of protein	20.10±0.51	20.57±0.50	20.77±0.11	20.63±0.71
Mass fraction of ash	0.96±0.01	0.96±0.01	0.97±0.01	0.97±0.02

N o t e. Control group was fed with basal diet (BD); test group I received BD + extract of Quercus corte; test group II received BD + extract of Quercus cortex + enzyme preparation; test group III received BD + enzyme preparation.

* Differences with the control group are statistically significant at $p \leq 0.05$

3. Fatty-acid profile (%) of the pectoral muscle tissue of 42-day-old broiler chickens of Smena 8 cross fed with dietary oak bark extract and biopreparation with amylolitic and cellulolytic activity ($M \pm$SEM, $n = 11$, the experiment in vivarium conditions)

Fatty acids	Group			
	control	I	II	III
Unsaturated fatty acids in total	69.78	70.09	69.49	70.18
of which the monounsaturated fatty acids	36.52	36.09	37.23	36.99
Saturated fatty acids in total	29.36	30.69	30.09	30.17
Myristic $C_{14:0}$	0.63±0.23	0.53±0.15*	0.50±0.10*	0.59±0.17
Myristolein $C_{14:1}$	0.13±0.13	0.10±0.13	0.10±0.12*	0.12±0.15
Palmitic $C_{16:0}$	19.03±1.69	20.13±0.35	20.76±0.73	19.81±0.98
Palmitoleic $C_{16:1}$	2.46±0.61	3.53±0.51*	4.10±0.54*	3.77±0.71
Stearic $C_{18:0}$	9.70±1.02	10.03±0.58	8.83±0.97	9.77±0.83
Oleic $C_{18:1}$	33.93±0.89	32.46±0.27	33.03±0.69	33.10±0.77
Linoleic $C_{18:2}$	1.50±0.15	1.50±0.21	1.56±0.19	1.49±0.18
Linolenic $C_{18:3}$	31.76±1.43	32.50±0.32**	30.70±0.79	31.70±0.97

N o t e. See the groups' description in Table 1.

* Differences with the control group are statistically significant at $p \leq 0.05$

** Differences with the experimental group II are statistically significant at $p \leq 0.05$

The energy content in the diet and feed intake are the most important factors influencing the accumulation of lipids in the muscle tissue. The additional energy in the diets of the experimental groups contributed to the increase of the feed utilization efficiency and could be deposited as fat in the muscles. The analysis of the fatty-acid profile of the broiler muscle tissue (Table 3) showed that in the control and test groups the sum of unsaturated fatty acids was almost

the same. At the same time, the peculiarities of accumulation of some fatty acids have been revealed. In particular, the decrease in the content of monounsaturated myristoleic acid by 23.1% both in the test group I ($p \leq 0.05$) and in II ($p \leq 0.05$) upon the increase of the share of palmitoleic acid ($p \leq 0.05$) by 43.5 and 66.7% respectively was the most obvious. The content of polyunsaturated long-chain fatty acids has not being changing significantly. Previously, the reduction of the share of monounsaturated fatty acids in the bird muscle tissue upon the inclusion in the diet of phenolic compounds (tannin, gallic acid, thymol) has been noted by other authors [34]. There is the data on the increasing of the content of polyunsaturated fatty acids in the broiler chicken muscle after the addition of thymol, carvacol and other polyphenols to the feed [11, 35]. Our data are consistent with this, i.e. the content of linolenic acid in test group I was 2.3-5.8% higher ($p \leq 0.05$) compared to control and other test groups. The content of saturated fatty acids in muscle tissues of the control and test groups also remained almost unchanged. It should be noted that such leveling of the content occurred mostly owing to the long-chain fatty acids vs. a 15.8-20.6% reduction of myristic acid in test groups ($p \leq 0.05$). The metabolites of phenolic compounds (tannins and other substances) including those contained in the extract of Quercus cortex have antioxidant properties [36-38] that could influence the fatty-acid profile of the muscle tissue.

It is quite probably that some of the biologically active substances contained in the plant extracts and performing protective functions in the plant tissues may ambiguously influence birds [39, 40], in particular, assimilation of microelements, therefore we investigated the elemental status of the broiler muscle tissue (Table 4).

4. Content (μg/g) **of essential elements in pectoral muscle tissue of 42-day-old broiler chickens of Smena 8 cross fed with dietary oak bark extract and biopreparation with amylolitic and cellulolytic activity** (M±SEM, n = 11, the experiment in vivarium conditions)

Element	Group			
	control	I	II	III
Ca	202±20	151±15*	156±16*	172±15
P	2608±261	2741±274	2515±251	2687±270
K	4231±423	4556±456	4600±460*	4495±431
Mg	315±31	375±37*	344±34	361±29
Na	775±78	638±64	539±54*	597±65
Zn	17.1±1.71	9.54±0.95	7.26±0.73*	8.93±0.98
Mn	0.207±0.025	0.135±0.016*	0.138±0.017*	0.143±0.020
Cu	0.694±0.083	0.309±0.037*	0.317±0.038*	0.433±0.044
Fe	13.76±1.38	6.25±0.62*	6.85±0.68*	7.55±0.95
Co	0.0019±0.00037	0.0008±0.00023*	0.0007±0.00022*	0.0010±0.00033*
Se	0.181±0.022	0.148±0.018	0.134±0.016*	0.157±0.020
I	0.116±0.014	0.039±0.006*	0.011±0.002*	0.088±0.007

N o t e. See the groups' description in Table 1.

* Differences with the control group are statistically significant at $p \leq 0.05$.

After the inclusion of the Quercus cortex extract in the diet, the accumulation of magnesium in test group I increased significantly ($p \leq 0.05$). This is probably related to its high concentration in the extract itself (1628 rg/g), as well as to the ability to form the weak complexes with chemical elements in the gastrointestinal tract and to more efficient reabsorption [41].

In test groups the calcium content in muscle tissue decreased ($p \leq 0.05$) compared to control. This can be explained by the formation of tannin-calcium complexes in the gastrointestinal tract of chickens that is implicitly confirmed by the literature data [42]. The calcium oxalate formation was found to be inhibited in laboratory animals fed with the extract of *Sargassum wightii* Greville ex J. Agardh, which also contains phenolic metabolites and tannins.

Magnesium and calcium are antagonists, and the increase in consumption of one of them causes the increased excretion of another one; moreover, a lot of researches have shown that as the calcium consumption increases, its absorption decreases [41]. In our experiment, the calcium concentration in the extract of Quercus cortex was the highest, 0.012 mg/g.

In test groups, the accumulation of three microelements, iron, zinc and copper, decreases ($p \leq 0.05$) that coincides with the data about decreasing of the zinc and copper content in the liver of monogastric animals fed with vegetable products (extract of grape marc) containing polyphenolic substances [43]. In other research [44], the authors suggested that the absorption of Ca, P, Mg, Na, K, Fe, and Co decreases when a high tannin content in the diet (1.36% of the dry matter of the diet). The results of our experiment agree with this. In addition, it is known that tannin fixes iron in the intestinal lumen that, in turn, affects the growth of microorganisms [45]. The fact of interaction with each other of the microelements contained in the broiler diets is no less important [46, 47].

Exogenous enzymes in the broiler diet had no apparent effect on the muscle tissue's chemical composition.

In the starting period, the digestibility of crude protein in test group I was 4.0% lower than in the control, but in the second growing period, this parameter, on the contrary, increased by 4.2%. The highest digestibility in both periods was noted in group II, i.e. in the first period, the difference with control was in raw protein 3.6%, in crude fiber 0.7%, in crude fat 5.7% ($p \leq 0.05$), in the second period these were 1.7; 7.7 and 3.8%, respectively. The tannin ability to bind with enzymes is known, and differences in the chemical structure of these polyphenols can influence on such interactions [48] and, as a result, on metabolic processes which are being changing during different periods of poultry growing. At a certain dose, tannins have a positive effect on productivity [49]. A similar effect (fluctuations in the digestibility of substances during growth) is observed in our experiment.

The use of the oak bark extract in the diet led to the changes in the quantitative composition of microorganisms in the small intestine of broiler chickens. The increase in the number of the gram-negative, non-sporeforming anaerobic rod-shaped bacteria of the *Bacteroidetes* phylum (by 5.1%) and of the bacteria of *Firmicutes* phylum, which have mostly gram-positive cell wall type (by 4.0%) and decrease in the number of the *Proteobacteria* group (by 3.2%) were noted. The changes were mainly related with the increase in the number of microorganisms of *Bacteroidia* (by 5.2%) and *Bacilli* (by 6.5%) classes and with the decrease in the representation of the *Gammaproteobacteria* (by 3.3%) and *Clostridia* (by 4.3%) classes. The investigation of the species composition showed the increase in the number of bacteria of the *Bacteroides* (by 4.9%), *Clostridium* (by 8.4%) and *Lactobacillus* (by 7%) genera as compared to control.

The enzyme additive caused the increase in the number of microorganisms of the *Bacteroidetes* (by 7.3%), *Firmicutes* (by 6.5%) and *Proteobacteria* (by 5.8%) phyla. The changes were mainly related with the increase in the number of representatives of the *Bacteroidia* (by 6.1%), *Bacilli* (by 14.4%) and *Clostridia* (by 17.1%) classes. The counts of bacteria of *Bacteroides* and *Clostridium* genera increased by 9.5% and 7.8%, respectively.

The combination of the enzyme additive with the oak bark extract increases the number of the *Actinobacteria* phylum bacteria (by 9.0%) and reduces *Bacteroidetes* phylum microorganisms (by 6.7%) that is natural when changing of the population of *Actinobacteria* and *Bacteroidia* classes. In the *Firmicutes* taxon, the increase in the number of the *Bacilli* bacteria (by 35.5%) and the decrease in the number of *Clostridia* microorganisms (by 36.8%) were observed. The number

of bacteria of *Lactobacillus* genus increased by 34.7%, of *Corynebacterium* increased by 9.0% and of *Clostridium* genus increased by 5.7%. The other investigations have also demonstrated the inhibition of the clostridia growth by the tannin containing substances [31] and the large changeability of the microbiome of the broiler' small intestine influenced by tannins [7[.

5. Representation of bacterial taxa in the small intestine of 42-day-old broiler chickens of Smena 8 cross fed with dietary oak bark extract and biopreparation with amylolitic and cellulolytic activity (M±SEM, n = 5, the experiment in vivarium conditions)

Phylum, class	Family	Genus
	Control	
Phylum *Bacteroidetes* (8.8±0.12 %):		
Bacteroidia (8.8±0.06 %)	*Bacteroidaceae* (7.95±0.11 %)	*Bacteroides* (7.95±0.09 %)
Phylum *Actinobacteria* (7.89±0.22 %):		
Actinobacteria (7.89±0.12 %)	*Microbacteriaceae* (2.86±0.08 %)	No data
	Nitriliruptoraceae (4.6±0.13 %)	*Nitriliruptor* (4.6±0.10 %)
Phylum *Firmicutes* (76.1±0.13 %):		
Clostridia (56.0±1.02 %)	*Lachnospiraceae* (16.3±0.23 %)	No data
	Ruminococcaceae (21.1±0.17 %)	*Ruminococcus* (15.8±0.14 %)
	Clostridiaceae (17.5±0.17 %)	*Clostridium* (3.2±0.12 %)
		Faecalibacterium (4.2±0.44 %)
Bacilli (19.6±0.3 %)	*Lactobacillaceae* (18.7±0.08 %)	*Lactobacillus* (18.7±0.13 %)
Phylum *Proteobacteria* (6.13±0.56 %):		
Gammaproteobacteria (5.7±0.87 %)	*Moraxellaceae* (5.64±0.01 %)	*Acinetobacter* (5.6±0.12 %)
	Group I	
Phylum *Firmicutes* (80.1±0.23 %):		
Bacilli (26.1±0.14 %)	*Lactobacillaceae* (25.7±0.18 %)	*Lactobacillus* (25.7±0.13 %)
Clostridia (51.7±0.12 %)	*Clostridiaceae* (25.7±0.81 %)*	*Faecalibacterium* (4.54±0.13 %)
		Clostridium (8.7±0.33 %)
	Ruminococcaceae (14.6±0.11 %)	*Pseudoflavonifractor* (5.03±0.10 %)
		Ruminococcus (3.02±0.08 %)
	Lachnospiraceae (11.1±0.14 %)	*Blautia* (2.06±0.06 %)
Phylum *Proteobacteria* (2.95±0.09 %)*:		
Gammaproteobacteria (2.35±0.14 %)*	No data	No data
Phylum *Bacteroidetes* (14±0.12 %):		
Bacteroidia (14.0±0.02 %)	*Bacteroidaceae* (12.9±0.11 %)	*Bacteroides* (12.9±0.19 %)
Phylum *Actinobacteria* (2.73±0.17 %)*:		
Actinobacteria (2.23±0.05 %)*	No data	No data
	Group II	
Phylum *Firmicutes* (74.3±0.25 %):		
Bacilli (55.1±0.42 %)*	*Lactobacillaceae* (53.4±1.21 %)	*Lactobacillus* (53.4±0.98 %)
	Aerococcaceae (2.34±0.12 %)	No data
Clostridia (19.2±0.09 %)*	*Clostridiaceae* (13.1±0.18 %)	*Clostridium* (8.9±0.35 %)*
	Lachnospiraceae (2.28±0.13 %)	No data
Phylum *Actinobacteria* (16.9±0.12 %)*:		
Actinobacteria (16.9±0.44 %)	*Corynebacteriacea* (17.9±0.11 %)	*Corynebacterium* (16.8±0.22 %)
Phylum *Proteobacteria* (5.2±0.23 %):		
Gammaproteobacteria (5.2±0.75 %)	*Peptostreptococcaceae* (5.4±0.12 %)	*Romboutsia* (2.63±0.51 %)
Phylum *Bacteroidetes* (2.18±0.78 %)*:		
Bacteroidia (2.18±0.41 %)*	*Bacteroidaceae* (2.18±0.19 %)	*Bacteroides* (2.18±0.74 %)
	Group III	
Phylum *Bacteroidetes* (16.1±0.05 %)*:		
Bacteroidia (14.9±0.12 %)	*Bacteroidaceae* (9.9±0.31 %)	*Bacteroides* (17.4±0.09 %)
Phylum *Actinobacteria* (8.9±0.16 %):		
Actinobacteria (7.8±0.18 %)	*Microbacteriaceae* (3.8±0.12 %)	No data
	Nitriliruptoraceae (4.8±0.77 %)	*Nitriliruptor* (2.3±0.04 %)
Phylum *Firmicutes* (82.6±0.12 %):		
Clostridia (73.1±0.17 %)	*Lachnospiraceae* (14.1±0.09 %)	No data
	Ruminococcaceae (21.1±0.08 %)	*Ruminococcus* (11.6±0.12 %)
	Clostridiaceae (29.5±0.07 %)*	*Clostridium* (11±0.06 %)*
		Faecalibacterium (5.0±0.10 %)
Bacilli (34.0±0.11 %)*	*Lactobacillaceae* (28.3±0.08 %)	*Lactobacillus* (19.9±0.22 %)
Phylum *Proteobacteria* (11.9±0.08 %)*:		
Gammaproteobacteria (5.7±0.07 %)	No data	No data

N o t e. See the groups' description in Table 1.

* Differences with the control group are statistically significant at $p \leq 0.05$.

Thus, the slaughter parameters of broilers improve both upon the addition of the Quercus cortex extract to the diet and when using it with enzyme containing diet. The content of some saturated and unsaturated fatty acids (pal-

mitoleic and linolenic) in the muscle tissue increases when feeding the birds with the said extract, but the fatty acid profile remains almost the same. Also, in the test groups, the content of dry matter and fats in the pectoral muscle increases, and the feed digestibility changes depending on the growing period. The detected decrease in the amount of certain macro- and microelements (Ca, Fe, Zn, Cu, Co and I) in the muscle tissue is conditioned by the peculiarities of the extract composition and dosage as well as by the synergistic interactions of chemical elements. After the inclusion of the Quercus cortex extract in the diet, the number of *Bacteroidetes* and *Firmicutes* phyla microorganisms in the thin intestine increases, and the count *Proteobacteria* decreases. Combination of the extract and enzyme containing diet increases the abundance of *Actinobacteria* phylum, and the enzyme containing diet without adding the extract increases abundance of *Bacteroidetes, Firmicutes* and *Proteobacteria* phyla.

REFERENCES

1. Redondo L.M., Chacana P.A., Dominguez J.E., Fernandez Miyakawa M.E. Perspectives in the use of tannins as alternative to antimicrobial growth promoter factors in poultry. *Front. Microbiol.*, 2014, 5: 118 (doi: 10.3389/fmicb.2014.00118).
2. Artem'eva O.A., Pereselkova D.A., Fomichev Yu.P. Dihydroquercetin, the bioactive substance, to be used against pathogenic microorganisms as an alternative to antibiotics. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2015, 50(4): 513-519 (doi: 10.15389/agrobiology.2015.4.513eng).
3. Shuhao W., Lin Z., Jiaolong L., Jiahui C., Feng G., Guanghong Z. Effects of dietary marigold extract supplementation on growth performance, pigmentation, antioxidant capacity and meat quality in broiler chickens. *Asian-Australas. J. Anim. Sci.*, 2017, 30(1): 71-77 (doi: 10.5713/ajas.16.0075).
4. Van Parys A., Boyen F., Dewulf J., Haesebrouck F., Pasmans F. The use of tannins to control *Salmonella typhimurium* infections in pigs. *Zoonoses Public Health*, 2010, 57: 423-428 (doi: 10.1111/j.1863-2378.2009.01242.x).
5. Anderson R.C., Vodovnik M., Min B.R., Pinchak W.E., Krueger N.A., Harvey R.B., Nisbet D.J. Bactericidal effect of hydrolysable and condensed tannin extracts on *Campylobacter jejuni* in vitro. *Folia Microbiol.*, 2012, 57: 253-258 (doi: 10.1007/s12223-012-0119-4).
6. Huyghebaert G., Ducatelle R., Van Immerseel F. An update on alternatives to antimicrobial growth promoters for broilers. *Vet. J.*, 2011, 187(2): 182-188 (doi: 1016/j.tvjl.2010.03.003).
7. Tosi G., Massi P., Antongiovanni M., Buccioni A., Minieri S., Marenchino L., Mele M. Efficacy test of a hydrolysable tannin extract against necrotic enteritis in challenged broiler chickens. *Italian Journal of Animal Science,* 2013, 12(3): e62 (doi: 10.4081/ijas.2013.e62).
8. Castaneda M., Hirschler E., Sams A. Skin pigmentation evaluation in broilers fed natural and synthetic pigments. *Poultry Sci.*, 2005, 84: 143-147 (doi: 10.1093/ps/84.1.143).
9. Duskaev G.K., Kazachkova N.M., Ushakov A.S., Nurzhanov B.S., Rysaev A.F. The effect of purified Quercus cortex extract on biochemical parameters of organism and productivity of healthy broiler chickens. *Veterinary World*, 2018, 11(2): 235-239 (doi: 10.14202/vetworld.2018.235-239).
10. Qari M.K., Masood A., Mian M.A., Muhammad S., Muddassar Z., Zafar I., Faqir M., Anwar M.I. Studies on *Emblica officinalis* derived tannins for their immunostimulatory and protective activities against coccidiosis in industrial broiler chickens. *The Scientific World Journal*, 2014, 2014: Article ID 378473 (doi: 10.1155/2014/378473).
11. Suriya K.R., Idrus Z., Nordiana A.A.R., Mahdi E., Meng G.Y. Effects of two herbal extracts and Virginiamycin supplementation on growth performance, intestinal microflora population and fatty acid composition in broiler chickens. *Asian-Australas. J. Anim. Sci.*, 2014, 27(3): 375-382 (doi: 10.5713/ajas.2013.13030).
12. Patel A.P., Bhagwat S.R., Pawar M.M., Prajapati K.B., Chauhan H.D., Makwana R.B. Evaluation of *Emblica officinalis* fruit powder as a growth promoter in commercial broiler chickens. *Veterinary World*, 2016, 9(2): 207-210 (doi: 10.14202/vetworld.2016.207-210).
13. Kassa S., Mengistu U., Animut G. Effect of different levels of *Lepidium sativum* L. on growth performance, carcass characteristics, hematology and serum biochemical parameters of broilers. *SpringerPlus*, 2016, 5(1): 1441 (doi: 10.1186/s40064-016-3118-0).
14. Shokraneh M., Ghalamkari G., Toghyani M., Landy N. Influence of drinking water containing Aloe vera (*Aloe barbadensis* Miller) gel on growth performance, intestinal microflora, and humoral immune responses of broilers. *Veterinary World*, 2016, 9(11): 1197-1203 (doi: 10.14202/vetworld.2016.1197-1203).
15. Shirzadegan K., Falahpour P. The physicochemical properties and antioxidative potential of raw

thigh meat from broilers fed a dietary medicinal herb extract mixture. *Open Vet. J.*, 2014, 4(2): 69-77.
16. Bourre J. Where to find omega-3-fatty acids and how feeding animals with diet enriched in omega-3-fatty acids to increase nutritional value derived products for human: What is actually useful? *J. Nutr. Health Aging*, 2005, 9(4): 232-242.
17. Wood J.D., Enser M., Nute G., Richardson R., Sheard P. Manipulating meat quality and composition. *Proc. Nutr. Soc.*, 1999, 58(2): 363-370 (doi: 10.1017/S0029665199000488).
18. Yalcin S., Onbaslar I., Sehu A., Yalcin S. The effect of dietary garlic powder on the performance, egg traits and blood serum cholesterol of laying quails. *Asian-Australas. J. Anim. Sci.*, 2007, 20: 944-950 (doi: 10.5713/ajas.2007.944).
19. Nechipurenko L.I., Dyukarev V.V. *Byulleten' VNII fiziologii, biokhimii i pitaniya sel'skokhozyaistvennykh zhivotnykh*, 1973, 2(28): 26-29 (in Russ.).
20. Miles R.D., Brown R.D. Jr., Comer C.W., Oelfke E. Influence of an enzyme and an antibiotic on broiler performance. *Journal of Applied Animal Research*, 1996, 9(2): 105-117 (doi: 10.1080/09712119.1996.9706112).
21. Andronov E.E., Pinaev A.G., Pershina E.V., Chizhevskaya E.P. *Nauchno-metodicheskie rekomendatsii po vydeleniyu vysokoochishchennykh preparatov DNK iz ob"ektov okruzhayushchei sredy* [Methodological recommendations on the isolation of highly purified DNA preparations from environmental objects]. St. Petersburg, 2011 (in Russ.).
22. Zhang J., Kobert K., Flouri T., Stamatakis A. PEAR: A fast and accurate Illumina Paired-End reAd merger. *Bioinformatics*, 2014, 30(5): 614-620 (doi: 10.1093/bioinformatics/btt593).
23. Edgar R.C., Flyvbjerg H. Error filtering, pair assembly and error correction for nextgeneration sequencing reads. *Bioinformatics*, 2015, 31(21): 3476-3482. (doi: 10.1093/bioinformatics/btv401).
24. Edgar R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, 2010, 26(9): 2460-2461 (doi: 10.1093/bioinformatics/btq461).
25. Edgar R.C., Haas B.J., Clemente J.C., Quince C., Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, 2011, 27(16): 2194-2200 (doi: 10.1093/bioinformatics/btr381).
26. Edgar R.C. UPARSE: highly accurate OTU sequences from microbial amplicon reads *Nature Methods*, 2013, 10(10): 996- 998 (doi: 10.1038/nmeth.2604).
27. Huse S.M., Mark Welch D.B., Voorhis A., Shipunova A., Morrison H.G., Eren A.M., Sogin M.L. VAMPS: a website for visualization and analysis of microbial population structures. *BMC Bioinformatics*, 2014, 15: 41 (doi: 10.1186/1471-2105-15-41).
28. Fisinin V.I., Egorov I.A., Okolelova T.M., Imangulov Sh.A. *Kormlenie sel'skokhozyaistvennoi ptitsy* [Feeding of poultry]. Sergiev Posad, 2010 (in Russ.).
29. Duskaev G.K., Deryabin D.G., Karimov I., Kosyan D.B., Notova S.V. Assessment of (in vitro) toxicity of Quorum Sensing inhibitor molecules of Quercus cortex. *Journal of Pharmaceutical Sciences and Research*, 2018, 10(1): 91-95.
30. Karimov I., Duskaev G., Inchagova K., Kartabaeva M. Inhibition of bacterial Quorum sensing by the ruminal fluid of cattle. *International Journal of GEOMATE*, 2017, 13(40): 88-92 (doi: 10.21660/2017.40.65948).
31. Schiavone A., Guo K., Tassone S., Gasco L., Hernandez E., Denti R., Zoccarato I. Effects of a natural extract of chestnut wood on digestibility, performance traits, and nitrogen balance of broiler chicks. *Poultry Sci.*, 2008, 87(3): 521-527 (doi: 10.3382/ps.2007-00113).
32. Starčević K., Krstulović L., Brozić D., Maurić M., Stojević Z., Mikulec Ž., Bajić M., Mašek T. Production performance, meat composition and oxidative susceptibility in broiler chicken fed with different phenolic compounds. *J. Sci. Food Agric.*, 2015, 95(6): 1172-1178 (doi: 10.1002/jsfa.6805).
33. Amerah A.M., Romero L.F., Awati A., Ravindran V. Effect of exogenous xylanase, amylase, and protease as single or combined activities on nutrient digestibility and growth performance of broilers fed corn/soy diets. *Poultry Sci.*, 2017, 96(4): 807-816 (doi: 10.3382/ps/pew297).
34. Hashemipour H., Kermanshahi H., Golian A., Veldkamp T. Effect of thymol and karvacrol feed supplementation on performance, antioxidant enzyme activities, fatty acid composition, digestive enzyme activities, and immune response in broiler chickens.. *Poultry Sci.*, 2013, 92: 2059-2069 (doi: 10.3382/ps.2012-02685).
35. Okuda T. Systematics and health effects of chemically distinct tannins in medicinal plants. *Phytochemistry*, 2005, 66(17): 2012-2031 (doi: 10.1016/j.phytochem.2005.04.023).
36. Brenes A., Viveros A., Goci I., Centeno C., Sáyago-Ayerdy S.G., Arija I., Saura-Calixto F. Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. *Poultry Sci.*, 2008, 87(2): 307-316.
37. Chamorro S., Viveros A., Rebolé A., Rica B.D., Arija I., Brenes A. Influence of dietary enzyme addition on polyphenol utilization and meat lipid oxidation of chicks fed grape pomace. *Food Research International*, 2015, 73: 197-203 (doi: 10.1016/j.foodres.2014.11.054).
38. Hashemi S.R., Davoodi H. Herbal plants and their derivatives as growth and health promoters in animal nutrition. *Vet. Res. Commun.*, 2011, 35(3): 169-180 (doi: 10.1007/s11259-010-9458-2).
39. Smulikowska S., Pastuszewska B., Święch E., Ochtabińska A., Mieczkowska A., Nguyen V.C., Buraczewska L. Tannin content affects negatively nutritive value of pea for monogastrics. *J.*

Anim. Feed Sci., 2001, 10(3): 511-523 (doi: 10.22358/jafs/68004/2001).
40. Oberlis D., Kharland B., Skal'nyi A. *Biologicheskaya rol' makro- i mikroelementov u cheloveka i zhivotnykh* [The biological role of macro- and microelements in humans and animals]. St. Petersburg, 2008 (in Russ.).
41. Sujatha D., Kiranpa Singh, Mursalin Vohra, Kumar K.V., Sunitha S. Antilithiatic activity of phlorotannin rich extract of *Sarghassum wightii* on calcium oxalate urolithiais — in vitro and in vivo evaluation. *Int. Braz. J. Urol.*, 2015, 41(3): 511-520 (doi: 10.1590/S1677-5538.IBJU.2014.0357).
42. Fiese A., Ehrmann M, Geßner D.K., Most E., Eder K. Effects of polyphenol-rich plant products from grape or hop as feed supplements on iron, zinc and copper status in piglets. *Archives of Animal Nutrition*, 2015, 69(4): 276-84 (doi: 10.1080/1745039X.2015.1057065).
43. Hassan I.A., Elzubeir E.A., El Tinay A.H. Growth and apparent absorption of minerals in broiler chicks fed diets with low or high tannin contents. *Tropical Animal Health and Production*, 2003, 35(2): 189-196 (doi: 10.1023/A:1022833820757).
44. Chung K.-T., Lu Z., Chou M.W. Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria. *Food and Chemical Toxicology*, 1998, 36(12): 1053-1060 (doi: 10.1016/S0278-6915(98)00086-6).
45. Andjelkovia M., Van Camp J., De Meulenaer B., Depaemelaere G., Socaciu C., Verloo M., Verhe R. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chem.*, 2006, 98(1): 23-31 (doi: 10.1016/j.foodchem.2005.05.044).
46. Bao Y.M., Choct M., Iji P.A., Bruerton K. Trace mineral interactions in broiler chicken diets. *Brit. Poultry Sci.*, 2010, 51(1): 109-117 (doi: 10.1080/00071660903571904).
47. Mondal S., Haldar S., Saha P., Ghosh T.K. Metabolism and tissue distribution of trace elements in broiler chickens' fed diets containing deficient and plethoric levels of copper, manganese, and zinc. *Biol. Trace Elem. Res.*, 2010, 137(2): 190-205 (doi: 10.1007/s12011-009-8570-z).
48. Hofmann T., Glabasnia A., Schwarz B., Wisman K.N., Gangwer K.A., Hagerman A.E. Protein binding and astringent taste of a polymeric procyanidin, 1,2,3,4,6-penta-O-galloyl-beta-D-glucopyranose, castalagin, and grandinin. *J. Agric. Food Chem.*, 2006, 54(25): 9503-9509 (doi: 10.1021/jf062272c).
49. Schiavone A., Guo K., Tassone S., Gasco L., Hernandez E., Denti R., Zoccarato I. Effects of a natural extract of chestnut wood on digestibility, performance traits, and nitrogen balance of broiler chicks. *Poultry Sci.*, 2008, 87(3): 521-527 (doi: 10.3382/ps.2007-00113).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 811-819
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.52/.58:591.132:612.343

doi: 10.15389/agrobiology.2018.4.811eng
doi: 10.15389/agrobiology.2018.4.811rus

THE EXOCRINE PANCREATIC FUNCTION IN CHICKEN (*Gallus gallus* L.) FED DIETS CONTAINING DIFFERENT INGREDIENTS

V.I. FISININ, V.G. VERTIPRAKHOV, A.A. GROZINA

Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Federal Agency of Scientific Organizations, 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail fisinin@land.ru, vertiprakhov63@mail.ru (✉ corresponding author), alena_fisinina@mail.ru
ORCID:
Fisinin V.I. orcid.org/0000-0003-0081-6336
Grozina A.A. orcid.org/0000-0002-3088-0454
Vertiprakhov V.G. orcid.org/0000-0002-3240-7636
The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Program on study of the mechanisms of digestive adaptation of mammals and poultry to different diets (Resolution of the Presidium RAS № 132 dated 05.07.2017)
Received April 17, 2018

Abstract

The ability of animal pancreas to adjust its exocrine function to the composition of a diet is still a controversial concept and open question. A bulk of experimental data is published to the present evidencing the ability of the pancreas to modify the composition of its secretion in accordance with the composition of the digested feed both in mammals (I.P. Pavlov, 1950; A.D. Sineshchokov, 1965) and poultry (P.P. Berdnikov, 1990; Ts.Zh. Batoev, 2001; V.G. Vertiprakhov, 2012). The supporters of the theory of non-parallel enzyme secretion argue that the pancreas can rapidly change enzymatic profile of the secretion in response to a change in the ingested feed composition (i.e., the ratio of protein, carbohydrates, and lipids). The problem of the pancreatic adaptation to feed composition for more effective digestion is especially urgent for intense commercial poultry production and highly productive commercial poultry crosses. The productive potential of these crosses cannot be effectively realized without detailed and comprehensive knowledge of the digestive function in poultry. There were the attempts of sampling of pure pancreatic juice by U.S. scientists (T.F. Degolier et al., 1999); however, their approach (cannulation of pancreatic ducts) did not find broad acceptance. The intestinal activity of the digestive enzymes is extensively studied in China (L.Q. Ren et al., 2012; H. Sun et al., 2013); however, their research did not elucidate the exact mechanism(s) of the adaptation of pancreatic secretion to feed quality. This paper was aimed at the investigation of exocrine pancreatic function in Hisex White chicken fed diets with different composition. The unique technique of transplantation of the pancreatic duct into the isolated duodenal section (allowing the collection of pancreatic juice during sampling periods and redirection of the juice into the duodenum at other times) enabled the sampling of pure pancreatic juice from live and healthy birds and the studying of its composition and changes induced by diet shifts. This technique of chronic fistulation provides new knowledge on the responses of enzymatic activity and chemical composition of the juice to the changes in diets. It was found that the pancreas can precisely adjust the enzyme-secreting activity to the composition of feed digested: large increase in dietary crude fat (by 20.8 %) resulted in a 33.8 % increase in the pancreatic lipase activity as compared to the basal level in starved birds. A moderate increase in dietary crude protein (by 3.3 %) and amino acids (by 2.1 %) resulted in substantial increase in proteolytic activity in the juice (by 28.1 %). The long-term adaptation of exocrine pancreas to a diet shift is related mostly to the basal levels of secretion, e.g. the basal level of lipase activity in pancreatic juice increases by 37.7 % after the increase in dietary crude fat. The short-term adaptation occurs in postprandial periods and involves both complex-reflex and neurochemical phases of the regulation of enzyme-secreting activity, e.g. the increases in lipase secretion at these phases after the shift to higher dietary crude fat level were 46.6 and 93.7 %, respectively. The concentration of total protein in pancreatic juice tended to increase in fed birds, in parallel to the increases in the secretion of individual enzymes.

Keywords: exocrine pancreatic function, chicken, Hisex White, adaptation of pancreas, feed composition, pancreatic enzymes, non-parallel secretion

Until now, the issue of animal pancreas ability to adapt to the diet com-

position is still controversial. There is a popular opinion about the identical changes in the activity of pancreatic juice enzymes. It is based mainly on findings for dogs which are being losing the pancreatic juice. In such animals, the pancreas ability to adapt the enzymatic composition of the secretion to the diet composition is largely impaired (1). In diseases of the digestive tract, as well as in overfeeding with certain food substances, in particular with fats, the pancreas ability to adapt its enzymes is also impaired [2-4].

There is a hypothesis that the pancreas responds to any food, regardless of its components, by releasing enzymes in the same proportions [5]. At the same time, there are a large experimental data pointing out the pancreas ability to change the composition of its secret according to the composition of consumed food [6-9], including data concerning poultry [10-14]. The supporters of such concepts postulate the possibility of an urgent change in the enzymes spectrum of the secret, depending on the type of consumed food. That is, carbohydrate food causes a predominant increase in exogenous amylase, protein food in proteases, and fatty food in lipase. In terms of morphology and physiology, the pancreas adaptation is ensured by changing the percent ratio of the enzymes in the course of synthesis, transportation and release of zymogenic granules in both individual acinar cells and in acini of topographically different areas of the gland [15]. The peculiarities of the induction of pancreatic secretion in birds as compared to mammals are discussed [16, 17]. Thus, basing on the analysis of the dose-dependent induction of amylase in vitro on isolated acini, the authors [17] have made the conclusion on the importance of the neuronal regulation, while the contribution of intestinal hormones, in their opinion, is not physiologically significant.

The studying of the adaptation of pancreas's secretion to the diet composition shall be based on the function of the nervous system and the humoral factors involved in the regulation of digestion. Thence, the optimal approach is to study the pancreatic secretion in a chronic experiment, conducted on a healthy animal pre-operated in a manner to exclude permanent loss of pancreatic juice. Due to the methodological difficulty of obtaining pure pancreatic juice in birds, the data on the adaptation of the pancreas secretory function in the scientific literature are scarce [18]. The studying of the digestive function basing on the activity of digestive enzymes is performed in the intestinal contents of fistulated bird [19-22], in pancreas tissue homogenates [23, 24] or by investigation of the digestibility of nutrients using the ileal availability method [25, 26], but such data do not allow studying mechanisms of complex reflex and neurohumoral phases of pancreas adaptation to the quality of food consumed.

Owing to the unique surgical transplantation of the pancreatic duct into the isolated piece of intestine, we have the possibility to obtain the pure pancreatic juice from the healthy chickens in the chronic experiment (Fig.) and to compare its properties as the diet changes.

The mechanisms of regulation of such process have not been studied fully, and further researches open up the perspectives for targeted enzymatic correction of the disorders of adaptability of the pancreas exocrine function in case of various diseases [27].

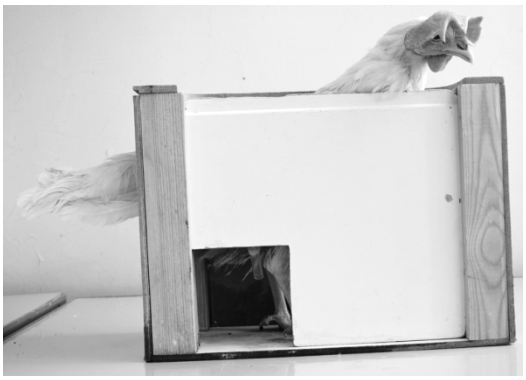
Our paper presents the first data on the chemical composition of the chicken pancreatic juice when using the diets with the different set of amino acids and other ingredients and on the indicators to character pancreatic enzyme activity.

The objective of the work was to study the exocrine function of the chicken pancreas under different dietary ingredients.

Techniques. The experiments were conducted on two 1-year old Hisex

White hens (*Gallus gallus* L.) which have been operated according to the method of Ts.Zh. Batoev and S.Ts. Batoeva [28]. For this, the 4-5 cm long piece was cut out from the duodenum and the main pancreatic duct was transplanted into it with the implantation of two L-shaped fistulas and formation of the external anastomosis which makes it possible to return the pancreatic juice to the duodenum in the period between the experiments.

The physiological tests were started in the morning when the hens were on an empty stomach after the 14-hour starvation. The birds were placed into the stall where they were kept for 3 hours. The microtube for collecting the pancreatic juice was attached to the fistula made of the isolated piece using the special rubber adapter. During the first 30 min, the juice was collected after the starvation, then the birds were given the portion of food (30 g) after that the secret was collected every 30 min during 180 min.



The hen with the chronic fistula of pancreatic duct during the experiment.

In biochemical analysis of the secret performed in 2 replications amylase was determined according to the Smith-Rhoy method in the modification for a high enzyme activity [29], proteases was determined according to Hammersten basing on the hydrolysis of purified casein under colorimetric control(λ = 450nm, KFK-3, Zagorsk Optic-Mechanical Plant OJSC, Russia) [11], and lipase was quantitated using a BS-3000P semi-automatic biochemical analyzer (SINNOWA Medical Science & Technology Co., Ltd., China) with a kit of veterinary diagnostic reagents for determining the lipase concentration in the blood of animals (DIACON-VET company, Russia).

1. Composition (%) and quality of used combined feeds

Ingredient, parameter	Control	Test
Wheat	58.224	55.781
Sunflower oilcake	5.000	21.026
Extracted soybean	19.784	8.912
Limestone (36%)	9.137	9.045
Soybean oil	1.936	3.026
Wheat bran	3.847	Not used
Monocalcium phosphate	1.149	1.233
Sodium salt	0.250	0.250
Lysine 98	0.073	0.214
Sodium sulfate	0.205	0.183
Fodder methionine 98	0.214	0.151
Mineral blend (0.08%)	0.080	0.080
Choline chloride	0.080	0.080
Vitamin blend (0.02%)	0.020	0.020
Nutritional value of 100 g of feed:		
metabolic energy, kcal	270.00	270.00
crude fat, g	6.72	8.12
crude fiber, g	4.89	5.92
crude protein, g	16.70	17.25

The amount of amino acids in the combined feed was determined by ion-exchange chromatography using postcolumn derivatization with the ninhydrin reagent and subsequent detection at λ = 570nm (λ = 440 nm for proline). A YL 9100 HPLC System for high-performance liquid chromatography (Young Lin Instrument Co., Ltd., Korea) which consists of YL9110 quaternary gradient pump, YL9101 vacuum degasser, YL9120 UV/VIS detector and YL9150 auto-sampler (Pinnacle PCX postcolumn derivator, ion-exchange column Na+ 4.0×150 mm, 5 μm, precolumn Na+ 3.0×20 mm, 5 μm) (Pickering Laboratories, Inc., USA) was used.

The statistical processing was performed with MS Excel software package; the mean values (*M*) and standard errors of the mean (±SEM) were cakculated. The significance of differences was evaluated by Student's *t*-test. The differences

were considered statistically significant at $p < 0.05$.

2. Amino acids content (%) in the used combined feeds

Amino acids	Control	Test	Change relative to control, %
Asparaginic acid	1.10	1.14	+3.6
Threonine	0.53	0.59	+11.3
Serine	0.67	0.69	+2.9
Glutamine	3.63	3.64	+0.3
Proline	1.09	0.98	–10.1
Glycine	0.69	0.81	+17.4
Alanine	0.63	0.69	+9.5
Cystine	0.28	0.28	Not changed
Valine	0.72	0.77	+6.9
Methionine	0.59	0.50	–17.3
Isoleucine	0.62	0.65	+4.8
Leucine	1.10	1.12	+1.8
Tyrosine	0.49	0.45	–8.2
Phenylalanine	0.74	0.75	+1.3
Lysine	1.15	1.14	–0.9
Histidine	0.41	0.43	+4.9
Arginine	1.06	1.10	+3.8
Total	15.50	15.83	

Results. The combined feed in the diets of hens (Table 1) differed in the content of crude fat and fiber, in test it was respectively 20.8 and 21.1% higher than in the control. The amount of crude protein in test differed from that in control insignificantly, but for protein quality the combined feed was selected to provide prevailing extracted soybean in the control, and sunflower oilcake in the test.

Total amino acids in the experiment were 2.1% more than in the control (Table 2). At the same time, the limiting methionine and lysine amino acids reduced by 17.3 and 0.9%, respectively, compared to the control but remained within the limits of relevant demands [30].

Table 3 presents the results of investigations of the pancreas secretory function. The obtained data show that upon the replacement of the control feed with the test sample, the lipase activity increased by 33.8% compared to control. This was due to a 20.8% increase in the amount of crude fat in the diet. The content of proteases increased by 28.1% as resulted from respectively 3.3% and 2.1% increase in crude protein and total amino acids in the feed. That is to say that the secretory function of the pancreas accurately adapts to the quality of the consumed food..

3. Secretory function of the pancreas of Hisex White chickens (*Gallus gallus* L.) under different compositions of feeds in the control and test periods of chronic experiment (M±SEM, $n = 20$)

Index	Control period	Test period	Change as to control,%
Amount of pancreatic juice during the experiment, ml	8.4±0.32	7.6±0.24	–9.5
Activity per 1 ml of pancreatic juice:			
amylase, mg/(ml · min)	4620±253.1	4855±290.0	+5.1
lipase, μmol/(ml · min)	6.5±0.51	8.7±0.62*	+33.8
proteases, mg/(ml · min)	267±17.9	342±61.3*	+28.1
Total protein, g/l	31.4±0.83	33.0±1.70	+5.1
Calcium, mmol/l	2.7±0.03	2.8±0.03	+3.7
Phosphorus, mmol/l	0.9±0.06	0.9±0.05	Not changed

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

To understand the mechanism of adaptation to the new feed, it is necessary to consider the time-course of the pancreatic juice secretion and of the enzyme activity after feeding (Table 4). The experimental data demonstrated that the time-course of the pancreatic juice secretion when using different feeds in the diet is different in the first (0-30th minute) and fourth (90-120th minute) periods of the chronic experiment. This corresponds to the concepts of the complex-reflex and neurochemical phases of regulation of the pancreas's exocrine function [11]. The matters of interaction of nerve and humoral mechanisms including those with the participation of trypsin in combination with nitrosyl iron complexes are still studied little and are the subject of our researches [31].

The obtained data show that the changes of lipase activity is most pronounced (see Table 4), There was a 37.7% increase before feeding, 46.6% in-

crease during the third period that was conditioned by the complex reflex phase of regulation of the pancreatic secretion, and 93.7% increase during minutes 120-150 which is related with the neurochemical phase of the regulation of secretion. This points out to the long-term adaptation to the second feed which causes the increase of lipolytic activity mainly in the phase of neurochemical regulation, i.e. in the period when the food masses digested in the stomach enter the duodenum thereby stimulating the release of secretin and cholecystokinin. When using the second feed, the proteolytic activity increased in the second, third and fourth periods of the experiment which correspond to the complex-reflex phase of the regulation of pancreatic secretion. During these periods, a stronger secretory response of the pancreas to the feed was observed (proteolytic activity increased 3.8 times compared to tthe basal level) that is apparently due to better taste of the second feed.

4. Release of pancreatic juice and enzyme activity in Hisex White chickens (*Gallus gallus* L.) under different compositions of feeds in the control and test periods of chronic experiment (M±SEM, n = 20)

Stage of the experiment in minutes	Pancreatic juice, ml	Activity		
		amylase, mg/(ml · min)	lipase, μmol/(ml · min)	proteases, mg/(ml · min)
0-30 (before feeding)	1.0±0.07	2570±434.0	5.3±0.65	108±17.5
	0.6±0.06*	3070±441.7	7.3±0.58*	92±20.8
30-60 (feeding)	1.4±0.13	4600±266.7	8.1±1.24	240±16.5
	1.3±0.09	4880±372.3	8.8±0.57	347±25.3*
60-90	1.5±0.07	5101±216.5	7.3±0.95	291±20.2
	1.6±0.09	5240±494.3	10.7±0.92*	416±37.7*
90-120	1.6±0.06	4954±398.5	6.6±1.35	308±22.7
	1.3±0.08*	4880±377.0	9.7±1.16	417±31.5*
120-150	1.5±0.08	5018±453.2	4.8±1.60	308±24.5
	1.5±0.08	5181±353.3	9.3±0.69*	394±32.5
150-180	1.4±0.07	5479±246.0	6.7±1.32	344±25.2
	1.3±0.07	6300±363.7	10.2±1.08	422±28.3

N o t e. Indices in the control and test periods are shown above and below the line, respectively.
* Differences with the control are statistically significant at $p < 0.05$.

S.S. Rothman [32] for the first time morphologically described the phenomenon of differences in secretion after the effect of nervous, humoral or food stimuli and called it non-parallel secretion, however many researchers did not agree with his findings. Meanwhile, as pointed out by S.S. Rothman [33, 34], the studying of this problem would not only contribute to the disclosure of the regulation mechanisms, but also would made it possible to manage the specific secretion of enzymes in various states of the organism. Later, in a number of studies performed on animals, such type of enzyme secretion in response to the endogenously released or exogenous stimulating or inhibiting substances has been confirmed [35-37]. Currently, the concept of “non-parallel” postprandial secretion of pancreatic enzymes has been recognized by most scientists [38-40]. Our experimental data are also in line with the hypothesis of such type of pancreatic enzyme secretion by. It should be noted that bulls showed the decrease of proteolytic activity of the pancreatic juice upon the increase of dietary protein has been discovered and described [41].

In our research, the pure pancreatic juice of chickens has been obtained owing to the unique operation for transplantation of the pancreatic duct to another place of the intestine; therefore, in the scientific literature similar data on the physicochemical properties of pancreatic juice are few [42]. In this work, we estimated several indices of pancreatic secretion. The analysis of total protein, calcium and phosphorus in the pancreatic juice has not revealed any differences between the effect of the control and the test feed (see Table 3), but studying dynamics of these indices after the feeding has made it possible to disclose some reg-

ularities of the secretion (Table 5). It is clear from the presented data that the total protein of the pancreatic juice tends to increase after the food intake (by 15.2-19.2%, $p < 0.05$). Similar changes occur in the activity of pancreas's enzymes. Cognate pattern of the content of organic matters in chicken pancreatic juice has been detected by S.G. Smolin [42]. In our test, the phosphorus content, on the contrary, slightly decreased ($p > 0.05$) 30 min after the feeding.

5. Changes in the content of total proteins, calcium and phosphorus in pancreatic juice of Hisex White chickens (*Gallus gallus* L.) under different compositions of feeds in the control and test periods of chronic experiment (M±SEM, $n = 20$)

Stage of the experiment in minutes	Control period			Test periods		
	total protein, g/l	calcium, mmol/l	phosphorus, mmol/l	total protein, g/l	calcium, mmol/l	phosphorus, mmol/l
0-30 (before feeding)	25.9±0.93	2.7±0.08	1.0±0.17	28.3±2.73	2.9±0.03	1.0±0.15
30-60 (feeding)	30.8±1.50*	2.6±0.06	0.7±0.06	32.6±1.57	2.8±0.03	0.8±0.09
60-90	31.3±0.87*	2.7±0.03	0.7±0.08	36.1±2.24*	2.8±0.03	0.9±0.21
90-120	32.3±1.22*	2.8±0.08	0.7±0.11	34.4±1.97*	2.8±0.04	1.0±0.19
120-150	33.7±1.02*	2.7±0.04	0.8±0.08	33.1±1.87	2.9±0.03	0.9±0.09
150-180	34.3±1.91*	2.7±0.03	1.4±0.19	33.7±1.56*	2.9±0.04	0.9±0.10

* Differences with the value prior feeding are statistically significant at $p < 0.05$.

Thus, basing on the obtained experimental data, we can conclude the following. The secretory function of chicken pancreas accurately adapts to the quality of the consumed food. Upon the increase of the crude fat content in the diet by 20.8%, the activity of the pancreatic juice lipase increases by 33.8%; a slight increase in crude protein (by 3.3%) and in the amino acids (by 2.1%) in the feed causes the 28.1% increase in proteolytic activity vs. the background level. The long-term adaptation of the pancreatic secretion to a new feed is mainly reflected in the basal secretion which increases by 37.7% upon the increase of the crude fat content in the feed. The urgent adaptation is well expressed in the postprandial period (complex reflex and neurochemical phases of the regulation of pancreatic secretory activity) when the lipase activity increases by 46.6 and 93.7%, respectively. The tendency to the increase in the total protein content in the pancreatic juice after feeding are noted that occurs simultaneously with the increase in the activity of pancreatic enzymes in the juice.

REFERENCES

1. Fomina L.S. V sbornike: *Fiziologiya pishchevareniya* /Pod redaktsiei A.V. Solov'eva [Physiology of digestion. A.V. Solov'ev (ed.)]. Leningrad, 1974: 360-369 (in Russ.).
2. Thomas T., Mah L., Barreto S.G. Systematic review of diet in the pathogenesis of acute pancreatitis: a tale of too much or too little? *Saudi J. Gastroenterol.*, 2012, 18(5): 310-315 (doi: 10.4103/1319-3767.101124).
3. Keller J., Layer P. Human pancreatic exocrine response to nutrients in health and disease. *Gut,* 2005, 54: 1-28.
4. Meyer J.H., Jones R.S. Canine pancreatic responses to intestinally perfused fat and products of fat digestion. *Am. J. Physiol. Gastrointest. Liver Physiol.,* 1974, 226: 1178-1187.
5. Morisset J. Seventy years of pancreatic physiology: take a look back. *Pancreas*, 2014, 43(8): 1172-1184 (doi: 10.1097/MPA.0000000000000226).
6. Lhoste E.F., Fiszlewicz M., Gueugneau A.-M., Corring T. Adaptation of exocrine pancreas to dietary proteins: effect of the nature of protein and rat strain on enzyme activities and messenger RNA levels. *The Journal of Nutritional Biochemistry*, 1994, 5(2): 84-94 (doi: 10.1016/0955-2863(94)90021-3).
7. Huguet A., Savary G., Bobillier E., Lebreton Y., Le Huërou-Luron I. Effects of level of feed intake on pancreatic exocrine secretions during the early postweaning period in piglets. *J. Anim. Sci.*, 2006, 84(11): 2965-2972 (doi: 10.2527/jas.2006-044).
8. Guilloteau P., Vitari F., Metzinger-Le Meuth V., Le Normand L., Romé V., Savary G., Delaby L., Domeneghini C., Morisset J. Is there adaptation of the exocrine pancreas in wild animal? The case of the Roe Deer. *BMC Veterinary Research*, 2012, 8: 70 (doi: 10.1186/1746-6148-8-70).
9. Harmon D.L. Impact of nutrition on pancreatic exocrine and endocrine secretion in ruminants: a review. *J. Anim. Sci.*. 1992, 70(4): 1290-1301.

10. Perepelkina L.I., Berdnikov P.P., Samsonenko I.A. *Vestnik Altaiskogo gosudarstvennogo agrarnogo universiteta*, 2012, 7(93): 67-68 (in Russ.).
11. Batoev Ts.Zh. *Fiziologiya pishchevareniya ptits* [Physiology of digestion in birds]. Ulan-Ude, 2001 (in Russ.).
12. Tesarivskaya T.B. *Uchenye zapiski ZabGGPU im. N.G. Chernyshevskogo (Chita)*, 2011, 1(36): 122-129 (in Russ.).
13. Vertiprakhov V.G. Pancreatic exocrine function in hens with addition of lysine and methionine to their ration. *Russian Agricultural Sciences*, 2015, 41(2-3): 171-174 (doi 10.3103/S1068367415020275).
14. Tsukanova E.S. *Sibirskii vestnik sel'skokhozyaistvennoi nauki*, 2009, 3: 129-132 (in Russ.).
15. Korot'ko G.F. *Fizicheskaya kul'tura i sport — nauka i praktika*, 2013, 1: 51-57 (in Russ.).
16. Wang B.J., Cui Z.J. How does cholecystokinin stimulate exocrine pancreatic secretion? From birds, rodents, to humans. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 2007, 292(2): R666-R678 (doi: 10.1152/ajpregu.00131.2006).
17. Murai A., Satoh S., Okumura J., Furuse M. Factors regulating amylase secretion from chicken pancreatic acini in vitro. *Life Sci.*, 2000, 66(7): 585-591 (doi: 10.1016/S0024-3205(99)00631-1).
18. DeGolier T.F., Place A.R., Duke G.E., Carraway R.E. Neurotensin modulates the composition of pancreatic exocrine secretions in chickens. *Journal of Experimental Zoology*, 1999, 283: 455-462 (doi: 10.1002/(SICI)1097-010X(19990301/01).
19. Ren L.Q., Zhao F., Tan H.Z., Zhao J.T., Zhang J.Z., Zhang H.F. Effects of dietary protein source on the digestive enzyme activities and electrolyte composition in the small intestinal fluid of chickens. *Poultry Sci.*, 2012, 91(7): 1641-1646 (doi: 10.3382/ps.201102081).
20. Sun H., Tang J.W., Yao X.H., Wu Y.F., Wang X., Feng J. Effects of dietary inclusion of fermented cottonseed meal on growth, cecal microbial population, small intestinal morphology, and digestive enzyme activity of broilers. *Trop. Anim. Health. Prod.*, 2013, 45: 987-993 (doi: 10.1007/s11250-012-0322-y).
21. Angel C.R., Saylor W., Vieira S.L., Ward N. Effect of a monocomponent protease on performance and protein utilization in 7- to 22-day-old broiler chickens. *Poultry Sci.*, 2011, 90(10): 2281-2286 (doi: 10.3382/ps.2011-01482).
22. Ai X.J., Han Z.K. Effect of cysteamine on the pancreatic secretion and enzymatic activity in geese. *Zhongguo Ying Yong Sheng Li Xue Za Zhi*, 2002, 18(3): 297-300.
23. Routman K.S., Yoshida L., Frizzas de Lima A.C., Macari M., Pizauro J.M., Jr. Intestinal and pancreas enzyme activity of broilers exposed to thermal stress. *Braz. J. Poult. Sci.*, 2003, 5(1): 23-27 (doi: 10.1590/S1516-635X2003000100003).
24. Fisinin V.I., Ocmanyan A. K., Mahdavi R., Egorov I.A. Nutrient density of prestarter diets from 1 to 10 days of age affects intestinal morphometry, enzyme activity, serum indices and performance of broiler chickens. *Anim. Nutr.*, 2017, 3(3): 258-265 (doi: 10.1016/j.aninu.2017.06.005).
25. Erdaw M.M., Perez-Maldonado R.A., Iji P.A. Apparent and standardized ileal nutrient digestibility of broiler diets containing varying levels of raw full-fat soybean and microbial protease. *Journal of Animal Science and Technology*, 2017, 59: 23 (doi: 10.1186/s40781-017-0148-2).
26. Gilani G.S., Xiao C.W., Cockell K.A. Impact of antinutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality. *Brit. J. Nutr.*, 2012, 108(52): 315-332 (doi: 10.1017/S0007114512002371).
27. Mozheiko L.A. *Novosti mediko-biologicheskikh nauk*, 2017, 15(1): 72-76 (in Russ.).
28. Vertiprakhov V.G., Egorov I.A. The influence of feed intake and conditioned reflex on exocrine pancreatic function in broiler chicks. *Open Journal of Animal Sciences*, 2016, 6(4): 298-303 (doi: 10.4236/Ojas.2016.64034).
29. Merina-Gluzkina V.M. *Laboratornoe delo*, 1965, 3: 142-146 (in Russ.).
30. Fisinin V.I., Egorov I.A., Lenkova T.N. et al. *Rukovodstvo po optimizatsii retseptov kombikormov dlya sel'skokhozyaistvennoi ptitsy* [Guide to optimize feed recipes for agricultural poultry]. Sergiev Posad. 2014: 3-4 (in Russ.).
31. Fisinin V.I., Vertiprakhov V.G., Grozina A.A. *Rossiiskaya sel'skokhozyaistvennaya nauka*, 2018, 1: 49-53 (in Russ.).
32. Rothman S., Liebow C., Grendell J. Nonparallel transport and mechanisms of secretion. *Biochim. Biophys. Acta*, 1991, 1071(2): 159-173 (doi: 10.1016/0304-4157(91)90023-P).
33. Rothman S.S., Isenman L.D. Secretion of digestive enzyme derived from two parallel intracellular pools. *Am. J. Physiol.*, 1974, 226(5): 1082-1087 (doi: 10.1152/ajplegacy.1974.226.5.1082).
34. Rothman S.S. The digestive enzymes of the pancreas: a mixture of inconstant proportions. *Annu. Rev. Physiol.*, 1977, 39: 373-389 (doi: 10.1146/annurev.ph.39.030177.002105).
35. Rossi J., Santamaki P., Airaksinen M.S., Herzig K.H. Parasympathetic innervation and function of endocrine pancreas the glial cell line-derived factor family receptor alpha2 (GFRalpha2). *Diabetes*, 2005, 54(5): 1324-1330 (doi: 10.2337/diabetes.54.5.1324).
36. Lindsay T.H., Halvorson K.G., Peters C.M., Ghilardi J.R., Kuskowski M.A., Wong G.Y., Mantyh P.W. A quantitative analysis of the sensory and sympathetic innervation of the mouse pancreas. *Neuroscience*, 2006, 137(4): 1417-1426 (doi: 10.1016/j.neuroscience.2005.10.055).
37. Fisinin V.I., Egorov I.A., Vertiprakhov V.G., Grozina A.A., Lenkova T.N., Manukyan V.A., Ego-

rova T.A. Activity of digestive enzymes in duodenal chymus and blood in broilers of parental lines and the meat cross depending on dietary bioactive additives. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2017, 52(6): 1226-1233 (doi: 10.15389/agrobiology.2017.6.1226eng).
38. Maouyo D., Morisset J. Modulation of pancreatic secretion of individual digestive enzymes in octreotide (SMS 201-995)-infused rats. *Pancreas*, 1997, 14(1): 47-57.
39. Layer P., Keller J. Pancreatic enzymes: secretion and luminal nutrient digestion in health and disease. *J. Clin. Gastroenterol.*, 1999, 28(1): 3-10.
40. Czaky L., Hajnal F., Németh J., Lonovics J. Assessment of pancreatic enzyme secretory capacity by a modified Lundh test. *International Journal of Pancreatology*, 2000, 27(1): 13-19 (doi: 10.1385/IJGC:27:1:13).
41. Aliev A.A., Alieva Z.M. Exocrinous function of liver, pancreas and sperm production in bulls-sires at the various rations. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2010, 2: 40-44 (in Russ.).
42. Smolin S.G. *Fiziko-khimicheskie pokazateli i aktivnost' fermentov soka podzheludochnoi zhelezy u kur, svinei i sobak* [Physico-chemical indices and activity of pancreatic juice enzymes in chickens, pigs and dogs]. Krasnoyarsk, 2008 (in Russ.).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 820-830
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Functional biochemistry

UDC 636.52/.58:591.111

doi: 10.15389/agrobiology.2018.4.820eng
doi: 10.15389/agrobiology.2018.4.820rus

THE AGE DYNAMICS OF BIOCHEMICAL BLOOD INDICES IN BROILER CHICKEN (*Gallus gallus* L.)

I.A. EGOROV, A.A. GROZINA, V.G. VERTIPRAKHOV, T.N. LENKOVA, V.A. MANUKYAN, T.A. EGOROVA, M.V. KOSHCHEYEVA

Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Federal Agency of Scientific Organizations, 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail Olga@vnitip.ru, alena_fisinina@mail.ru, Vertiprakhov63@mail.ru ✉, dissovet@vnitip.ru, manukyan@vnitip.ru, eta164@mail.ru, vlk.733@mail.ru

ORCID:
Egorov I.A. orcid.org/0000-0001-9122-9553
Grozina A.A. orcid.org/0000-0002-3088-0454
Vertiprakhov V.G. orcid.org/0000-0002-3240-7636
Lenkova T.N. orcid.org/0000-0001-8026-3983
Manukyan V.A. orcid.org/0000-0003-4564-4427
Egorova T.A. orcid.org/0000-0002-5102-2248
Koshcheyeva M.V. orcid.org/0000-0002-0744-1883

The authors declare no conflict of interests

Acknowledgements:

Supported financially by Russian Science Foundation, grant № 16-16-04089 "Digestion physiology and microbiology in meat chickens during embryonic and postembryonic periods to develop feeding technologies for ensuring maximum poultry performance"

Received April 19, 2018

Abstract

Biochemical blood indices are important characteristics of physiological and health status of agricultural animals and poultry. In the past decades, the advanced measuring methodology and equipment including the development of automatic biochemical analyzers with standardized reagent kits have led to the significantly faster procedures and higher accuracy of analysis. The reference values of the biochemical indices determined by classic methods should therefore be redefined. In this study we report for the first time the revised reference values of biochemical blood indices including circulatory activity of blood digestive enzymes in growing broiler chicks (*Gallus gallus* L.) during early ontogenesis (1 to 35 days of age). In the study, the hybrid chicks of an experimental broiler cross Smena 8 (line B59) were compared to their parental lines (Cornish line B5 and Plymouth Rock line B9) selected at the Smena Center for Genetic Selection (60 birds per genotype).The hybrids were fed ad libitum; parental lines were restricted in feed since day 15 of age. Mortality and live bodyweight were determined at 1, 7, 14, 21, 28, and 35 days of age. Two replicates of 10 birds per genotype were randomly taken for biochemical blood analyses. Blood was sampled from the jugular vein of decapitated chicks. Biochemical blood indices were measured by a flow semi-automatic biochemical analyzer Sinnowa BS3000P (Sinnowa Medical Science & Technology Co., Ltd., China) using reagent kits DIACON-VET (Russia). At 1 day of age in all studied genotypes concentrations of total protein, triglycerides, and calcium in blood serum were lower while concentrations of total phosphorus and total cholesterol were higher as compared to all subsequent ages. The most substantial increase in biochemical blood indices occurred during the first 7 days of age. This was due, among other factors, to the intensive growth of the body and the functional formation of the digestive system for this age period. The total protein concentration in blood serum increased with age in all genotypes because of proceeded body growth and the enhanced ability of protein synthesis. Concentration of total cholesterol tended to decrease with age, especially after 21 days of age. Concentration of triglycerides in serum increased to 14 days of age by 41.6-57.1 % as compared to the previous ages. There were no significant differences between the genotypes in total blood protein until 35 days of age. The hybrid chicks and their parental lines differed in the blood concentration of calcium, total phosphorus, and total cholesterol at 14 and 28 days of age, and triglycerides at 7 days of age. The blood digestive enzyme activities per live bodyweight decreased from day 1 to day 35 of age, most notably for trypsin (501-fold in hybrids, 453-fold in the Cornish line, and 442-fold in the Plymouth Rock line). Blood amylase activity decreased 100-, 52-, 50-fold, and lipase activity decreased 31-fold, 33-fold, and 35-fold, respectively.

Keywords: broiler chicken, total protein in blood serum, lipid exchange, calcium and phosphorus in serum, digestive enzymes in serum

Transportation and regulatory function of blood ensures multiway interaction between the digestive system and metabolism. It manifests itself not only in fine coordination of digestion and metabolism attributable to neural and hormonal regulation, but also to existence of specific functions of gastro-intestinal tract that facilitate chemical processes in tissues. Our experimental data prove the scientific hypothesis [1, 2] about the cycle of digestive enzymes and their existence in blood serum [3-5]. Our prior papers demonstrated the age-related dynamics of pancreatic enzymes in the pancreatic tissue and blood serum of broiler chicks [6].

Blood composition in mammals and birds is stable, since stability of inner environment of the body alone may ensure smooth and precise operation of its systems. Biochemical blood indices are important characteristics of physiological and health status of agricultural animals and poultry. Biochemical indices of chicken blood are known to change with age [7, 8].

Over the past few decades, scientific laboratories have considerably upgraded their equipment. Classical methods made way for semi- and fully-automatic biochemical analyzers with commercial reagent kits, which have lead to much faster and higher accuracy of the studies. Consequently, reference values of the studied indices needed to be redefined. Professional literature offers expansive albeit rather contradictory data on biochemical properties of blood in broiler chicks [9-11], including application of various additives [12-14] and correlation with the age [15]. However, comparative data obtained from reference broiler lines and hybrids are virtually non-existent in freely available publications. Details concerning the changes in biochemical blood indices and digestive enzymes will help trace the evolving metabolic processes in broilers of diverse lines and hybrids during their postembryonic life. This is particularly important in light of new cross lines breeding, as it helps define periods of the effective growth and make sure that the bird is getting adequate diet so as to increase conversion of feed stock into marketable product.

This study reports for the first time reference values of biochemical blood indices, including circulatory activity of blood digestive enzymes in experimental broiler cross and their parental lines during the ontogenesis. Activity of blood digestive enzymes against the live body weight has been found to go down between day 1 and day 35 of life, with biggest changes established for trypsin (501-fold in hybrids, 453-fold in chicks of parental line and 442-fold in chicks of maternal line). Activity of amylase decreased 100, 52 and 50-fold respectively, and lipase decreased 31, 33 and 35-fold.

This paper aimed to study biochemical blood indices in broilers (*Gallus gallus* L.) cross Smena 8 in post-embryogenesis and to compare the same with the birds of reference lines.

Techniques. Studies were carried out in 2017 on reference lines (Cornish Line B5 and Plymouth Rock Line B9) and their hybrids (cross Smena 8 B59) bred at the Smena Center for Genetic Breeding. During the trial period, chicks were kept at the animal facility of the All-Russia Research and Technological Institute of Poultry Farming (60 chicks in each group) in conditions of feeding and upkeep recommended for the relevant cross and parental lines. Ten chicks were taken out of each group for testing, and the tests were run in two replicates. The hybrids were fed *ad libitum*; parental lines were restricted in feed since day 15 of age. Mortality and live bodyweight were determined at day 1, day 7, day 14, day 21, day 28, and day 35 of age.

Blood was sampled from the jugular vein of decapitated chicks. Freshly made sodium citrate solution was added to the test tubes, blood samples were given a centrifuge ride at 5000 rev/min for 5 minutes at a time; the resulting se-

rum was then studied on the flow semi-automatic analyzer Sinnowa BS3000P (SINNOWA Medical Science & Technology Co., Ltd, China) using biochemical reagent kits (DIACON-VET, Russia) to measure total protein, total cholesterol, triglycerides, calcium and phosphorus.

Blood serum was tested for amylase and lipase activity on Chem well 2900 (T) (Awareness Technology, USA) using the appropriate reagent kits (Human GmbH, Germany). During the measurement of pancreatic amylase and lipase activity, 200 μl of buffered solution (Goods buffer, NaCl, $MgCl_2$, α-Glucosidase, monoclonal antibodies against salivary amylase, sodium azide, pH 7.15 for amylase, and Goods buffer, aurodesoxycholate, Desoxycholate, Calcium ions, colipase sodium azide, pH 8.0 for lipase) were mixed with 4.0 μl of blood serum and incubated at 37 °C for 3 and 5 min, respectively. Thereafter, 50 μl of substrate (Goods buffer, EPS-G7, sodium azide, pH 7.15 for amylase, and Tartrate buffer, lipase substrate, propan-1-ol, pH 4.0 for lipase) were added and incubated for 2 min, whereupon $\lambda = 405$ nm and $\lambda = 580$ nm filters were used respectively to measure absorbance (A) within 1, 2 and 3 min (for amylase) and 1 and 2 minutes (for lipase), followed by calculation of mean values for ΔA/min. Trypsin activity was measured by semi-automatic biochemical analyzer Sinnowa BS-3000P (SINNOWA Medical Science & Technology Co., Ltd, China) [15].

Two trials covering each age group involved at least 20 studies by each indicator. Statistical processing of the findings included calculation of the mean value (*M*) and the standard error of mean (±SEM) in Microsoft Excel 2010. Reliability was estimated by the Student's *t*-test. Differences are considered statistically significant at $p < 0.05$.

Results. Broiler lines and crosses are improved and created at the Smena Center for Genetic Breeding using the latest methods and selection techniques and study aids for evaluation and selection of the young and grown birds [16, 17]. Birds selected for trials were kept on the relevant diets (see composition in Table 1 below).

1. Composition (%) and feed quality indices for broiler chicks (*gallus gallus* l.) cross Smena 8 and parental lines in various ages

Ingredient, indicator	Broiler chicks			Parental line chicks
	days 1-14	days 15-21	days 22-41	days 1-49 сут
Corn	22.000	20.566	17.000	10.000
Wheat	27.806	36.292	40.355	49.480
Soy bean meal	20.107	8.526	2.950	–
Sunflower cake	15.000	20.000	25.000	18.580
Wheat bran	–	–	–	17.310
Corn gluten	7.171	5.709	4.064	–
Soy bean oil	4.000	5.000	6.977	–
Kitchen salt	0.216	0.219	0.217	0.250
Monocalcium phosphate	0.864	0.780	0.617	1.040
Limestone	1.536	1.508	1.470	2.500
Lysine	0.300	0.400	0.350	0.350
Premix	1.000	1.000	1.000	1.000
In 100 g of compound feed:				
metabolizable energy, kcal	305.000	311.000	320.000	255.000
crude protein, g	24.840	21.378	19.786	15.590
crude fiber, g	5.029	4.996	5.211	6.730
calcium, g	0.938	0.889	0.809	1.000
phosphorus, total, g	0.773	0.728	0.683	0.800
phosphorus. digestible, g	0.499	0.469	0.429	–

N o t e. Dashes mean that component does not exist in the diet.

Proteins comprise a most important part of blood serum [18]. Approx. 60% of blood proteins are represented by albumin that plays crucial role in maintaining the oncotic blood pressure and performs transport and nutritional functions. The balance is represented by α- and β-globulins and other serum

proteins, including enzymes (trypsin, amylase, lipase). Concentration of protein in blood serum of chicks 1 day of age was much smaller than in all other periods of their life (Table 2), which can be explained by low protein biosynthesis function [18].

2. Biochemical blood indices and live bodyweight for broiler chicks (*Gallus gallus* L.) of parental lines and their hybrids broken down by age (n = 20, M±SEM)

Original lines and hybrid	Index					
	live bodyweight, g	total protein, g/l	cholesterol, mmol/l	triglycerides, mmol/l	calcium, mmol/l	phosphorus, mmol/l
			Day 1 of life			
Line 5	44.8±0.25	27.2±0.36	5.3±0.04	2.3±0.05	3.0±0.10	3.5±0.13
Line B9	43.2±0.23	27.6±0.70	5.2±0.06	2.4±0.03	3.1±0.01	3.7±0.25
Hybrid B59	44.7±0.14	28.9±0.68	5.2±0.09	2.4±0.08	3.1±0.01	4.1±0.27
			Day 7 of life			
Line 5	136.1±1.67	38.9±0.92	5.3±0.05	2.1±0.02	4.3±0.11	2.1±0.03
Line B9	121.8±1.36*	41.0±0.84	5.3±0.02	2.2±0.04	4.3±0.05	2.2±0.04
Hybrid B59	136.1±1.55	38.7±0.81	5.2±0.05	2.4±0.03	4.3±0.05	2.4±0.23
			Day 14 of life			
Line 5	278.0±4.91	33.8±1.18	5.2±0.03	3.3±0.07	4.3±0.15	2.6±0.07**
Line B9	252.3±4.32**	33.1±1.27	5.2±0.04	3.3±0.04	5.5±0.12*	2.6±0.11**
Hybrid B59	313.4±7.62	34.1±0.48	5.9±0.10	3.4±0.10	4.6±0.06	2.1±0.09
			Day 21 of life			
Line 5	578.1±13.93**	35.2±0.67	3.7±0.03	3.2±0.04	3.8±0.11	1.8±0.09
Line B9	499.0±11.41**	34.8±1.14	3.8±0.04	3.1±0.04	4.1±0.13	1.8±0.05
Hybrid B59	677.5±19.50	33.7±0.49	3.8±0.02	3.2±0.06	4.0±0.09	1.7±0.12
			Day 28 of life			
Line 5	967.1±24.31**	38.7±1.04	3.8±0.04	3.1±0.02	3.5±0.19**	1.9±0.07*
Line B9	796.0±2.62**	40.6±0.82	3.8±0.04	3.1±0.02	4.5±0.13	1.9±0.05*
Hybrid B59	1146.1±38.43	39.8±1.27	4.2±0.12	3.2±0.06	4.3±0.06	2.4±0.21
			Day 35 of life			
Line 5	1609.2±26.91**	41.4±1.31	4.6±0.12	3.3±0.11	2.8±0.05	2.3±0.04
Line B9	1394.0±30.62**	41.7±0.81	4.4±0.05	2.9±0.02	3.0±0.03	2.2±0.05
Hybrid B59	1996.3±98.31	40.7±2.04	4.5±0.07	3.2±0.18	3.1±0.22	2.3±0.04

*, ** Differences with hybrid birds are statistically significant at $p \le 0.05$ and $p \le 0.001$ respectively.

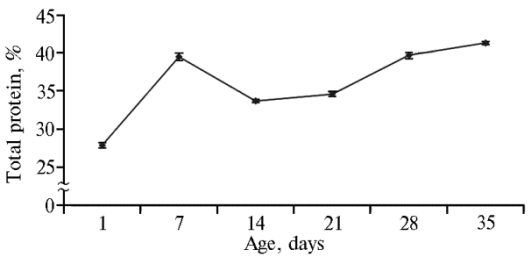


Fig. 1. Total protein content in blood serum of broiler chicks (*Gallus gallus* L.) of cross Smena 8 birds and parental lines depending on age (mean indices by the three groups, n = 30).

By day 7, total blood protein would increase sharply (by 33.9%-48.5%) and reach the peak value. In chicks of parental line B9, concentration of protein in blood serum positively exceeded the same index in hybrids and chicks of parental line B5 by 5.4% ($p < 0.1$). By day 14, protein content would drop to approx. 33.1 g/l in each group and then keep stable for the next week of life. By day 28, it would grow up to 40.6-41.7 g/l ($p < 0.05$) and then stabilize until day 35 (Fig. 1).

Lipid metabolism in birds is an important issue, since lipids serve as energy-intensive substrate: oxidation of 1.0 g of fat generates 2.2-fold more energy than proteins and carbohydrates. Fats mobilize calcium out of the intracellular depot, regulate many biological processes in blood, stimulate digestive function of pancreatic gland and increase the lipase content in pancreatic fluid. Neutral fats occur in the body in the form of storage and protoplasmic fat that includes phospholipids and lipoproteids. Cholesterol is cyclic mono alcohol that occurs in the outer cell membranes [19]. It serves for the synthesis of pregnenolon — precursor of all steroids (aldosterone, cortisol, corticosterone, progesterone, estradiol, testosterone, choleric and other bile acids, a D vitamins). Triglycerides (true fats) are the derivatives of tri-alcohol glycerin and higher fatty acids [19].

Cholesterol and triglycerides content in one-day-old chicks did not differ

from adult birds (see Table 2). On day 21, cholesterol content would drop by 30.2% in chicks of parental line and by 27.0% in chicks of maternal line and broiler chicks compared to 1 day of age. This index would remain unchanged until day 35 when the indices would grow by 21.0%, 15.8% and 7.1% respectively against prior period. Triglycerides content in blood would stay virtually unchanged until day 14 of age, after which it would grow by 43.5% in chicks of parental line, 37.5% in chicks of maternal line and 41.7% in broiler chicks. This index would stay until day 35.

Minerals take part in keeping the body properly hydrated and maintaining normal pH balance, distributing fluids in the body, triggering the nerves and muscles, promoting conductivity of nerve impulses in nerve fibers, etc. [20]. They occur in supporting tissues (calcium) and compounds rich in energy (sulfur, phosphorus), affecting the enzyme activity and functions of a live body. Considerable role here belongs to calcium, phosphorus, magnesium and a number of microelements [20]. What makes mineral metabolism in chicks particularly unique is that intake and elimination of minerals are not balanced, which influences productivity indices [20].

About 50% of blood serum calcium is ionized, 45% is tied with albumins and approx. 5% with phosphates and citrates. Calcium content in blood is defined by the balance between the intestinal absorption, distribution between cellular spaces in the body, bone metabolism and renal elimination. These processes are controlled by parathyroid hormone (parathormone), thyrocalcitonin and the active form of vitamin D [21]. All metabolic processes in the body are inextricably intertwined with phosphoric acid transformation. Phosphorus mostly occurs in the form of anion PO_4^{3+}. Its involvement includes the energy supply to the body and participation in metabolic processes [21].

Newly hatched chicks had considerably low calcium content in their blood serum, but within a single week of their postembryonic life it would grow by 38.7% and stay on that level (with slight fluctuations) until day 35. on day 14, one would detect differences in blood serum calcium content in hybrids and line B9 chicks: in the latter, calcium content was considerably higher than in hybrids (by 19.6%, $p < 0.001$) and line B5 chicks (21.8%, $p < 0.01$). By day 28, this index would grow in hybrids compared to line B5 chicks by 18.6%. On day 35, calcium content would drop down to 2.8±0.05 and 3.0±0.03 mmol/l in chicks of parental lines and 3.1±0.22 mmol/l in hybrids.

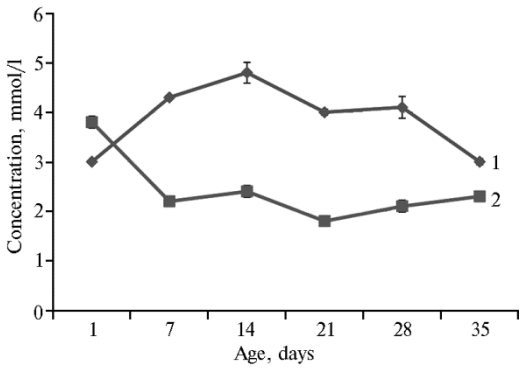


Fig. 2. Calcium (1) **and phosphorus** (2) **content in blood serum of broiler chicks (*Gallus gallus* L.) of cross Smena 8 and birds of parental lines, depending on their age** (mean index for three groups, $n = 30$).

Phosphorus content was high in chicks 1 day of age, but by day 7 it would go down 1.7-fold (Fig. 2). Slight decline was observed until day 21, followed by an upward trend on day 28, which continued up to day 35. Differences in concentration of phosphorus in blood serum were observed in 14 days of age (in chicks of parental lines higher by 23.8% than in hybrids) and 28 days of age (20.8% higher in hybrids than in chicks of parental lines).

In chicks 1 day of age, the calcium/phosphorus ratio was 1:0.8-1:0.7 due to fetal life and the appropriate mineral metabolism. By day 7, the ratio shifted toward the increased calcium, up to 1.9:1, and by day 14 it would grow

up to 2.2:1 in hybrids and 2.1:1 in chicks of parental line B9, and would go down to 1.6:1 in chicks of parental line B5. At the age of 28 days, calcium/phosphorus ratio would shift upwards for chicks of line B9 (2.4:1), and at the age of 35 days it would go down in hybrids (1.3:1), chicks of line B9 (1.4:1) and line B5 (1.2:1).

In addition to transportation, blood also performs regulatory function due to availability of hormones, peptides, minerals and enzymes. Digestive enzymes are known to come to the blood stream and perform regulatory function [1, 2, 22]. Therefore, biochemical analysis of blood would be incomplete without data on age-related changes in digestive enzymes in broiler chickens' blood serum. By day 21 of life of the chicks of parental and maternal lines, the amylase activity would go down by 53.8% and 52.1% respectively. By day 35 of life, however, it would grow by 15.5% and 17.2% respectively against the indices for day 1 of life (Table 3). The amylase activity per unit (g) of live bodyweight would go down steadily with age: between day 1 and day 35 – 58-fold in chicks of parental line, and 50-fold in chicks of maternal line. The amylase activity would grow in hybrids between day 1 and day 14 by 93.1%, followed by the abrupt decline in blood serum enzyme activity, by 55.6% compared to chicks on their day 1 of life. Over 35 days of growing, the amylase activity per unit of live bodyweight of broilers would drop 100-fold, i.e. much harder (almost twice) than in the species of parental lines.

Between day 1 and day 7 of life, the lipase activity would grow by 66.7% (parental line, $p < 0.001$), 53.3% (maternal line, $p < 0.001$) and 64.3% (hybrids, $p < 0.001$). By day 28 of life, these indices would drop 2.5-2.3-fold in all three groups, however bouncing back by day 35 of life to the indices recorded for chicks 7 days of age. The lipase activity per unit of the bird's weight between day 1 and day 35 of life would go down in chicks of parental line 33-fold, in maternal line 35-fold, and in hybrids 31-fold.

3. Activity of digestive enzymes in blood serum and their ratio per unit of live bodyweight of broiler chicks of parental lines and hybrids depending on their age (n = 20, M±SEM)

Original lines and hybrid	Amylase activity		Lipase activity		Trypsin activity	
	in blood serum, U/l	relative to bird's weight (g)	in blood serum, U/l	relative to bird's weight (g)	in blood serum, U/l	relative to bird's weight (g)
			Day 1 of life			
Line 5	827±132.9	18.45	15±0.8	0.33	203±12.7	4.53
Line B9	929±92.9	21.50	15±0.9	0.35	191±11.2	4.42
Hybrid B59	671±50.5	15.00	14±0.3	0.31	224±12.1	5.01
			Day 7 of life			
Line 5	704±66.3	5.17	25±1.7	0.18	29±5.2	0.21
Line B9	926±107.9	7.60	23±1.5	0.19	21±1.9	0.17
Hybrid B59	1001±21.4	7.35	23±1.5	0.17	19±4.3	0.14
			Day 14 of life			
Line 5	583±56.6	2.10	20±3.7	0.07	30±3.7	0.11
Line B9	832±136.3	3.29	21±2.3	0.08	30±1.4	0.12
Hybrid B59	1296±358.3	4.13	16±1.7	0.05	29±3.5	0.09
			Day 21 of life			
Line 5	382±39.0	0.66	15±0.5	0.02	29±3.6	0.05
Line B9	445±103.7	0.89	14±1.3	0.03	23±3.2	0.05
Hybrid B59	525±95.0	0.77	18±2.2	0.03	23±2.1	0.03
			Day 28 of life			
Line 5	564±124.0	0.58	10±1.4	0.01	18±1.2	0.02
Line B9	642±82.3	0.81	11±0.6	0.01	20±1.4	0.02
Hybrid B59	516±86.7	0.45	10±1.4	0.01	19±1.4	0.02
			Day 35 of life			
Line 5	510±71.3	0.32	22±2.2	0.01	16±1.1	0.01
Line B9	605±86.3	0.43	18±1.1	0.01	12±2.5	0.01
Hybrid B59	298±28.2	0.15	20±2.8	0.01	23±3.6	0.01

High trypsin activity was observed in blood serum of one-day-old chicks,

which could be attributed to production of sufficient amounts of substances inhibiting the enzyme activity over the period of embryogenesis. By day 7 of life, trypsin activity dropped in chicks of parental line 7.0-fold; maternal line — 9.1-fold; and in hybrids — 11.8-fold. Consequently, this index would go down steadily both in terms of absolute values and relative to live bodyweight. Over 35 days of trial, relative trypsin activity index per unit of live bodyweight went down in chicks of parental line 453-fold; maternal line — 442-fold; and in hybrids — 501-fold. This may be attributed to inhibition of proteolytic activity of embryo in the egg by the existence of high absorption proteins that are used for building of body tissues [23]. Therefore, relative decrease in activity of digestive enzymes in blood serum with age is indicative of decreasing intensity of metabolic processes in the body and likewise is indicative of regulatory functions of enzymes, especially trypsin.

In our trial, blood serum protein content in one-day-old chicks was much smaller than in any subsequent period of their life. Similar phenomenon is observed in children and is attributed to low protein production on this stage of ontogenesis [18]. Total protein growth index in broilers is observed between day 14 and day 42 [24]. Total protein content in the blood of birds is contingent on such factors as fatty deposits [25] and stress [26]. The identified dynamics of total protein concentration in blood serum of chicks appear to be consistent with age-related trypsin activity modulations [23], changing in the course of formation of digestive function in early ontogenesis oand depending on the quantity and quality of protein in feed [27].

The observed fluctuations in concentration of cholesterol and triglycerides were consistent with age-related fluctuations in activity of lipase in broilers and chicks of parental lines: we have earlier detected the decreasing enzyme activity in pancreatic gland tissue and in blood serum after day 21 of life [23]. One should keep in mind certain ambiguity of academic papers regarding the content of cholesterol and triglycerides in blood serum of the birds. For example, according to S.Y. Gulyushin et al. [28], cholesterol content in the blood of broiler chicks cross Cobb Avian 48 on day 36 of life was 2.8 mmol/l. According to S.A. Yermolina et al. [29], concentration of cholesterol in broiler chicks cross Smena 7 went as high up as 5.8±0.10 mmol/l. According to O.S. Kotlyarova [9], concentration of cholesterol in blood serum of broilers cross ISA Hubbard F15 depends on the age: for chicks on day 12-day 14 of life 2.1-3.8, day 18-day 20 4.5-4.6, day 24-day 26 2.7-4.9, and day 34-day 36 3.2-3.6 mmol/l. A.G. Koshchayev [30] mentions cholesterol index of 0.25 mmol/l. It is common knowledge [31] that intensity of lipid metabolism is affected by controlled feeding of broiler chickens in the early period of ontogenesis.

According to Y.N. Nazarova [9], content of triglycerides in broilers cross Smena 7 would vary with age (in one-day-old chicks 0.69 mmol/l; on day 7 0.77 mmol/l; on day 14 0.66 mmol/l; on day 21 0.68 mmol/l; on day 28 0.59 mmol/l; on day 35 0.61 mmol/l). Based on the obtained data, the author concludes that bile acids inhibit lipids absorption in gastrointestinal tract, thereby causing fat transit through the digestive system, which results in decreasing concentration of lipids in blood serum. According to A.G. Koshchayev [30], triglycerides content in the blood of broiler chicks was 0.02 mmol/l. In cross Hubbard broilers, concentration of triglycerides in blood serum would vary between 0.28 and 0.51 mmol/l [20].

Therefore, our findings in the context of the patterns of protein, lipid and mineral metabolism in broiler chickens in ontogenesis and in comparative aspect (parental lines and their hybrids) expand the concept of biological peculiarities of formation of digestive function in birds. The obtained results help iden-

tify critical growth periods of functional genesis and adaptation of all systems of the body. On these stages birds need more favorable feeding and treatment, which will have considerable impact on livability and productivity of the species. Our data further expand the available information on digestive enzymes in blood serum of the birds. It should be noted, studies like this are quite sparse [32] and data on the age-related dynamics of enzyme activity in broiler lines and hybrids are virtually non-existent.

To sum up, total protein, triglycerides and calcium content in blood serum of one-day-old broiler chicks of parental lines Cornish, Plymouth Rock and their hybrids cross Smena 8 is low, while phosphorus and cholesterol content is rather high. The key blood-related indices are likely to peak by day 7 of life due to intensive growth and functional formation of the digestive system. Total protein in hybrids and chick of parental lines keeps growing with age due to the development of body and improvement of protein production function. Lipid metabolism indices go down as the bird grows older especially after day 21. Triglyceride content dynamics are characterized by the reverse tendency: by day 14 the content grows by 41.6%-57.1% compared to prior indices. Until day 35, hybrids and chicks of parental lines do not considerably differ in terms of total protein. However, there are differences in concentrations of calcium (14 and 28 days of age), phosphorus (14 and 28 days of age), cholesterol (14 and 28 days of age) and triglycerides (7 days of age). Indices of activity of digestive enzymes in blood in relation to live bodyweight of broilers go down with age, with most considerable changes occurring to the content of trypsin: 501-fold in hybrids, 453-fold in chicks of parental line, and 442-fold in chicks of maternal line (from day 1 to day 35). Amylase activity in terms of the live bodyweight of the bird goes down 100-fold, 52-fold and 50-fold respectively, and lipase goes down 31-fold, 33-fold and 35-fold.

REFERENCES

1. Rothman S., Liebow C., Isenman L. Conservation of digestive enzymes. *Phys. Rev.*, 2002, 82: 1-18 (doi: 10.1152/physrev.00022.2001).
2. Korot'ko G.F. *Fizicheskaya kul'tura, sport — nauka i praktika*, 2013, 1: 51-57 (in Russ.).
3. Vertiprakhov V.G., Grozina A.A., Dolgorukova A.M. The activity of pancreatic enzymes on different stages of metabolism in broiler chicks. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2016, 51(4): 509-515 (doi: 10.15389/agrobiology.2016.4.509eng).
4. Fisinin V.I., Osmonyan A.K., Mahdavi R., Egorov I.A. Nutrient density of prestarter diets from 1 to 10 days of age affects intestinal morphometry, enzyme activity, serum indices and performance of broiler chickens. *Animal Nutrition*, 2017, 3(3): 258-265 (doi: 10.1016/j.aninu.2017.06.005).
5. Egorov I.A., Vertiprakhov V.G., Lenkova T.N., Manukyan V.A., Grozina A.A., Egorova T.A.. *Ptitsevodstvo*, 2017, 2: 23-29 (in Russ.).
6. Gerasimenko V.V., Katkova T.V., Nazarova E.A. *Fundamental'nye issledovaniya*, 2011, 8-1: 88-90 (in Russ.).
7. Cherkasova V.V., Zelenskii K.S. *Izvestiya Orenburgskogo gosudarstvennogo agrarnogo universiteta*, 2009, 4(24-1): 60-63 (in Russ.).
8. Ponomarev V.A., Pronin V.V., Kletikova L.V., Malovichko L.V., Yakimenko N.N. *Klinicheskie i biokhimicheskie pokazateli krovi ptits* [Clinical and biochemical parameters of blood of birds]. Ivanovo, 2014: 69-132 (in Russ.).
9. Alagawany M., Attia A.I., Ibrahim Z.A., Mahmoud R.A., El-Sayed S.A. The effectiveness of dietary sunflower meal and exogenous enzyme on growth, digestive enzymes, carcass traits, and blood chemistry of broilers. *Environ. Sci. Pollut. R.*, 2017, 24(13): 12319-12327 (doi: 10.1007/s11356-017-8934-4).
10. Lala A.O., Ajayi O.L., Okwelum N., Oso A.O., Fakorede T.V., Adebayo T.A., Jagbojo J.E. Haematological, biochemical and organ changes in broiler chickens fed varying levels of *Morinda lucida* (brimstone) leaf meal supplementation in the diets. *Trop. Anim. Health Pro.*, 2018, 50(5): 1005-1010 (doi: 10.1007/s11250-018-1524-8).
11. Dong X.Y., Yin Z.Z., Ma Y.Z., Cao H.Y., Dong D.J. Effects of rearing systems on laying performance, egg quality, and serum biochemistry of Xianju chickens in summer. *Poultry Sci.*, 2017, 96(11): 3896-3900 (doi: 10.3382/ps/pex155).

12. Kamely M., Karimi Torshizi M.A., Rahimi S. Blood biochemistry, thyroid hormones, and performance in broilers with ascites caused by caffeine. *Poultry Sci.*, 2016, 95(11): 2673-2678 (doi: 10.3382/ps/pew227).
13. Toghyani M., Mosavi S.K., Modaresi M., Landy N. Evaluation of kefir as a potential probiotic on growth performance, serum biochemistry and immune responses in broiler chicks. *Animal Nutrition*, 2015, 1(4): 305-309 (doi: 10.1016/j.aninu.2015.11.010).
14. Mikhailova A.G., Khairullin R.F., Demidyuk I.V., Kostrov S.V., Grinberg N.V., Burova T.V. , Grinberg V.Y., Rumsh L.D. Cloning, sequencing, expression, and characterization of thermostability of oligopeptidase B from *Serratia proteamaculans*, a novel psychrophilic protease. *Protein Expres. Purif.*, 2014, 93: 63-76 (doi: 10.1016/j.pep.2013.10.011).
15. Kierończyk B., Sassek M., Pruszy ska-Oszmałek E., Kołodziejski P., Rawski M., Świątkiewicz S., Józefiak D. The physiological response of broiler chickens to the dietary supplementation of the bacteriocin nisin and ionophore coccidiostats. *Poultry Sci.*, 2017, 96(11): 4026-4037 (doi: 10.3382/ps/pex234).
16. Egorova A. *Ptitsevodstvo*, 2012, 12: 8-10 (in Russ.).
17. Emanuilova Zh.V., Efimov D.N., Tuchemskii L.I., Egorova A.V. *Rossiiskaya sel'skokhozyaistvennaya nauka*, 2014, 2: 48-51 (in Russ.).
18. Kuznik B.I. *Fiziologiya i patologiya sistemy krovi* [Physiology and pathology of blood]. Chita, 2002 (in Russ.).
19. Metreveli T.V. *Biokhimiya zhivotnykh* /Pod redaktsiei N.S. Sheveleva [Animal biochemistry. N.S. Shevelev (ed.)]. St. Petersburg, 2005 (in Russ.).
20. Azarnova T.O., Naidenskii M., Bobyl'kova A. *Zhivotnovodstvo Rossii*, 2012, 7: 13-15 (in Russ.).
21. Bessarabov B.F., Alekseeva S.A., Kletikova L.V. *Laboratornaya diagnostika klinicheskogo i immunobiologicheskogo statusa u sel'skokhozyaistvennoi ptitsy* [Laboratory diagnostics of clinical and immunobiological status of poultry]. Moscow, 2008 (in Russ.).
22. Laporte J.C., Tremolieres J. Regulation hormonale de la sécrétion enzymatique du pancréas exocrine. *Comptes rendus de l'Académie des Sciences*, ser. D, 1971, 273: 1205-1207.
23. Egorov I.A., Vertiprakhov V.G., Grozina A.A., Laptev G.Yu., Nikonov I.N., Novikova N.I., Il'ina L.A., Iyldyrym E.A., Filippova V.A., Dubrovin A.V., Manukyan V.A., Lenkova T.N. Age dynamics of pancreas secretory function and intestinal microbiota in meat broiler chicks and theier parental lines. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2017, 52(4): 757-766 (doi: 10.15389/agrobiology.2017.4.757rus).
24. Piotrowsska A., Burlikowska K., Szymeczko R. Changes in blood chemistry in broiler chickens during the fattening period. *Folia biologica (Krakyw)*, 2011, 59(3-4): 183-187 (doi: 10.3409/fb59).
25. Dong J.Q., Zhang H., Jiang X.F., Wang S.Z., Du Z.Q., Wang Z.P., Leng L., Cao Z.P., Li Y.M., Luan P., Li H. Comparison of serum biochemical parameters between two broiler chicken lines divergently selected for abdominal fat content. *J. Anim. Sci.*, 2015, 93(7): 3278-3286 (doi: 10.2527/jas.2015-8871).
26. Hu H., Bai X., Shah A.A., Wen A.Y., Hua J.L., Che C.Y., He S.J., Jiang J.P., Cai Z.H., Dai S.F. Dietary supplementation with glutamine and γ-aminobutyric acid improves growth performance and serum parameters in 22- to 35-day-old broilers exposed to hot environment. *J. Anim. Physiol. An. N.*, 2016, 100(2): 361-370 (doi: 10.1111/jpn.12346).
27. Somova O.V. *Uchenye zapiski UO VGAVM*, 2012, 1(48): 142-145 (in Russ.).
28. Gulyushin S.Yu., Zernov R.A. Methyl group donors — promising means for prevention of chronic mycotoxicosis. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2011, 2: 21-31.
29. Ermolina S.A., Buldakova K.V., Sozinov V.A. *Uspekhi sovremennogo estestvoznaniya*, 2014(9): 34-37 (in Russ.).
30. Koshchaev A.G. *Biotekhnologiya proizvodstva i primeneniya funktsional'nykh kormovykh dobavok dlya ptitsy. Avtoreferat doktorskoi dissertatsii* [Biotechnology of production and application of functional feed additives for poultry. DSc Thesis]. Krasnodar, 2008 (in Russ.).
31. Yang X., Zhuang J., Rao K., Li X., Zhao R. Effect of early feed restriction on hepatic lipid metabolism and expression of lipogenic genes in broiler chickens. *Res. Vet. Sci.*, 2010, 89(3): 438-444 (doi: 10.1016/j.rvsc.2010.04.003).
32. Amann O., Visschers M.J.M., Dorrestein G.M., Westerhof I., Lumeij J.T. Exocrine pancreatic insufficiency in pigeons. *Avian Pathol.*, 2006, 35(1): 58-62 (doi: 10.1080/03079450500465791).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 831-841
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.2:619:618.3:577.1

doi: 10.15389/agrobiology.2018.4.831eng
doi: 10.15389/agrobiology.2018.4.831rus

ANTIOXIDANT STATUS AND FUNCTIONAL CONDITION OF RESPIRATORY SYSTEM OF NEWBORN CALVES WITH INTRAUTERINE GROWTH RETARDATION

V.A. SAFONOV[1], V.I. MIKHALEV[2], A.E. CHERNITSKIY[2]

[1]*Vernadskii Institute of Geochemistry and Analytical Chemistry RAS,* Federal Agency of Scientific Organizations, 19, ul. Kosygina, Moscow, 119991 Russia, e-mail safonovbio@gmail.com;
[2]*All-Russian Research Veterinary Institute of Pathology, Pharmacology and Therapy* RAAS, Federal Agency of Scientific Organizations, 114-b, ul. Lomonosova, Voronezh, 394087 Russia, e-mail vnivipat@mail.ru, cherae@mail.ru (✉ corresponding author)
ORCID:
Safonov V.A. orcid.org/0000-0002-5040-6178
Mikhalev V.I. orcid.org/0000-0001-9684-4045
Chernitskiy A.E. orcid.org/0000-0001-8953-687X
The authors declare no conflict of interests
Received March 21, 2018

Abstract

Intrauterine fetal and embryo growth retardation (IUGR), defined as a discrepancy of embryo forming and fetus size and their gestation terms, is a common pathology among farm animals. Respiratory dysfunctions in newborns with IUGR are among the factors leading to animal death from birth to weaning. The immature antioxidant defense system (AOS) of newborns with IUGR predisposes to oxidative stress progression and associated pathologies. We show in this paper the lack of enzymatic and non-enzymatic links of antioxidant protection, an increased concentration of malonic dialdehyde in blood and in exhaled air, and higher expiration of enzymes of different subcellular localization, i.e. alanine aminotransferase, γ-glutamyl transferase, aspartate aminotransferase, indicating damage to the respiratory tract cells. These data contribute to elucidating mechanisms of respiratory dysfunctions as influenced by IUGR. A comparative study of AOS indicators, functional state of respiratory organs of newborn calves and the respiratory disease progression in the neonatal period was carried out at a large dairy complex (Agrotech-Garant Nashchekino Co. Ltd, Anninsky Region, Voronezh Province) in 2013. A total of 53 red-motley calves were examined, including 28 calves with IUGR in history and 25 ones whose mothers had physiological course of pregnancy (control group). In 24 hours after the calves' birth, switch tail hair samples, blood and exhaled breath condensate (EBC) were collected for biochemical studies, the heart rate (HR) and frequency of respiratory rate (RR) per minute, the ratio of HR/RR (Hildebrandt index), tidal volume (TV) and respiratory minute volume (RMV), the volume of EBC produced per minute (V1) and from 100 liter of exhaled air (V2) were determined. The hair concentrations of iron, copper, zinc, manganese, selenium and cobalt were determined by atomic absorption spectrophotometry (Shimadzu AA6300, Japan); the activity of catalase, selenium-dependent glutathione peroxidase (GPO), superoxide dismutase (SOD) in blood, the blood concentration of malonic dialdehyde (MDA), the serum (plasma) content of vitamin A, α-tocopherol, L-ascorbic acid and total antioxidant activity (AOA) were studied spectrophotometrically (Shimadzu UV-1700, Japan). The MDA concentration (Shimadzu UV-1700, Japan), intensity of iron-induced chemiluminescence (BHL-07, Russia), the activity of alanine aminotransferase (ALAT), γ-glutamyltransferase (GGT) and aspartate aminotransferase (ASAT) (Hitachi-902, Japan) were examined in EBC of calves. In the calves with IUGR, as compared to the control group, blood catalase activity reduced by 14.4 % ($P < 0.001$), GPO by 14.0 % ($P < 0.001$) and SOD by 33.8 % ($P < 0.001$), blood serum content of vitamin A decreased by 36.7 % ($P < 0.05$) and α-tocopherol by 38.3 % ($P < 0.001$), while blood plasma AOA was higher by 18.6 % ($P < 0.01$), hair concentration of copper decreased by 28.3 % ($P < 0.001$), zinc by 10.7 % ($P < 0.001$), manganese by 9.4 % ($P < 0.001$), selenium by 26.4 % ($P < 0.001$) and cobalt by 36.8 % ($P < 0.001$), the MDA level in blood and EBC increased by 26.8 % ($P < 0.001$) and 119.5 % ($P < 0.001$), respectively, also, intensity of chemiluminescence outbreak Imax and the light sum of chemiluminescence S of EBC were higher by 36.2 % ($P < 0.01$) and 40.6 % ($P < 0.01$), respectively. An increase in the ratio of S/tg2α in EBC of calves with IUGR (by 35.5 % compared to the control group, $P < 0.01$) indicated imbalance of oxidative and antioxidant activity of EBC and oxidative stress progression. Structural and functional damage of respiratory tract under oxidative stress of IUGR calves was accompanied by an increase in ALAT expiration by 105.9 % ($P < 0.001$), GGT by 416.1 % ($P < 0.001$), ASAT by

62.5 % ($P < 0.001$), and respiratory moisture release (V2) by 67.3 % ($P < 0.001$) compared to the control group. An increase in Hildebrandt index of calves with IUGR (by 7.9 % compared to the control group, $P < 0.05$) indicates the autonomic regulation disorder and the cardiorespiratory functional system overstrain. A statistically significant relationship was found between the risk of bronchopneumonia development and the S/tg2α ratio which reflects the balance of EBC oxidative and antioxidant activity ($r_{\tau\text{-K}} = +0.58$, $P < 0.01$), and also the blood activity of catalase ($r_{\tau\text{-K}} = -0.68$, $P < 0.01$), GPO ($r_{\tau\text{-K}} = -0.36$, $P < 0.05$) and SOD ($r r_{\tau\text{-K}} = -0.62$, $P < 0.01$).

Keywords: intrauterine fetal and embryo growth retardation, newborn calves, antioxidant defense system, oxidative stress, exhaled breath condensate, respiratory diseases, bronchopneumonia

Intrauterine fetal and embryo growth retardation (IUGR), defined as discrepancy of embryo forming a fetus size and their gestation terms is still a serious problem of animal farming [1-3]. Academic interest toward IUGR is caused not only by the fact that it is a common syndrome affecting pregnant animals [1, 4], but also by its negative impact on postnatal growth and health of the offspring [1, 5-7]. Postnatal hypoglycemia, hypoxemia [1, 8], hypersensitivity to hypothermia resulting from dysfunctional thermoregulatory functions and low energy stores of the body [8-10] are common in newborns with IUGR. Respiratory dysfunctions in newborns with IUGR are among the factors leading to animal death in the period between the birth and weaning [1, 5, 11].

Although clinical condition of such animals may appear normal, their internal organs are morphologically and functionally immature [12-14]. According to the study on swine [15], immature antioxidant defense system in newborns with IUGR predisposes to oxidative stress progression and postnatal metabolic disorders.

We have first conducted comparative studies of the antioxidant protection system indicators and functional state of respiratory organs of newborn calves during physiological pregnancy and the IUGR. Newborn calves with IUGR appeared to have deficient enzymatic and non-enzymatic links of the antioxidant protection system, as well as increased concentration of malonic dialdehyde in blood and in exhaled breath condensate, and higher expiration of enzymes of various sub-cellular localization (alanine aminotransferase, γ-glutamyltransferase, aspartate aminotransferase), indicative of damage to respiratory tract cells in conditions of oxidative stress.

The study aims to get an insight into antioxidant status indicators and functional state of respiratory system of the newborn calves with intrauterine growth retardation and the impact of these dysfunctions on the respiratory disease progression over the neonatal period.

Techniques. A total of 53 red-motley calves were examined, including 28 calves with IUGR in history and 25 calves whose mothers had physiological course of pregnancy (control group); the studies were carried out in 2013 at Agrotech-Garant Nashchekino Co. Ltd. (Anninsky Region, Voronezh Province). The cows were diagnosed for IUGR by way of transrectal palpation and ultrasonic scanning with the help of Easi-Scan-3 (BCF Technology Ltd., U.K.) with 4.5-8.5 MHz linear array probe. Embryo and fetus growth retardation criteria included the crown-rump length and body diameter on day 38-day 45 following insemination and conception — less than 16 mm and 9 mm respectively; on day 60-day 65 — less than 45 mm and 16 mm respectively; on day 110-day 115 — horn-uterus diameter less than 15 cm and placentoma less than 17 mm [2, 4].

Biochemical studies were conducted on samples of hair, blood and exhaled breath condensate (EBC) collected from calves 24 hours after their birth. Hair samples were taken from switch tails, blood samples were drawn from the jugular vein using commercial vacuum blood sampling systems (with EDTA as anticoagulant). EBC samples were collected from calves using our proprietary de-

vice [16]. Blood serum samples were obtained through centrifugal process that took 10 minutes at room temperature and without addition of anticoagulant (4000 rpm, UC-1612, ULAB, China). Immediately upon collection, the serum and EBC samples would be frozen and stored in liquid nitrogen at –196 °C until biochemical studies.

Hair samples were prepared by wet ashing technique and the content of minor elements (selenium, copper, zinc, iron, cobalt and manganese) was determined using the atomic absorption spectrophotometry (Shimadzu AA6300, Japan).

Assessment of the state of enzymatic link of the antioxidant protection system in blood involved measurement of catalase activity (EC 1.11.1.6) and selenium-dependent glutathione peroxidase (GPO, EC 1.11.1.9) using the relevant methods described by M.I. Retsky et al. (17); and activity of superoxide dismutase (SOD, EC 1.15.1.1) in blood was measured by the rate of inhibition of adrenaline autoxidation [18]. The state of non-enzymatic link of the antioxidant protection system was measured by the content of vitamin A [19], α-tocopherol [17], L-ascorbic acid [20] in blood serum and by total antioxidant activity (AOA) of blood serum [21]. Concentration of malonic dialdehyde (MDA) in blood and EBC was measured spectrophotometrically (Shimadzu UV-1700, Japan) by reaction with thiobarbituric acid [17].

Intensity of iron-induced chemiluminescence in EBC was studied with the help of biochemiluminometer BHL-07 (Medozones, Ltd., Russia) by the methods described by Y.G. Voronkova et al. [22], with slight modifications. EBC (0.2 ml), 0.4 ml of 0.02 M potassium-phosphate buffer (pH 7.5) and 0.4 ml of 0.01 M iron (II) sulfate solution were added into the measuring cell successively. The cell with the resulting blend was then placed in the measuring slot of the device, quickly adding 0.2 ml of 2% of hydrogen peroxide, and moving the cell into the measuring position. Free radical process was registered for 30 seconds, chemiluminescence was measured on the basis of the kinetic curve by the following indicators: maximum intensity of the outbreak (that characterizes the intensity of free radical oxidation) — Imax, mV; chemiluminescence light sum (representing the oxidative activity) — S, mV × sec; kinetic curve slope ratio against the time axis (characterizing the antioxidant activity) — tg2α. To determine the balance between oxidative and antioxidant activity, we calculated the ratio of S/tg2α [23]. Activity of alanine aminotransferase (ALAT), γ-glutamyl transferase (GGT) and aspartate aminotransferase (ASAT) in EBC were studied on biochemical analyzer Hitachi-902 (Roche Diagnostics, Japan).

The heart rate (HR) and frequency of respiratory rate (RR) per minute were measured in calves; external respiration functions (tidal volume and respiratory minute volume) were studied with the help of SSP lung tester (KPO Medapparatura, Ukraine) and a mask with valves; Hildebrandt index was calculated as the HR/RR ratio. Animals were under continuous clinical supervision from their birth to day 30 inclusively: their condition in the course of respiratory disease was rated under WI system [24], recording the time of first clinical indications and the height of bronchitis, morbidity (0 — no pathology, 1 — mild, 2 — moderately severe and 3 — severe disease), complication in the form of bronchial pneumonia.

Statistical data processing was done in Statistica 8.0 (StatSoft, Inc., USA) and IBM SPSS Statistics 20.0 (IBM Corp., USA) software. The results represent mean arithmetic and standard deviation ($M \pm SD$), minimum (min), maximum (max) and median values (*Me*). The significance of difference between median values of the samples was measured by means of nonparametric Wilcoxon test. Correlations between the values were identified by means of Spearman rank

correlation coefficient (r_S) and Kendall's τ ($r_{\tau\text{-K}}$). Zero hypothesis was disregarded in all statistical processing methods at $p < 0.05$.

Results. According to the study of blood of claves with IUGR, activity of catalase was found to be down by 14.4% ($p < 0.001$), GPO by 14,0% ($p < 0.001$) and SOD by 33.8% ($p < 0.001$); vitamin A content in blood serum was down by 36.7% ($p < 0.05$) and α-tocopherol down by 38.3% ($p < 0.001$); blood serum AOA was up by 18.6% ($p < 0.01$) compared to control group (Table 1). No statistically significant differences in L-ascorbic acid concentrations in blood serum of various groups of calves were observed.

1. Indicators of antioxidant system in blood (serum) of newborn red-motley calves — normal and with intrauterine fetal growth retardation (Agrotech-Garant Nashchekino Co. Ltd., Anninsky Region, Voronezh Province, 2013

Indicator	*M*±SD	min-max	*Me*
Catalase, μmol H_2O_2/(l · min)	26.7±1.4	23.6-28.7	26.9***
	31.2±3.0	27.3-35.3	30.6
GPO, μmol GSH/(l · min)	6.84±0.70	5.56-7.84	6.90***
	7.95±0.71	6.71-8.72	8.31
SOD, conventional units	0.53±0.07	0.43-0.65	0.53***
	0.80±0.09	0.62-0.92	0.81
Vitamin A, μmol/l	0.57±0.22	0.27-0.87	0.54*
	0.90±0.35	0.60-1.60	0.73
A-tocopherol, μmol/l	5.0±2.2	2.8-8.4	4.0***
	8.1±1.6	5.5-9.9	8.7
L-ascorbic acid, μmol/l	17.8±6.8	10.9-30.6	17.3
	23.4±5.1	17.3-36.6	22.8
AOA,%	42.5±7.7	27.9-48.0	44.4**
	52.2±4.2	47.6-61.0	51.7

N o t e. GSH — reduced glutathione, GPO — glutathione peroxidase, SOD — superoxide dismutase, AOA — total antioxidant activity in blood serum. Above the line — group of calves with intrauterine growth retardation ($n = 28$), below the line — group of calves whose mothers had physiological course of pregnancy ($n = 25$).
*, **, *** Differences between the groups are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Concentration of virtually every microelement (excluding iron) covered by this study was found to be down in the hair of newborn calves with IUGR: copper by 28.3% ($p < 0.001$), zinc by 10.7% ($p < 0.001$), manganese by 9.4% ($p < 0.001$), selenium by 26.4% ($p < 0.001$) and cobalt by 36.8% ($p < 0.001$) compared to control group (Table 2).

Correlation analysis detected statistically significant correlation between copper content in calves' hair and SOD activity in blood ($r_S = +0.55$ at $p < 0.05$), as well as selenium content in hair and GPO activity in blood ($r_S = +0.84$ at $p < 0.01$).

2. Microelements content in tail hair of newborn red-motley calves — normal and with intrauterine fetal growth retardation (Agrotech-Garant Nashchekino Co. Ltd., Anninsky Region, Voronezh Province, 2013)

Indicator	*M*±SD	min-max	*Me*
Iron, mg/kg	31.7±8.6	20.3-47.0	30.4
	37.5±13.0	20.3-65.0	34.4
Copper, mg/kg	6.52±1.30	3.15-8.0	7.01*
	9.09±1.01	7.4-10.6	9.35
Zinc, mg/kg	105.6±14.2	88.7-127.3	98.8*
	118.2±15.8	91.3-138.1	121.6
Manganese, mg/kg	8.55±0.27	8.11-9.01	8.50*
	9.44±1.22	8.11-12.8	9.19
Selenium, μg/kg	345.0±67.4	261.0-447.0	317.5*
	468.5±69.4	398.0-595.0	457.5
Cobalt, μg/kg	42.5±10.1	25.4-56.0	42.9*
	67.3±15.2	51.2-96.8	62.7

N o t e. Above the line — group of calves with intrauterine growth retardation ($n = 28$), under the line — group of calves whose mothers had physiological course of pregnancy ($n = 25$).
* Differences between the groups are statistically significant at $p < 0.001$.

Increase of MDA concentration in blood and EBC of calves with IUGR

by 26.8% ($p < 0.001$) and 119.5% ($p < 0.001$) respectively compared to control group (Fig. 1) was indicative of the increasing systemic and local (lungs) intensity of lipids peroxidation associated with functional deficiency of enzymatic and non-enzymatic links of the antioxidant protection system.

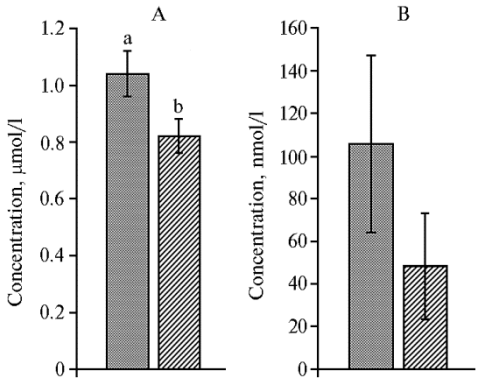


Fig. 1. Malonic dialdehyde (MDA) **content in blood** (A) **and in exhaled breath condensate** (B) **of newborn red-motley calves with intrauterine fetal growth retardation** (a) **and physiological course of pregnancy of cows** (b) (*M*±SD, Agrotech-Garant Nashchekino Co. Ltd., Anninsky Region, Voronezh Province, 2013).

Studies of the iron-induced chemiluminescence EBC (Table 3) in newborn calves with IUGR registered the chemiluminescence outbreak Imax and the light sum S EBC by 36.2% ($p < 0.01$) and 40.6% ($p < 0.01$) respectively compared to control group, which was indicative of the increased oxidative activity in EBC. The Imax and S/tg2α indicators correlated with MDA and EBC concentrations: $r_S = +0.78$ ($p < 0.01$) and $r_S = +0.48$ ($p < 0.01$) respectively. No significant differences between the groups of calves in terms of tg2α have been detected. The increasing ratio S/tg2α in EBC of calves with IUGR (by 35.5% compared to control group, $p < 0.01$) is indicative of imbalanced oxidative and antioxidant activity of EBC the development of oxidative stress. Statistically significant correlations were found between S/tg2α in EBC and the activity of antioxidant enzymes in blood, i.e. catalase ($r_S = -0.54$ at $p < 0.01$), GPO ($r_S = -0.49$ at $p < 0.01$) and SOD ($r_S = -0.85$ at $p < 0.01$).

3. Indicators of iron-induced chemiluminescence in exhaled breath condensate of newborn red-motley calves — normal and with intrauterine fetal growth retardation (Agrotech-Garant Nashchekino Co. Ltd., Anninsky Region, Voronezh Province, 2013)

Indicator	*M*±SD	min-max	*Me*
S, mV × sec	417.3±37.6	374.0-472.0	411.5*
	296.9±49.1	248.0-422.0	282.0
Imax, mV	65.8±4.7	61.0-73.0	64.5*
	48.3±10.0	40.0-73.0	43.0
tg2α	20.3±1.7	18.0-22.5	20.3
	19.5±2.4	18.0-25.5	18.0
S/tg2α	20.6±1.1	19.2-22.1	20.6*
	15.2±1.0	13.4-16.5	15.7

N o t e. Above the line — group of calves with intrauterine growth retardation ($n = 28$), under the line — group of calves whose mothers had physiological course of pregnancy ($n = 25$).
* Differences between the groups are statistically significant at $p < 0.01$.

In EBC of calves IUGR, there was an increase in activity of ALAT by 105.9% ($p < 0.001$), GGT by 416.1% ($p < 0.001$), and ASAT by 62.5% ($p < 0.001$) compared to control group (Fig. 2). Increased activity of enzymes of various subcellular localization (cytoplasmic ALAT, membrane-bound GGT, mitochondrial ASAT) in EBC of calves with intrauterine growth retardation reflects the extent of structural and functional damage to the respiratory tract cells (from disruption of biomembrane permeability to cytolysis) and is caused by the occurrence of these components in epithelial lining fluid [26, 27]. We have established statistically significant correlation between the GGT activity in EBC and the intensity of free radical oxidation Imax ($r_S = +0.51$ at $p < 0.01$), ratio S/tg2α reflecting the balance between the oxidative and anti-

oxidant activity EBC (r_S = +0.74 at p < 0.01), as well as GPO (r_S = –0.55 at p < 0.01) and SOD (r_S = –0.53 at p < 0.01) activity in blood. Similar correlations have been found between the ASAT activity in EBC and the S/tg2α ratio (r_S = +0.41 at p < 0.05), GPO (r_S = –0.52 at p < 0.01) and SOD (r_S = –0.38 at p < 0.05) activity in blood, and between the ALAT activity in EBC and the value of antioxidant activity EBC tg2α (r_S = –0.39 at p < 0.05).

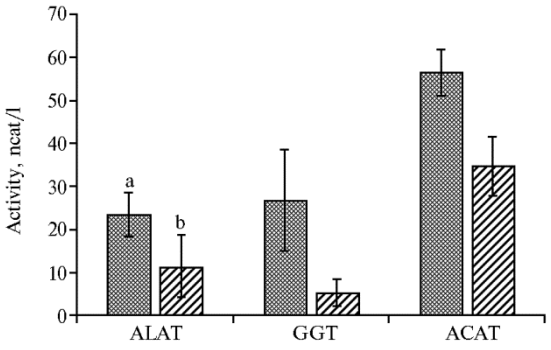


Fig. 2. Enzyme activity in the breath condensate exhaled by newborn red-motley calves: a — with intrauterine fetal growth retardation, b — physiological course of pregnancy; ALAT — alanine aminotransferase, GGT — γ-glutamyl transferase, ASAT — aspartate aminotransferasea (*M*±SD, Agrotech-Garant Nashchekino Co. Ltd., Anninsky Region, Voronezh Province, 2013).

Increased intensity of respiratory moisture was detected in calves with IUGR against the background of the respiratory minute volume decrease by 34.0% (p < 0.001) and the breathing capacity by 20.9% (p < 0.001): the amount of EBC produced over the period of 1 minute out of 100 liters of the exhaled air would increase compared to control group by 28.6% (p < 0.01) и 67.3% (p < 0.001) respectively (Table 4). We have detected inverse relationship between the amount of condensate produced out of 100 liters of the exhaled air and the depth of breathing (r_S = –0.61 at p < 0.01). The increase in Hildebrandt index in calves with IUGR (by 7.9% against the control group, p < 0.05) was indicative of the autonomic imbalance and cardio-respiratory system overload [25].

4. Respiratory and moisture producing function of the lungs of red-motley calves — normal and with intrauterine fetal growth retardation (Agrotech-Garant Nashchekino Co. Ltd., Anninsky Region, Voronezh Province, 2013)

Indicator	*M*±SD	min-max	*Me*
RR, min^{-1}	46.3±8.1	23.6-28.7	44.0*
	57.7±15.7	27.3-35.3	52.0
RMV, l	9.5±2.2	7.4-12.5	8.7**
	14.4±2.8	10.6-19.1	13.4
TV, ml	210.7±55.8	153-284	195.0**
	266.3±88.3	156-445	254.0
V1, ml	0.09±0.03	0.06-0.12	0.10*
	0.07±0.02	0.05-0.09	0.07
V2, ml	0.92±0.31	0.59-1.42	0.84**
	0.55±0.09	0.39-0.72	0.53

N o t e. RR — respiratory rate, RMV — respiratory minute volume, TV — tidal volume; V1 and V2 — volume of exhaled breath condensate produced over the period of 1 minute out of 100 liters of exhaled air. Above the line — group of calves with intrauterine growth retardation (n = 28), below the line — group of calves whose mothers had physiological course of pregnancy (n = 25).
*, ** Difference between the groups is statistically significant at p < 0.01 and p < 0.001 respectively.

Respiratory diseases were registered in 48.0% of calves whose mothers had physiological course of pregnancy and in 100% calves with IUGR; severe bronchitis developing into bronchial pneumonia occurred in 12.0% and 85.7% of animals respectively. We have identified statistically significant relationship between the potential development of bronchial pneumonia in calves and the S/tg2α ratio that reflects the balance of pro- and antioxidant activity of EBC ($r_{\tau\text{-}K}$ = +0.58 at p < 0.01), and the activity of catalase in blood ($r_{\tau\text{-}K}$ = –0.68 at p < 0.01), GPO ($r_{\tau\text{-}K}$ = –0.36 at p < 0.05) and SOD ($r_{\tau\text{-}K}$ = –0.62 at p < 0.01).

Rapid evolution of fetus from intrauterine hypoxic environment to normoxia and pulmonary respiration at the time of birth is accompanied by con-

siderable stress on every functional system of the body [28-30]. Beginning of pulmonary respiration is associated with the increased generation of reactive oxygen intermediates (ROI) and oxidative stress development [28, 31]. In turn, the oxidative stress is associated with a number of pathological conditions in the newborn animals — cardiovascular and pulmonary disorders, lactic acidosis, reduced absorption and passive passage of nutrients and immunoglobulins in the intestinal tract [15, 28, 31]. Excess ROI generation is compensated by the adaptive changes in the antioxidant protection system [32-35], mostly enzymatic part thereof [31, 32, 35].

As the cattle tail switch hair begins to grow on month 7 of pregnancy [36, 37], concentration of chemicals in the hair samples collected from this part of the body (entire length of the hair stem) on day 1 after the birth may be considered as the integral indicator of minerals supply in the calves over the last 3 months of intrauterine development. In the period of 10%-15% of the term preceding the completion of gestation, the activity of antioxidant enzymes in fetal tissues is known to increase by 150%-200% [33, 38]. According to the findings of our studies, unlike the calves whose mothers had physiological course of pregnancy, calves with intrauterine growth retardation had copper concentration in their tail hair smaller by 28.3% ($p < 0.001$), zinc by 10.7% ($p < 0.001$), manganese by 9.4% ($p < 0.001$), selenium by 26.4% ($p < 0.001$), and cobalt by 36.8% ($p < 0.001$). According to the obtained data, in the event of IUGR, the enzymatic part of the antioxidant protection system in fetus develops in conditions of deficient amount of copper, zinc, manganese, selenium and cobalt. In calves with IUGR, the catalase activity in blood was down by 14.4% ($p < 0.001$), GPO by 14.0% ($p < 0.001$), and SOD by 33.8% ($p < 0.001$) compared to the control group. Strong relationship between the deficient microelements and functional disorder of enzymatic part of the antioxidant protection system in newborn calves was confirmed by correlation between copper concentration in the hair and the SOD activity in blood ($r_S = +0.55$ at $p < 0.05$), and selenium concentration in the hair and GPO activity in blood ($r_S = +0.84$ at $P < 0.01$). D. Shukla et al. [39] prove the importance of cobalt in antioxidant protection of the lungs, so deficit of cobalt in calves with IUGR takes on particular significance.

Considerable drop in concentration of vitamin A in blood serum, α-tocopherol and overall antioxidant activity of blood serum has been detected in calves with IUGR (see Table 1). Oxidative stress development in the lungs, however, appeared to be mostly tied to deficient enzymatic part of the antioxidant protection system, which has been confirmed by statistically significant relationship between S/tg2α in EBC and the catalase activity ($r_S = –0.54$ at $p < 0.01$), GPO ($r_S = –0.49$ at $p < 0.01$) и SOD ($r_S = –0.85$ at $p < 0.01$) in blood, as well as by the findings of the studies conducted by other authors [33, 40, 41].

In calves with IUGR, we have observed the increase in MDA concentration in blood and EBC (by 26.8% at $p < 0.001$ and 119.5% at $p < 0.001$ respectively, compared to control group), indicative of the increasing systemic and local (lungs) intensity of peroxidation of lipids against the deficient enzymatic and non-enzymatic links of the antioxidant protection system. Our data correlate with the findings of Z. Hracsko et al. [42], which demonstrated significant increase in blood MDA against the decreased activity of catalase, GPO, SOD and the reduced glutathione concentration in the newborns with IUGR compared to children born by mothers who had physiological course of pregnancy.

Structural and functional damage to respiratory tract cells in conditions of oxidative stress in calves with IUGR was accompanied by the increased exhalation of enzymes of various subcellular localization (cytoplasmic ALAT, membrane-bound GGT, mitochondrial ASAT) and intensified release of respiratory

moisture. According to prior studies [29], the increase of respiratory moisture release occurs in newborn calves in the event of hypoxia and acidosis and is caused by metabolic and respiratory adaptation disorder. The increased Hildebrandt index in calves with IUGR (by 7.9% compared to control group, $p < 0.05$) is indicative of the autonomic regulation disorder and cardiorespiratory functional system overstrain [25]. A statistically significant relationship was found between the risk of development of bronchial pneumonia in calves and the S/tg2α ratio which reflects the balance of EBC oxidative and antioxidant activity ($r_{\tau\text{-K}} = +0.58$ at $p < 0.01$), and also the blood activity of catalase ($r_{\tau\text{-K}} = -0.68$ at $p < 0.01$), GPO ($r_{\tau\text{-K}} = -0.36$ at $p < 0.05$) and SOD ($r_{\tau\text{-K}} = -0.62$ at $p < 0.01$). Severe course of bronchitis evolving into bronchial pneumonia in calves with IUGR within neonatal period was registered 7.14 times more frequently ($p < 0.001$) than in calves born by mothers with physiological course of pregnancy.

Therefore, according to the findings of this study, the lack of enzymatic and non-enzymatic links of the antioxidant protection in newborn calves with intrauterine growth retardation causes the oxidative stress, accumulation of toxic products of peroxidation of lipids in blood and epithelial lining fluid, structural and functional damage to respiratory tract cells, and considerably increases the risk of bronchial pneumonia in neonatal period.

REFERENCES

1. Wu G., Bazer F.W., Wallace J.M., Spencer T.E. Board-invited review: Intrauterine growth retardation: implications for the animal sciences. *J. Anim. Sci.*, 2006, 84: 2316-2337 (doi: 10.2527/jas.2006-156).
2. Nezhdanov A.G., Mikhalev V.I, Klimov N.T., Smirnova E.V. *Veterinariya*, 2014, 3: 36-39 (in Russ.).
3. Wang J., Feng C., Liu T., Shi M., Wu G., Bazer F.W. Physiological alterations associated with intrauterine growth restriction in fetal pigs: causes and insights for nutritional optimization. *Molecular Reproduction & Development*, 2017, 84(9): 897-904 (doi: 10.1002/mrd.22842).
4. Nezhdanov A., Shabunin S., Mikhalev V., Klimov N., Chernitskiy A. Endocrine and metabolic mechanisms of embryo and fetal intrauterine growth retardation in dairy cows. *Turk. J. Vet. Anim. Sci.*, 2014, 38(6): 675-680 (doi: 10.3906/vet-1405-12).
5. Rossdale P.D., Ousey J.C. Fetal programming for athletic performance in the horse: Potential effects of IUGR. *Equine Vet. Educ.*, 2003, 6: 24-37 (doi: 10.1111/j.2042-3292.2003.tb01811.x).
6. Gallo L.A., Tran M., Moritz K.M., Wlodek M.E. Developmental programming: variations in early growth and adult disease. *Clin. Exp. Pharmacol. Physiol.*, 2013, 40(11): 795-802 (doi: 10.1111/14401681.12092).
7. Gonzales-Bulnes A., Astiz S., Parraguez V.H., Garcia-Contreras C., Vazquez-Gomez M. Empowering translation research in fetal growth restriction: sheep and swine animal models. *Current Pharmaceutical Biotechnology*, 2016, 17(10): 848-855 (doi: 10.2174/1389201017666160519111529).
8. Greenwood P.L., Hunt A.S., Hermanson J.W., Bell A.W. Effects of birth weight and postnatal nutrition on neonatal sheep: I. Body growth and composition, and some aspects of energetic efficiency. *J. Anim. Sci.*, 1998, 76(9): 2354-2367 (doi: 10.2527/1998.7692354x).
9. Mellor D.J. Nutritional and placental determinants of foetal growth rate in sheep and consequences for the newborn lamb. *Brit. Vet. J.*, 1983, 139(4): 307-324 (doi: 10.1016/S0007-1935(17)30436-0).
10. Sharma D., Shastri S., Sharma P. Intrauterine growth restriction: antenatal and postnatal aspects. *Clinical Medicine Insights: Pediatrics*, 2016, 10: 67-83 (doi: 10.4137/CMPed.S40070).
11. Thornbury J.C., Sibbons P.D., van Velzen D., Trickey R., Spitz L. Histological investigations into the relationship between low-birth-weight and spontaneous bowel damage in the neonatal piglet. *Pediatric Pathology*, 1993, 13(1): 59-69 (doi: 10.3109/15513819309048193).
12. Ginther O.J., Douglas R.H. The outcome of twin pregnancies in mares. *Theriogenology*, 1982, 18(2): 237-244 (doi: 10.1016/0093-691X(82)90108-X).
13. Lipsett J., Tamblyn M., Madigan K., Roberts P., Cool J.C., Runciman S.I., McMillen I.C., Robinson J., Owens J.A. Restricted fetal growth and lung development: a morphometric analysis of pulmonary structure. *Pediatric Pulmonology*, 2006, 41(12): 1138-1145 (doi: 10.1002/ppul.20480).
14. Rozance P.J., Seedorf G.J., Brown A., Roe G., O'Meara M.C., Gien J., Tang J.-R., Abman S.H. Intrauterine growth restriction decreases pulmonary alveolar and vessel growth and causes pulmonary artery endothelial cell dysfunction in vitro in fetal sheep. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 2011, 301(6): L860-L871 (doi: 10.1152/ajplung.00197.2011).

15. Che L., Xuan Y., Hu L., Liu Y., Xu Q., Fang Z., Lin Y., Xu S., Wu D., Zhang K., Chen D. Effect of postnatal nutrition restriction on the oxidative status of neonates with intrauterine growth restriction in a pig model. *Neonatology*, 2015, 107(2): 93-99 (doi: 10.1159/000368179).
16. Chernitskii A.E., Retskii M.I., Zolotarev A.I. *Ustroistvo dlya sbora kondensata vydykhaemogo vozdukha u zhivotnykh. Pat. 134772 (RF) MPK A61B 5/00. Gosudarstvennoe nauchnoe uchrezhdenie Vserossiiskii nauchno-issledovatel'skii veterinarnyi institut patologii, farmakologii i terapii Rossiiskoi akademii sel'skokhozyaistvennykh nauk (RF). № 2013135753/14. Zayavl. 30.07.2013. Opubl. 27.11.2013. Byul. № 33* [Device for collecting condensate of exhaled air in animals. Patent 134772 (RF), IPC A61B 5/00. Appl. 30.07.2013. Publ. 27.11.2013. Bull. № 33] (doi: 10.13140/RG.2.1.4414.1283) (in Russ.).
17. Retskii M.I., Shabunin S.V., Bliznetsova G.N., Rogacheva T.E., Ermolova T.G., Fomenko O.Yu., Bratchenko E.V., Dubovtsev V.Yu., Kaverin N.N., Tsebrzhinskii O.I. *Metodicheskie polozheniya po izucheniyu protsessov svobodnoradikal'nogo okisleniya i sistemy antioksidantnoi zashchity organizma* [Methodical provisions for studying free radical oxidation and the system of antioxidant defense of the body]. Voronezh, 2010 (in Russ.).
18. Sirota T.V. *Voprosy meditsinskoi khimii*, 1999, 45(3): 263-272 (in Russ.).
19. Miller K.W., Yang C.S. An isocratic high-performance liquid chromatography method for the simultaneous analysis of plasma retinol, α-tocopherol and various carotenoids. *Anal. Biochem.*, 1985, 145(1): 21-26 (doi: 10.1016/0003-2697(85)90321-5).
20. Okamura M. An improved method for determination of L-ascorbic acid and L-dehydroascorbic acid in blood plasma. *Clinica Chimica Acta*, 1980, 103(3): 259-268 (doi: 10.1016/0009-8981(80)90144-8).
21. Erel O. A novel automated method to measure total antioxidant response against potent free radical reactions. *Clin. Biochem.*, 2004, 37(2): 112-119 (doi: 10.1016/j.clinbiochem.2003.10.014).
22. Voronkova Y.G., Popova T.N., Agarkov A.A., Skulachev M.V. Influence of 10-(6'-plastoquinonyl)decyltriphenylphosphonium (SKQ1) on oxidative status in rats with protamine sulfate-induced hyperglycemia. *Biochemistry Moscow*, 2015, 80(12): 1606-1613 (doi: 10.1134/S0006297915120093).
23. Andronov S.V., Lobanov A.A. *Vestnik Severo-Zapadnogo gosudarstvennogo meditsinskogo universiteta im. I.I. Mechnikova*, 2012, 4(1): 73-77 (in Russ.).
24. McGuirk S.M. Disease management of dairy calves and heifers. *Veterinary Clinics of North America: Food Animal Practice*, 2008, 24: 139-153 (doi: 10.1016/j.cvfa.2007.10.003) (in Russ.).
25. Fudin N.A., Sudakov K.V., Khadartsev A.A., Klassina S.Ya., Chernyshov S.V. *Vestnik novykh meditsinskikh tekhnologii*, 2011, 18(3): 244-248 (in Russ.).
26. Chernitskii A., Retskii M. *Kondensat vydykhaemogo vozdukha. Ispol'zovanie v diagnostike i prognozirovanii respiratornykh boleznei telyat* [Condensate of the exhaled air — use in the diagnosis and prediction of respiratory diseases of calves.]. LAP LAMBERT Academic Publishing GmbH & Co. KG, Saarbrücken, 2010 (in Russ.).
27. Chernitskii A.E., Efanova L.I., Zolotarev A.I., Shakhov A.G., Shabunin S.V., Retskii M.I. *Metodicheskoe posobie po prognozirovaniyu i rannei diagnostike respiratornykh boleznei u telyat* [Manual on prediction and early diagnosis of respiratory diseases in calves]. Voronezh, 2013 (doi: 10.13140/RG.2.2.11326.28481) (in Russ.).
28. Retskii M.I., Bliznetsova G.N., Shabunin S.V. *Metabolicheskie adaptatsii telyat v rannii postnatal'nyi period* [Metabolic adaptation of calves during early postnatal period]. Voronezh, 2010 (in Russ.).
29. Chernitskii A.E., Retskii M.I., Zolotarev A.I. Functional formation of respiratory system in neonatal calves with different viability. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2013, 4: 99-104 (doi: 10.15389/agrobiology.2013.4.99eng) (in Russ.).
30. Sharma A., Ford S., Calvert J. Adaptation for life: a review of neonatal physiology. *Anaesthesia & Intensive Care Medicine*, 2011, 12(3): 85-90 (doi: 10.1016/j.mpaic.2010.11.003).
31. Mutinati M., Pantaleo M., Roncetti M., Piccinno M., Rizzo A., Sciorsci R.L. Oxidative stress in neonatology. A review. *Reprod. Dom. Anim.*, 2014, 49(1): 7-16 (doi: 10.1111/rda.12230).
32. Retskii M.I. *Sistema antioksidantnoi zashchity u zhivotnykh pri stresse i ego farmakologicheskoi regulyatsii. Doktorskaya dissertatsiya* [Antioxidant protection in animals under stress and its pharmacological regulation. DSc Thesis]. Voronezh, 1997 (in Russ.).
33. Frank L., Sosenko I.R. Prenatal development of lung antioxidant enzymes in four species. *The Journal of Pediatrics*, 1987, 110(1): 106-110 (doi: 10.1016/S0022-3476(87)80300-1).
34. Harman A.W., McKenna M., Adamson G.M. Postnatal development of enzyme activities associated with protection against oxidative stress in the mouse. *Biol. Neonate*, 1990, 57(3-4): 187-193 (doi: 10.1159/000243190).
35. Surai P.F., Speake B.K., Noble R.C., Sparks N.H. Tissue-specific antioxidant profiles and susceptibility to lipid peroxidation of the newly hatched chick. *Biol. Trace Elem. Res.*, 1999, 68(1): 63-78 (doi: 10.1007/BF02784397).
36. Enukashvili A.I. *Mineral'nyi sostav volosyanogo pokrova krupnogo rogatogo skota v svyazi s vozrastom, polom, sezonom goda i fiziologicheskim sostoyaniem. Kandidatskaya dissertatsiya* [Mineral composition of hair in a relationship with cattle age, sex, season and physiological condition. PhD Thesis]. St. Petersburg, 1992 (in Russ.).
37. Alekhin Yu.N., Prigorodova O.V.. *Naukovii visnik veterinarnoï meditsini*, 2014, 13(108): 21-24 (in Russ.).

38. Rickett G.M., Kelly F.J. Developmental expression of antioxidant enzymes in guinea pig lung and liver. *Development*, 1990, 108(2): 331-336.
39. Shukla D., Saxena S., Jayamurthy P., Sairam M., Singh M., Jain S.K., Bansal A., Ilavazaghan G. Hypoxic preconditioning with cobalt attenuates hypobaric hypoxia-induced oxidative damage in rat lungs. *High Altitude Medicine & Biology*, 2009, 10(1): 57-69 (doi: 10.1089/ham.2008.1028).
40. Prohaska J.R. Changes in Cu,Zn-superoxide dismutase, cytochrome c oxidase, glutathione peroxidase and glutathione transferase activities in copper-deficient mice and rats. *The Journal of Nutrition*, 1991, 121(3): 355-363 (doi: 10.1093/jn/121.3.355).
41. McElroy M.C., Postle A.D., Kelly F.J. Catalase, superoxide dismutase and glutathione peroxidase activities of lung and liver during human development. *Biochim. Biophys. Acta*, 1992, 1117(2): 153-158 (doi: 10.1016/0304-4165(92)90073-4).
42. Hracsko Z., Orvos H., Novak Z., Pal A., Varga I.S. Evaluation of oxidative stress markers in neonates with intra-uterine growth retardation. *Redox Report*, 2008, 13(1): 11-16 (doi:

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 842-850
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Veterinary virology, immunology

UDC 636.52/.58:619:578:575

doi: 10.15389/agrobiology.2018.4.842eng
doi: 10.15389/agrobiology.2018.4.842rus

DETECTION OF AVIAN LEUKEMIA VIRUS SUBGROUP K IN RUSSIA AND ITS MOLECULAR GENETIC ANALYSIS

A.M. BORODIN[1], Ya.I. ALEKSEEV[2, 3], N.V. KONOVALOVA[2, 3], E.V. TERENTYEVA[2, 3], D.N. EFIMOV[4], Zh.V. EMANUILOVA[4], S.V. SMOLOV[4], O.A. OGNEVA[4], V.I. FISININ[5]

[1]*Non-proffn Partnership Institute of Medico-Biological Research,* 10, ul. Studenaya, Nizhnii Novgorod, 603000 Russia, e-mail Aborodinm@sinn.ru;
[2]*All-Russian Research Institute of Agricultural Biotechnology,* Federal Agency of Scientific Organizations, 42, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail jalex@iab.ac.ru (✉ corresponding author);
[3]*LLC Syntol*, 42, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail jalex@syntol.ru;
[4]*Breeding and Genetic Center Smena*, Federal Agency of Scientific Organizations, pos. Bereznyaki, Moscow Province, 141327 Russia, e-mail Smena@tsinet.ru, dmi40172575@yandex.ru (✉ corresponding author);
[5]*Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS,* Federal Agency of Scientific Organizations, 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141315 Russia, e-mail olga@vnitip.ru, vnitip@vnitip.ru
ORCID:
Borodin A.M. orcid.org/0000-0002-1478-1261
Alekseev Ya.I. orcid.org/0000-0002-1696-7684
Konovalova N.V. orcid.org/0000-0003-4316-1077
Terentyeva E.V. orcid.org/0000-0003-2777-0948
Efimov D.N. orcid.org/0000-0002-4152-2476
Emanuilova Zh.V. orcid.org/0000-0002-8855-2947
Smolov S.V. orcid.org/0000-0001-6058-3672
Ogneva O.A. orcid.org/0000-0002-8698-1975
Fisinin V.I. orcid.org/0000-0003-0081-6336
The authors declare no conflict of interests
Acknowledgements:
The study was carried out with the equipment of «Biotechnology» center (All-Russian Research Institute of Agricultural Biotechnology).
Supported financially by Federal Agency of Scientific Organizations, the state assignment for Breeding and Genetic Center Smena № 007-01359-17-00 in the framework of the Federal Scientific and Technical Program of agricultural development for 2017-2025, Subprogram «Development of competitive national broiler meat cross»
Received November 27, 2017

Abstract

The Avian leukemia virus (ALV) belongs to the genus *Alpharetrovirus* (*Retroviridae*). The genome of the virus is a single-stranded RNA of more than 7,000 nucleotides in length. The ALV subgroups A, B, C, D, J, K and E are specific viruses of chicken. ALV classification is based on the type-specific envelope protein antigens, GP85. ALV is widely spread all over the world, causes various diseases, reduces productivity and leads to huge damage to poultry industry. The viruses of subgroup K were first discovered in Asian countries. Studies of the prevalence of ALV of subgroup K are few and have not been conducted in Russia before. Our goal was to study the spread of the ALV of this subgroup to the chickens of Russian poultry farms using a test system designed to identify the genome of the ALV subgroup K by real-time PCR and analysis of the properties of the ALV of subgroup K. The test of the real-time PCR test system was carried out on 5292 DNA samples of chickens of domestic broiler meat type of one of the farms in the Moscow Province and in chickens of meat and egg breeds from various regions of Russia. The ALV-K-specific gene *gp85* sequences were found in 177 (3.3 %) broilers of one of the farms in the Moscow Province. Sequencing of *gp85* gene fragments revealed the presence of two groups of different ALV subgroup K in the chickens of this farm: one had 96 % similarity to the ALV subgroup K strains Oki 009, GDFX0601, GDFX0602, GDFX0603, Km_5845 etc., and the other - up to 100 % similar to ALV subgroup K strain TW-3593. Using the reference sequence of the ALV subgroup K strain GD14LZ (KU605774), 22 full-length genomic sequences, belonging to ALV subgroup K, with 95 to 100 % similarity to the GD14LZ *gp85* gene sequences were detected in GenBank. The construction of phylogenetic trees based on the *gp85* gene sequences showed that subgroup K viruses form a separate group, distinct from the rest of the viruses, which can be divided into four clusters based on differences in long terminal repeats. The subgroup K viruses have a set of four different 3′UTR sequences belonging to both the pathogenic ALV subgroup J and the less pathogenic ALV subgroup E, and there are also ALV K and Oki, the ALV-K-specific 3′UTRs. In addition to the Moscow region, ALV subgroup K was found in the Kaliningrad, Leningrad, Sverdlovsk, and Novgorod regions of Russia. Thus, the

distribution of ALV subgroup K is not limited to the countries of Asia. Scientific publications show that ALV subgroup K can cause gliomas and myocarditis. Literature data allows us to say that ALV subgroup K can be pathogenic and theses viruses need a control program, because even the subclinical form of exogenous and endogenous ALV can lead to large economic losses.

Keywords: avian leukosis virus subgroup K, real time PCR, ALV subgroup K detection

Avian leukosis virus (ALV) belongs to the *Alpharetrovirus* genus of the *Retroviridae* family. The virus genome has the form of a single-stranded RNA of more than 7000 nucleotides in length. The A, B, C, D, J, K and E subgroups of ALV are specific for chickens. These subgroups differ with their antigenic regions of the GP85 coat protein [1-3]. ALV can cause not only lymphoid and myeloid leukosis, but also neoplasms of other tissues [4-6] including gliomas and neurofibrosarcomas [7, 8]. The virus subgroup can be identified basing on the analysis of the nucleotide sequence of the GP85 coat protein gene [3, 9, 10]. Depending on the way of infecting the host cells, ALV is divided into exogenous [1, 4] and endogenous viruses. The viruses of A, B, C, D, J and K subgroups belong to exogenous ones and are more pathogenic than the endogenous virus of E subgroup which has no or weak pathogenicity. The genome of endogenous viruses is integrated into the host's genome and is transmitted vertically like the rest genes of the host. Exogenous viruses are able to spread congenitally by infecting hens and the embryos form infected hens (a form of vertical transmission). Endogenous ALV can sometimes behave as exogenous ones infecting chickens horizontally. Usually, the chicken mortality caused by ALV infection does not exceed a few percent, but for active form of infection, the mortality rate may amount to more than 20% [9]. ALV is widely spread all over the world and can cause various pathologies, reducing bids' performance and leading to huge damage to commercial poultry.

The ALV of K subgroup was recently found in Asia, i.e. in China [3, 10, 11], Japan [8], and Taiwan [12]. The pathogenicity of these isolates is unclear. There is a danger of recombination of the K subgroup ALVs with the viruses of other subgroups, in particular with ALV-J that may lead to the emergence of a new subgroup of ALV with higher pathogenicity [10, 11]. The studies focusing the prevalence of the K subgroup of ALV are few and have not been conducted in Russia before [13].

This article reports the first identification of the K subtype of ALV in Russian industrial populations of chickens and presents the results of its molecular-genetic studies.

Our goal was to detect the K subgroup of ALV in poultry farms in Russia with using the developed testing system based on the real-time PCR (q-PCR) and the subsequent analysis of the properties of the K subgroup of ALV.

Techniques. The primers and probe for the RT-PCR and Sanger sequencing were synthesized in the Syntol LLC. They were selected so as to amplify the DNA of K subgroup ALVs without amplifying the known endoviruses. The carboxyfluorescein (6FAM) was used as a fluorescent tag, BHQ-1 dye was a fluorescence quencher, and phosphate (p) was used for the 3′ terminal modification of the probe.

The following primers and probe were used to identify the K subtype of ALV by the RT-PCR method:

ALVKF (5′→3′) — CGGAGCATTGACACGCTTTCAGA,

ALVKR (5′→3′) — GTGGTTGCGGCGGAGGAGGA,

KPL (5′→3′) — (6FAM)CCACCTCGTGAG(dT-BHQ-1)TGCGGCC-p.

The length of the synthesized amplicon is 72 bp, the fragment is a part of the *gp85* gene encoding the GP85 coat protein.

The primers which have been used for PCR and sequencing the DNA of

the K subtype of ALV:

ALVKF (5′→3′) — CGGAGCATTGACAAGCTTTCAGA,

SEQA-KR (5′→3′) — CGCGATCCCCACAAATGAGGAAA.

The length of the amplification product (part of the *gp85* gene encoding the GP85 coat protein) is 466 bp.

Typing of 3′ terminal fragments of *gp85* genes and of the variants of long terminal repeats (LTRs) has been performed using three reverse primers and SEQKF universal primer:

SEQKF (5′→3′) — GGCCGTTCATTTGCTGAAAGGA,

SEQKRKm (5′→3′) — CAGGCTAGGCACTTAAGTACAACA,

SEQKRJ (5′→3′) — GGGCACTTAAATACAGTATCTCTG,

SEQKROKI (5′→3′) — CAATCAGCATGCGCCACGATGAA.

To amplify the 3′ terminal fragments of the *gp85* gene and of the LTRs similar to those of the endovirus, we used the special pair of primers which preclude the amplification of the sequences of DNA of the E subgroup of ALV:

SEQKF12 (5′→3′) — GTGGCTCCTCCTCCGCCGCAA,

SEQKREV (5′→3′) — GCAGCTTATATAATCGTGCATAGC.

The tryout of the testing system was performed on 5292 DNA samples of chickens of broiler-type meat cross from a farm of the Moscow region.

DNA was extracted from feathers using M-Sorb kit (Syntol LLC, Russia, a Savraska-02 robotized complex for molecular-genetic researches, Syntol LLC, Russia). A 0.3-0.5 cm fragment of feather was put in a 1.5 ml test tube, then 400 μl of the lysing solution was added and incubated at 60 °C for 20 min with mixing. The lysate was precipitated in the high-speed Cyclotemp-902 microcentrifuge (Cyclotemp CJSC, Russia) for 3 min at 13000 rpm. The supernatant was transferred to a 1.5 ml test tube and the extraction was continued according to the standard protocol for the M-Sorb kit.

The mode for the PCR performing was universal, 1.5 μl of the extracted DNA was taken for the reaction. The primers concentration in the reaction mixture was 450 nM, the probe concentration was 150 nM. RT-PCR (detection channel FAM) was performed (an ANK-48 device, Analytical Instrumentation Institute RAS, Russia) according to the following program (45 cycles): denaturation at 93 °C for 10 s, annealing at 60 °C for 30 s. To amplify DNA, the reaction mixture (10 μl) for the RT-PCR was used (Cat. No. M-428, Synthol LLC, Russia).

The specificity of RT-PCR was confirmed by sequencing of the amplification products obtained with ALVKF and SEQA-KR primers (Nanofor 05 genetic analyzer, Analytical Instrumentation Institute RAS, Russia).

In phylogenetic analysis and selection of the specific DNA regions for typing the K subgroup of ALV and sequencing, the genomic sequences of ALV strains of different subgroups were used: A subgroup — MQNCSU-A (DQ365814); B subgroup — Schmidt-Ruppin B (AF052428); C subgroup — Prague C (J02342.1); D subgroup — Schmidt-Ruppin D (D10652); E subgroup — ev-1 (AY013303); J subgroup — HPRS103 J (Z46390); K subgroup — Km_6222 (AB764103), Km_5943 (AB669897), Km_5845 (AB670314), Km_5844 (AB670312), Km_6202 (AB764101), Km_5892 (AB682778), Km_6181 (AB764100), Km_6349 (AB764106), Km_6249 (AB764104), Km_6343 (AB764105), Sp-53 (AB617820), SD110503R (KF738251), Sp-40 (AB617819), JS14CZ02 (KY490696), GDFX0601 (KP686142), GDFX0602 (KP686143), TW-3593 (HM582658), GD14LZ (KU605774), JS11C1 (KF746200), GDFX0603 (KP686144), JS14CZ01 (KY490695), Oki 009 (AB669433) (in the parentheses are the numbers of the GenBank). The conservative regions of the ALV genome, which are specific for the K subgroup, were detected (ClustallW and BLAST

http://www.genome.jp/tools-bin/clustalw and http://www.ncbi.nlm.nih.gov/BLAST software was used). The phylogenetic trees for the ALVs of different subgroups were constructed with ClustallW by Rooted phylogenetic tree (UPGMA) algorithm.

Results. The selected regions of the ALV genome, which are specific for the K subgroup, have been used for the amplifying and sequencing the DNA. When designing of the system, the synthetic fragment of DNA corresponding to the estimated amplicon for the ALV of K subgroup was the positive control. The analytical sensitivity of the system was evaluated in the test with dilutions of the positive control. It amounted to 100 copies of the ALV genome of K subgroup or approximately 70 genome equivalents per 1 μl of the initial sample. The solution not containing the dilutions of the synthetic amplicon was the negative control.

Using the developed testing system, 5292 DNA samples from broilers of a farm in the Moscow region have been examined. The specific sequences of the *gp85* gene of the K subgroup ALVs were found in 177 birds that amounted to 3.3% of the total number. The samples collected in other regions of Russia have been also analyzed. As the result, the genomic sequences of the K subgroup ALVs were also found in Kaliningrad region (3 samples), Leningrad region (2 samples), Sverdlovsk region (5 samples) and Novgorod region (3 samples). Considering the fact that the poultry of one of the poultry farms of the Kaliningrad region, where the ALVs of K subgroups have been detected, has been supplied from Germany, the geography of distribution of this ALV type in Europe is probably not limited to Russia.

To confirm the specificity of the testing system, the fragments of *gp85* gene were sequenced using the ALVKF and SEQA-KR primers and DNA of 12 chickens from a farm of the Moscow region. The analysis revealed two variants of genomic sequences of the K subgroup ALV. In the first group, the virus had 96% similarity with the Oki 009, GDFX0601, GDFX0602, GDFX0603, Km_5845 and other ALV strains, in the second group the similarity was up to 100% with the TW-3593 strain. The sequencing showed that both variants of examined ALV have the LTRs similar to those of the E subgroup of ALV.

In analysis of geographic prevalence and relation of K subgroup ALV with the chicken diseases, we used the KU605774 sequence of the GD14LZ isolate genome of the K subgroup ALV as the reference sequence based on the GenBank data [3]. The presence of sequences similar to the GD14LZ genome has revealed the countries in which the ALV of K subgroup is widespread. A number of full-length genomic sequences of ALV with 95-100% similarity of the *gp85* gene sequences with the *gp85* gene sequence of the GD14LZ isolate has been found in the GenBank database. The variability of the viral genome of the J subgroup ALV is very high and may reach 94.9% within one organism [14[. The pool consisting of 22 full-length genomic sequences of ALV, which presumably belong to the K subgroup, was formed and analyzed (Table, Fig.).

The construction of phylogenetic trees based on the nucleotide sequences of *gp85* gene fragments showed that the ALV K subgroup forms a separate group which is different from the rest viruses (see Fig., A). The analysis of the 3′-UTR sequences of ALV K subgroup demonstrates viral heterogeneity and the fact that the K subgroup can be divided into four clusters (see Fig., B). The ALV K subgroup has four different 3′-UTR regions (see Table) similar to 3′-UTR of both the pathogenic strains of ALV J subgroup and less pathogenic E subgroup. There are also 3′-UTRs specific for the K subgroup (ALV K and Oki).

Different 3′-UTRs could be obtained as the result of recombination with other viruses, for example, the virus carrying *gp85* gene from the ALV A subgroup and the LTR from ALV J subgroup is known [15]. The ALV J subgroup

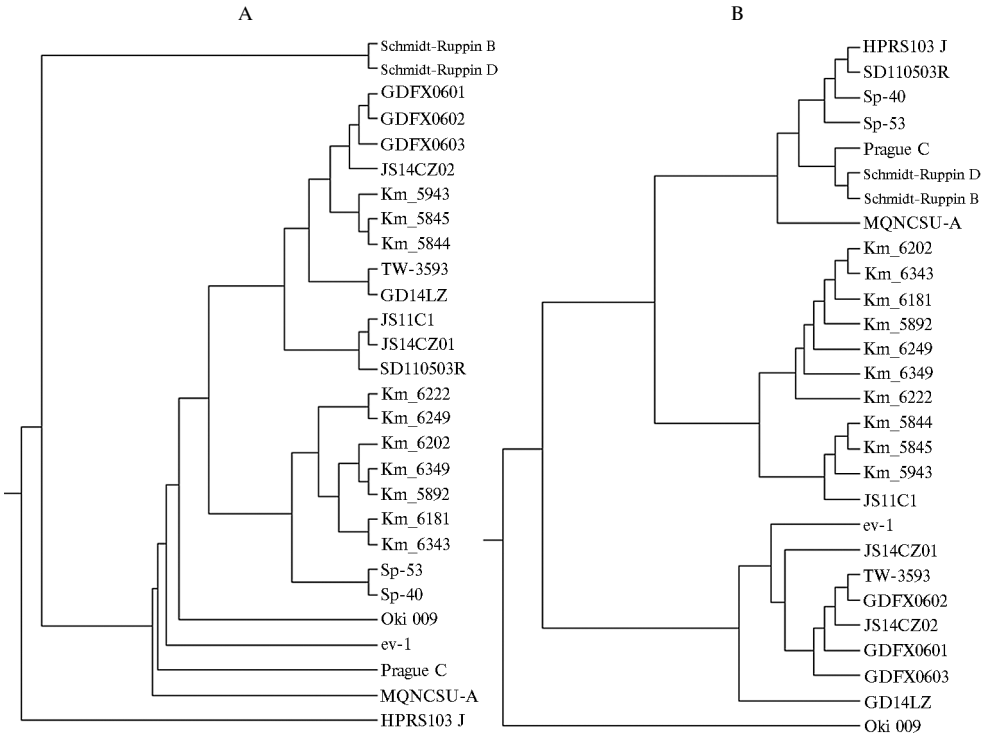
presumably results from recombination between endogenous and exogenous forms of the virus [16]..

Pathogenic properties and prevalence of K subgroup avian leukosis virus isolates described in the literature and represented in the GenBank (NCBI)

Isolate (area)	No. in GenBank	Author (reference)	Type of 3′-UTR	Pathology
SD110503R (China)	KF738251	Chen J. n.p.	ALV J	Unknown
Sp-40 (Japan)	AB617819	Nakamura S. et al. [8]	ALV J	Glioma
Sp-53 (Japan)	AB617820	Nakamura S. et al. [8]	ALV J	Glioma
Km_6222 (Japan)	AB764103	Nakamura S. n.p.	ALV K	Unknown
Km_6343 (Japan)	AB764105	Nakamura S. n.p.	ALV K	Unknown
Km_6181 (Japan)	AB764100	Nakamura S. n.p.	ALV K	Unknown
Km_5892 (Japan)	AB682778	Nakamura S. et al. [17]	ALV K	Myocarditis
Km_6249 (Japan)	AB764104	Nakamura S. n.p.	ALV K	Unknown
Km_6349 (Japan)	AB764106	Nakamura S. n.p.	ALV K	Unknown
Km_6222 (Japan)	AB764103	Nakamura S. n.p.	ALV K	Unknown
Km_5844 (Japan)	AB670312	Ochi A. et al. [18]	ALV K	Glioma
Km_5845 (Japan)	AB670314	Ochi A. et al. [18]	ALV K	Astrocyte proliferation
Km_5943 (Japan)	AB669897	Ochi A. et al. [18]	ALV K	Unknown
JS11C1 (China)	KF746200	Cui N. et al. [10]	ALV K	Unknown
JS14CZ01 (China)	KY490695	Shao H. et al. [11]	ALV E	Unknown
TW-3593 (Taiwan)	HM582658	Chang S.W. et al. [12]	ALV E	Unknown
GDFX0601 (China)	KP686142	Jianyong H. n.p.	ALV E	Unknown
GDFX0602 (China)	KP686143	Jianyong H. n.p.	ALV E	Unknown
GDFX0603 (China)	KP686144	Jianyong H. n.p.	ALV E	Unknown
JS14CZ02 (China)	KY490696	Shao H. et al. [11]	ALV E	Unknown
GD14LZ (China)	KU605774	Li X. et al. [3]	ALV E	Unknown
Oki 009 (Japan)	AB669433	Ochi A. et al. [18]	Oki	Unknown

N o t e. “n.p.” means the unpublished data (deposited in the GenBank).

The analysis of the literature shows that the ALV K subgroup can cause gliomas and myocarditis [8, 17, 18] which are atypical for the ALV pathology. In the case of avian leukosis virus, the mechanism of pathogenesis is not fully studied. It is assumed that the high variability of retroviruses may contribute to their virulence and pathogenesis [19]. In the LTR areas of some ALV strains, there is the E element which is not an oncogene but increases the pathogenicity of the viruses [20]. Some of the studied sequences of the ALV K subgroup have a similarity to the E element. The example is SD110503R (KF738251) strain from China (nothing is known about the pathogenicity of this strain because only the genomic sequence in the GenBank has been published). Two ALV strains causing glioma are described in Japan, these are Sp-40 (AB617819) and Sp-53 (AB617820). Their genomes contain a small fragment of 27 nucleotides in length which is similar to the E element [8] (see Table). Insertional mutagenesis is a mechanism in infection by avian leukosis virus [21, 22]. Also, the expression of the host cell genes may change due to additional transcription of ALV LTR [23, 24]. Much in the infection expression mechanism is still unclear because the spectrum of diseases caused by ALV is quite wide. For example, it is assumed that in the hemangiomas progression the leading role belongs to the insertion mutagenesis with changing of the *met* gene expression [21], other authors point out the importance of the participation of GP85 coat protein in the disease progression [24]. There is the evidence that the coat protein is the main determinant when lymphoid and myeloid leukosis [25]. It may be assumed that the presence of ALVK subgroup reduces the productive performance of poultry. Thus, it is believed that the ALVs having the LTRs similar to the LTRs of the ALV E subgroup do not have any noticeable pathogenicity. However, the metagenomic analysis of the possible infectious agent which has caused up to 20% loss of the chicken population [26] showed that the nucleotide sequences of this virus express 100% similarity with the *gp85* gene and the LTR of the ALV strain TW-3593 which has the LTR of the ALV E subgroup.



Phylogenetic trees constructed for the sequences of *gp85* gene of coat protein (A) **and 3′-UTRs** (B) **of strains of different avian leukosis virus subtypes from the GenBank**. See the description of these strains in the Techniques section

The exogenous and endogenous avian leukosis viruses may cause large economic losses even in subclinical infection [6, 27]. To study the pathogenic properties of the ALV K subgroup which we have found in the broiler-type meat cross, 123 infected birds at the age of 58 days were isolated from the main herd. We have not detected any neoplastic processes in 6 birds died at the age of 156 days.

So, the real-time PCR testing system has been developed for detecting the avian leucosis virus of K subgroup. Using this test we have detected the virus of this subgroup in Russia. Thus, the prevalence of the ALV K subgroup is not limited to Asian countries. The analysis of the literature data indicates possible pathogenicity of the K subgroup ALV, therefore, the program for its monitoring and control is needed.

REFERENCES

1. Lloyd Spencer J. Progress towards eradication of lymphoid leukosis viruses — a review. *Avian Pathol.*, 1984, 13(4): 599-619 (doi: 10.1080/03079458408418560).
2. Payne L.N., Brown S.R., Bumstead N., Howes K., Frazier J.A., Thouless M.E. A novel subgroup of exogenous avian leukosis virus in chickens. *J. Gen. Virol.*, 1991, 72: 801-807 (doi: 10.1099/0022-1317-72-4-801).
3. Li X., Lin W., Chang S., Zhao P., Zhang X., Liu Y., Chen W., Li B., Shu D., Zhang H., Chen F., Xie Q. Isolation, identification and evolution analysis of a novel subgroup of avian leukosis virus isolated from a local Chinese yellow broiler in South China. *Arch. Virol.*, 2016, 161(10): 2717-2725 (doi: 10.1007/s00705-016-2965-x).
4. Crittenden L.B. Exogenous and endogenous leukosis virus genes — A review. *Avian Pathol.*, 1981, 10(2): 101-112 (doi: 10.1080/03079458108418464).
5. Payne L.N., Fadly A.M. Leukosis/sarcoma group. In: *Diseases of poultry*. B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. Mc Dougald, Y.M. Saif (eds.). Iowa, 1997, V. 10: 414-466.
6. Payne L.N. Retrovirus-induced disease in poultry. *Poultry Sci.*, 1998, 77(8): 1204-1212 (doi: 10.1093/ps/77.8.1204).
7. Ochi A., Ochiai K., Nakamura S., Kobara A., Sunden Y., Umemura T. Molecular characteris-

tics and pathogenicity of an avian leukosis virus isolated from avian neurofibrosarcoma. *Avian Dis.*, 2012, 56(1): 35-43 (doi: 10.1637/9830-060711-Reg.1).
8. Nakamura S., Ochiai K., Hatai H., Ochi A., Sunden U., Umemura T. Pathogenicity of avian leukosis viruses related to fowl glioma-inducing virus. *Avian Pathol.*, 2011, 40(5): 499-505 (doi: 10.1080/03079457.2011.605783).
9. Gao L., Qin L.T., Pan W., Wang Y.Q., Lee Qi.X., Gao H.L., Wang X.M. Avian leukosis virus subgroup J in layer chickens, China. *Emerg. Infect. Dis.*, 2010, 16(10): 1637-1638 (doi: 10.3201/eid1610.100780).
10. Cui N., Su S., Chen Z., Zhao X., Cui Z. Genomic sequence analysis and biological characteristics of a rescued clone of avian leukosis virus strain JS11C1, isolated from indigenous chickens. *J. Gen. Virol.*, 2014, 95(Pt 11): 2512-2522 (doi: 10.1099/vir.0.067264-0).
11. Shao H., Wang L., Sang J., Li T., Liu Y., Wan Z., Qian K., Qin A., Ye A. Novel avian leukosis viruses from domestic chicken breeds in mainland China. *Arch. Virol.*, 2017, 162(7): 2073-2076 (doi: 10.1007/s00705-017-3344-y).
12. Chang S.W., Hsu M.F., Wang C.H. Gene detection, virus isolation, and sequence analysis of avian leukosis viruses in Taiwan country chickens. *Avian Dis.*, 2013, 57(2): 172-177 (doi: 10.1637/10387-092612-Reg.1).
13. Plotnikov V.A., Grebennikova T.V. Dudnikova E.K., SHul'pin M.I., Lazareva S.P., Nikonova Z.B., Men'shchikova A.E., Norkina S.N., Aliper T.I. About spreading the avian leukosis viruses in poultry farms in the Russian Federation. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2013, 6: 36-42 (doi: 10.15389/agrobiology.2013.6.36eng) (in Russ.).
14. Meng F., Li X., Fang J., Gao Y., Zhu L., Xing G., Tian F., Gao Y., Dong X., Chang S., Zhao P., Cui Z., Liu Z. Genomic diversity of the Avian leukosis virus subgroup J gp85 gene in different organs of an infected chicken. *J. Vet. Sci.*, 2016, 17(4): 497-503 (doi: 10.4142/jvs.2016.17.4.497).
15. Lupiani B., Hunt H., Silva R., Fadly A. Identification and characterization of recombinant subgroup J avian leukosis viruses (ALV) expressing subgroup A ALV envelope. *Virology*, 2000, 276(1): 37-43 (doi: 10.1006/viro.2000.0539).
16. Bai J., Payne L.N., Skinner M.A. HPRS-103 (exogenous avian leukosis virus, subgroup J) has an *env* gene related to those of endogenous elements EAV-0 and E51 and an E element found previously only in sarcoma viruses. *J. Virol.*, 1995, 69(2): 779-84 (doi: 10.1128/JVI.75.2.726-737.2001).
17. Nakamura S., Ochiai K., Ochi A., Yabushita H., Abe A., Kishi S., Sunden Y., Umemura T. Cardiac pathology and molecular epidemiology by avian leukosis viruses in Japan. *PLoS ONE*, 2014, 9(1): e86546 (doi: 10.1371/journal.pone.0086546).
18. Ochi A., Ochiai K., Kobara A., Nakamura S., Hatai H., Handharyani E., Tiemann I., Tanaka I.B. 3rd, Toyoda T., Abe A., Seok S.H., Sunden Y., Torralba N.C., Park J.H., Hafez H.M., Umemura T. Epidemiological study of fowl glioma-inducing virus in chickens in Asia and Germany. *Avian. Pathol.*, 2012, 41(3): 299-309 (doi: 10.1080/03079457.2012.684373).
19. Mansky L.M. Retrovirus mutation rates and their role in genetic variation. *J. Gen. Virol.*, 1998, 79: 1337-1345 (doi: 10.1099/0022-1317-79-6-1337).
20. Chesters P.M., Smith L.P., Nair V. E (XSR) element contributes to the oncogenicity of Avian leukosis virus (subgroup J). *J. Gen. Virol.*, 2006, 87(9): 2685-2692 (doi 10.1099/vir.0.81884-0).
21. Justice J. 4th, Malhotra S., Ruano M., Li Y., Zavala G., Lee N., Morgan R., Beemon K. The MET gene is a common integration target in avian leukosis virus subgroup J-induced chicken hemangiomas. *J. Virol.*, 2015, 89(9): 4712-4719 (doi: 10.1128/JVI.03225-14).
22. Hayward W.S., Neel B.G., Astrin S.M. Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. *Nature*, 1981, 290(5806): 475-480 (doi: 10.1038/290475a0).
23. Laimins L.A., Tsichlis P., Khoury G. Multiple enhancer domains in the 3′ terminus of the Prague strain of Rous sarcoma virus. *Nucleic Acids Res.*, 1984, 12(16): 6427-6442.
24. Maeda N., Fan H., Yoshikai Y. Oncogenesis by retroviruses: old and new paradigms. *Rev. Med. Virol.*, 2008, 18(6): 387-405 (doi: 10.1002/rmv.592).
25. Chesters P.M., Howes K., Petherbridge L., Evans S., Payne L.N., Venugopal K. The viral envelope is a major determinant for the induction of lymphoid and myeloid tumours by avian leukosis virus subgroups A and J, respectively. *J. Gen. Virol.*, 2002, 83: 2553-2561 (doi: 10.1099/0022-1317-83-10-2553).
26. Bande F., Arshad S.S., Omar A.R. Isolation and metagenomic identification of avian leukosis virus associated with mortality in broiler chicken. *Adv. Virol.*, 2016, 2016: Article ID 9058403 (doi: 10.1155/2016/9058403).
27. Bacon L.D., Fulton J.E., Kulkarni G.B. Methods for evaluating and developing commercial chicken strains free of endogenous subgroup E avian leukosis virus. *Avian Pathol.*, 2004, 33(2): 233-243 (doi: 10.1080/0307943042000195731).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 851-859
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.4:619:615.375

doi: 10.15389/agrobiology.2018.4.851eng
doi: 10.15389/agrobiology.2018.4.851rus

PORCINE BIFERON-C APPLIED TOGETHER WITH MEDICINAL PROPHYLAXIS IN COMMERCIAL PIG BREEDING PROVIDES IMMUNOSTIMULATION OF SOWS AND AN INCREASED VIABILITY OF THEIR PIGLETS

S.V. SHABUNIN, A.G. SHAKHOV, G.A. VOSTROILOVA, L.V. CHESKIDOVA, P.A. PARSHIN, T.I. ERMAKOVA, N.A. GRIGORYEVA

All-Russian Research Veterinary Institute of Pathology, Pharmacology and Therapy RAAS, Federal Agency of Scientific Organizations, 114-b, ul. Lomonosova, Voronezh, 394087 Russia, e-mail gvostroilova@mail.ru (✉ corresponding author), vnivipat@mail.ru, A.G.Shakhov@mail.ru, lvcheskidova@yandex.ru, doktor57@mail.ru, ermakova53@list.ru, kettbery@mail.ru

ORCID:
Shabunin S.V. orcid.org/0000-0002-2689-6998
Shakhov A.G. orcid.org/ 0000-0002-6177-8858
Vostroilova G.A. orcid.org/ 0000-0002-2960-038X
Cheskidova L.V. orcid.org/ 0000-0003-0196-1754
Parshin P.A. orcid.org/ 0000-0002-8790-0540
Ermakova T.I. orcid.org/ 0000-0003-1069-1223
Grigoryeva N.A. orcid.org/0000-0002-7593-1198

The authors declare no conflict of interests
Received February 8, 2018

Abstract

Immunodeficiency of pigs resulting from the effect of various infectious and invasive pathogens, mycotoxins, heavy metals, wide usage of chemotherapeutic agents and other xenobiotics, nutritional deficiencies, deficiency of some vitamins and microelements, stresses of various etiology is one of the main causes of high morbidity in breeder herds and the offspring. In this paper, the immune status of sows and safety of suckling piglets under the effect of recombinant alpha- and gamma-interferons, the main active substances of Biferon-C preparation (the Republic of Belarus), applied in combination with conventional measures for prophylaxis with antibiotics and chemoprophylaxis are studied for the first time. It is found that the treatment of sows improves their immune status with a 2-fold increase in safety of their piglets while the offspring of untreated sows shows only a 4 % increase in viability after injections of Biferon-C. For the experiment, two groups of 10 farrow sows each were formed (Zolotaya Niva, Znamenskii Region, Tambov Province, 2017). The animals of basic variant (group I) were treated after farrowing according to the scheme accepted in the farm: during the first 24 hours after farrowing and on day 3 and day 5, one time a day, sows were intramascularly injected with Metramag and treated intrauterinely with Iodopen. Sows of test group II were additionally injected intramuscularly with Biferon-C (10 ml per sow) 24 hours before farrowing and 2 days post farrowing. Newborn piglets of group I born from mother sows not treated with Biferon-C (basic variant, $n = 332$) were treated according to the scheme accepted in the farm, and the animals of test group II ($n = 333$) additionally received two intramascular injections of Biferon-C (0.1 ml/kg of body weight) at 24-hour intervals. Experimental sows and piglets were clinically observed till weaning (24-26 day), considering the percent of morbidity, mortality and growth dynamics of piglets. Blood samples were tested for morphological and immunological indices before the application of the preparations and at the end of the experiment. The basic level of immune indices showed that immune status of sows of all the groups practically did not differ and corresponded to their physiological state. Receiving recombinant proteins by sows led to a significant increase in relevant and absolute blood levels of monocytes, lymphocytes, T- and B-lymphocytes, complementary and lysozyme blood serum activity, phagocytosis. Higher immune status of sows promoted the prophylaxis of their postpartum pathologies and a 2-fold increase in viability of their litters. The injectionы of Biferon-C to piglets led to the decrease in intestinal infection frequency, increasing the safety of the herd.

Keywords: Biferon-C, recombinant proteins, immune status, sows, piglets, postpartum pathology, intestinal infections

One of the main reasons for the high incidence of pig breeding stock and their offspring is immunodeficiency caused by exposure to the various infectious and invasive pathogens [1, 2], mycotoxins [3, 4], heavy metals [5], widespread use of chemotherapeutic agents and other xenobiotics, deficiencies of certain

vitamins, nutrients and microelements [6, 7], stresses of various etiologies (8). Industrial pig farms often face postnatal pathology (acute postnatal purulent catarrhal endometritis, mastitis-metritis-agalactia, MMA) in breeding stock and diarrhoeal and respiratory disease in pigs caused by a variety of infectious agents on the background of reduced immune reactivity [9-13]. Different measures and methods have been offered for their prevention and therapy [14, 15].

In view of the etiology and pathogenesis of postpartum diseases in sows, a complex of prevention measures is promising with the immunostimulatory substances including recombinant interferon with a complex action resulting from the activation of the immune system. Interferons act against the reproduction of many viruses, express tissue specificity, and are insensitive to antiviral antibodies [16, 17]. Interferons possess antibacterial features. The bacteriostatic effect is due to the significant violation of bioenergetic processes in microorganisms by depleting tryptophan and a secondary bactericidal effect is due to the generation of nitric oxide and reactive oxygen species in macrophages [18-21]. The protective role of interferons in the body during bacterial infections is also associated with the activation of T-lymphocytes, macrophages, and natural killer cells, performing a protective function [22]. In the light of detected and investigated properties of interferons indicating their participation in maintaining homeostasis, the preparations are developed with antiviral and immunomodulatory properties [23-26].

Biferon-C is a marketed product with anti-viral and immune stimulatory activity, which is a mixture of porcine species-specific recombinant α and γ - interferons with the total antiviral activity not less than 1.0×10^4 TCD_{50}/cm^3. According to the pharmacological properties of the preparation, it affects the natural resistance (as an inductor bactericidal and lysozyme activity) and immune status (induction of cellular and humoral immunity of a system of endogenous cytokines) of sows and piglets. It should be noted that the literature contain limited data on the impact of Biferon-C. We know one work with the data on a study of the influence of recombinant bovine α- and γ-interferons of a biological product Biferon-B on pregnancy completion and the status of cows and calves after birth [27].

The accumulation of information on the use and effectiveness evaluation of recombinant proteins with species specificity is of obvious scientific interest and of practical importance. We were first to examine the change in the immune status of sows and the safety of suckling piglets influenced by recombinant α- and γ-interferons, the principal active ingredients of Biferon-C (Republic of Belarus) used with the accepted antibiotics and chemoprophylaxis. It was found that the treatment of pregnant sows twice increases the safety of pigs whereas the safety of pigs from non-treated sows is increased by Biferon-C by only 4%.

Objective was to evaluate the effect of Biferon-C using as an additive to the standard scheme of medical prophylaxis of postnatal pathologies of sows and to improve the immune status of suckling piglets in an industrial complex.

Techniques. For the study (Zolotaya Niva LLC, Znamensky district, Tambov Prvince, 2017), two groups were formed (n = 10 each) of crossbred sows (large white + landrace + Duroc breeds) at later stages of pregnancy. On day 107 of gestation after sanitizing the pigs were placed in individual pens in disinfected isolated box of the farrowing area. Microclimatic indices were optimal for the physiological condition of the animals (average temperature in box was 20-23 °C, relative humidity 65-71%). During the experiment, sows were fed with SC-2 feed (Russia), balanced for all the declared nutrients, according to the manufacturer.

Animals from group I (control) after the farrowing were treated according to the guidelines adopted at the farm: in day 1 after farrowing and next at

day 3 and day 5, Metramag (Mosagrogen, Russia), containing 4 IU/ml of oxytocin and 100 mg/ml of ciprofloxacin, was injected intramuscularly once a day and Iodopen (NITA-FARM, Russia), suppositories, each containing 1.5 g of Povidone-Iodine, were used. For sows from group II (experimental) the adopted basic scheme of veterinary processing was supplemented by 10 ml of Biferon-C intramuscularly (Scientific and production center ProBioTeh, LLC, Republic of Belarus; the preparation is a mixture of porcine species-specific recombinant α- and γ-interferons with the total antiviral activity not less than 1.0×10^4 TCD_{50}/cm^3) 24 h before and 2 days after the farrowing. Clinical observation (percentage of incidence, thermometry in the first 4 days after farrowing) of the sows was conducted prior to weaning piglets (days 24-26). Prior to the use of the preparations and at the end of the observation period (days 24-26), the blood was sampled from the animals in blanching Green Vac-Tube tubes (Green Cross, South Korea) to study the morphological and immunological parameters (whole blood and blood serum clinical study).

Newborn piglets from untreated sows also formed the two groups: group I (control; $n = 332$), and group II (experimental; $n = 333$). The control piglets day 1 after birth were docked with tusks removed, and on day 3 got Ursoferran-200 (VIC-Animal Health, Republic of Belarus) containing 200 mg of iron per 1 ml in the form of iron (III)-dextran-heptonic acid, and boar pigs were castrated. The experimental group was injected with Biferon-C intramuscularly at a dose of 0.1 ml/kg Bw on days 1 and 2. Clinical observation of the pigs performed for the weaning from sows (days 24-26) included a selective body temperature measurement, accounting percent of ill and dead animals, and the growth indices (by group weighing).

Blood morphology was studied on a hematologic analyzer AVH Micros 60 (ABX Diagnostics, France) with the determination of leukocyte formula according to recommendations (M.I. Reckij, A.G. Shakhov, V.I. Shushlebin, etc. Methodical Instructions. Voronezh, 2005).

Immune parameters, including complement (SCA) and lysozyme (SLA) activity, total immunoglobulins (Ig), circulating immune complexes (CIC) in blood serum (humoral serum factors of natural nonspecific resistance), phagocytic activity of leukocytes (PAL), phagocytic number (PN), phagocytic index (PI), T- and B-cells count (cell-mediated immunity), were determined using standardized and harmonized methods in accordance with the recommendations (A.G. Shakhov, Yu.V. Masjanov, M.I. Reckij, etc. Methodical recommendations on the evaluation and correction of the immune status of animals. Voronezh, 2005). Reserve function of oxygen-dependent bactericidal system of phagocytes (spontaneous and stimulating nitroblue tetrazolium test, spNBT and stNBT), reserve index (RI) and neutrophil activation index (NAI) were assessed by cytochemical reaction with the calculation of intracellular diformazane, an insoluble form of reduced nitroblue tetrazolium, deposits in accordance with the recommendations (Technique of human neutrophils functional activity assessment by nitroblue tetrazolium recovery test. Kazan, 1979) and description [28].

The data obtained were statistically processed using the Statistica 6.1 software (StatSoft, Inc., USA). The study results are presented as the arithmetic mean (M) and the arithmetic mean error (±SEM). The significance of the differences between the test and control groups was assessed by Student's t-test. Differences were considered statistically significant at $p < 0.05$.

Results. The background blood immunological research of sows in both groups showed that the majority of indices tested was consistent with the optimal values for the end of gestation, without no significant differences between

the groups.

Sows treated with Biferon-C showed at the end of the experiment (days 24-26) the increase of relative and absolute number of monocytes, causing an intensification of phagocytosis compared to the base case by 54.8% ($p < 0.001$) and 51.9% ($p < 0.01$), respectively. Such changes under the influence of interferons have occurred also in the lymphocytes content although to a lesser degree. The animals in the group II showed a tendency to increased relative and absolute lymphocyte count compared to the similar values in group I by 6.9% and 6.7%, respectively, while increasing of T-and B-lymphocytes numbers was significant: 57.5% ($p < 0.0001$) and 34.6% ($p < 0.01$), and 100% ($p < 0.001$) and 58.1% ($p < 0.01$), respectively, indicating the positive effect of Biferon-C on cells responsible for all the specific immune response (see Table 1).

1. Morphological blood indices and lymphocyte profile in crossbred sows influenced by Biferon-C on days 24-26 (*M*±SEM, Zolotaya Niva LLC, Tambov Province, 2017)

Indices		Group I (control, $n = 10$)	Group II (test, $n = 10$)
Erythrocytes, $\times 10^{12}$/L		4.82±0.30	5.17±0.08
Leucocytes, $\times 10^{9}$/L		12.70±0.66	12.70±0.56
Hemoglobin, g/L		122.20±2.17	125.20±3.54
Hematocrit, %		31.70±1.61	32.80±0.48
White blood cell differential count:			
bands	% of	4.10±0.16	2.20±0.31***
	overall	0.53±0.05	0.28±0.05***
segmented neutrophils	% of	45.3±1.63	47.50±2.79
	overall	5.97±0.49	6.00±0.42
eosinophils	% of	4.70±0.52	3.07±0.31*
	overall	0.60±0.07	0.39±0.06*
monocytes	% of	2.10±0.17	3.25±0.09**
	overall	0.27±0.04	0.41±0.03*
lymphocytes	% of	42.0±2.13	44.90±1.77
	overall	5.34±0.36	5.70±0.10
T lymphocytes, 92	% of	29.2±1.86	46.00±2.71***
	overall	1.82±0.17	2.45±0.19*
B-cells	% of	12.0±1.34	24.00±2.70**
	overall	0.74±0.09	1.17±0.11*

N o t e. Description of the groups is given in the Techniques section.
*, **, *** Differences with control are statistically significant at $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

2. Immune status in crossbred sows on days 24-26 influenced by Biferon-C (*M*±SEM, Zolotaya Niva LLC, Tambov Province, 2017)

Parameter	Group I (control, $n = 10$)	Group II (test, $n = 10$)
SCA, hemolysis in %	30.70±0.65	33.20±1.83
SLA, rg/mL	3.53±0.08	3.83±0.10*
PAL, %	83.80±1.91	85.40±1.29
PN	3.58±0.21	4.61±0.36*
PI	7.34±0.54	7.51±0.48
IG, g/L	25.10±0.83	25.20±1.08
CIC, g/l	0.26±0.01	0.17±0.01***
spNBT, %	33.20±2.08	43.20±1.93**
stNBT, %	47.60±3.04	67.80±2.96**
RI, units.	1.43±0.03	1.57±0.05*
IAN, units	0.30±0.01	0.36±0.01**

N o t e. Description of the groups is given in the Techniques section. SCA — serum complement activity; SLA — serum lysozyme activity; PAL — phagocytic activity of leukocytes; PN — phagocytic number; PI — phagocytic index; CIC — circulating immune complexes; spNBT — spontaneous NBT test; stNBT — stimulated NBT test; RI — reserve index; and NAI — neutrophils activation index.
*, **, *** Differences with control are significant at $p < 0.05$, $p < 0.01$, and $p < 0.0005$, respectively.

Use of Biferon-C enhanced the natural non-specific resistance against any infectious and non-infectious agents. So, the animals in the experimental group showed a steady tendency to increased activity (8.1% compared to control; Table 2) of complement system, the components of which bind bacteria, playing an important role in inflammation and in the development of resistance to infectious agents [29]. The serum content of lysozyme, antimicrobial protection factor (see Table 2), in sows of group II compared to those in group I increased by 8.5% ($p < 0.05$), indicating a higher lysozyme-synthesizing granulocytes, monocytes, and macrophages proliferative activity influenced by Bif-

eron-C comprising interferons.

Animals in the experimental group showed the neutrophils absorbency more significantly compared to the control, with the 18.8% higher phagocytic index ($p < 0.05$) (Table 2). The positive effect of interferon on phagocytosis system is shown by indices characterizing the cells digestive function. The spNBT test assessing the oxygen-dependent bactericidal properties of blood phagocytes in vitro [30] in group II was 30.1% ($p < 0.01$) higher than in the control, indicating the strengthening of phagocytes cytotoxicity under the influence of interferons injected (see Table 2). Under the influence of Biferon-C, stNBT test characterizing the phagocytic cells activity in the presence of an antigen and indicating their ability to the completed phagocytosis [30] was in the sows 42.4% higher ($p < 0.01$). Functional reserve of the cells (the difference between the number of activated and spontaneous diformazane-positive phagocytes) in animals from the experimental group increased by 9.8%, and NAI by 20.0% ($p < 0.01$). The results obtained allow us to postulate an increase of phagocytes metabolic reserve and digestive function under the influence of interferons from Biferon-C.

CIC (antigen-antibody reaction products involved in the homeostasis) in sows from both groups corresponded to the physiological value (less than 0.5 g/l). However, animals in the experimental group showed the CIC number 34.6% ($p < 0.0005$) less than in group I, apparently in association with declining impact of technological immunosuppressive factors and antigenic burden on the body influenced by interferons (see Table 2).

The positive effect of Biferon-C on the immune status of sows is caused by the presence of recombinant proteins [23]. α-Interferon, possessing mainly the anti-virus and antiproliferative effects, enhances the natural killer cells and T-helper cells activity, phagocytosis, B-cells differentiation, and the elimination of circulating immune complexes [31-33]. γ-Interferon synthesized by activated T-cells and NK cells is one link connecting the factors of innate and adaptive immunity [34, 35]. The stimulating effect of γ-interferon is associated with activation of phagocytic function of macrophages, the production of reactive oxygen and nitrogen species and prostaglandins. It can also activate T-helpers and T-cytotoxic lymphocytes, stimulate the differentiation of B-cells to immunoglobulin G production, and migration of lymphocytes in tissue due to adhesion to the endothelium, thus strengthening the immune cell reaction, and improve the functional activity of antigen-presenting cells [36-38].

3. Indicators of offspring preservation derived from crossbred sows treated with Biferon-C (*M* ±SEM, Zolotaya Niva LLC, Tambov Province, 2017)

Indicator	Group I (control, $n = 10$)	Group II (test, $n = 10$)
The number of sows, *n*	10	10
Piglets per sow	13.50±0.67	13.90±0.78
Piglet weight at birth, kg	1.26±0.03	1.24±0.04
Piglets weight at weaning, kg	7.68±0.56	7.95±0.49
Average daily weight gain, g/day	247.00±7.54	258.00±7.33
Death rate of piglets, %	4.20	2.15

N o t e. Description of the groups is given in the Techniques section.

The use of Biferon-C, increasing the immune status, had a positive effect on the clinical condition of sows and their piglets (Table 3), contributing to the prevention of postnatal diseases in breeding stock and improving the offspring safety. Sows from both groups showed no signs of postnatal pathology, and only some (10% less in experimental group than in control) showed a 0.1-0.3 °C increase in body temperature in the first days after farrowing. The increase of alive weight and safety of piglets obtained from sows of the experimental group also exceed those in control by 4.5% and almost 2-fold, respectively (see Table 3).

The use of Biferon-C in sicker piglets whose mothers did not receive this

preparation showed a generally positive impact on the animals' condition: their safety was 95.2% compared to 93.1% in those without using the preparation (control). Meanwhile, the incidence of gastrointestinal infections in the experimental group decreased by 4.0% and the death rate 1.4 times (Table 4).

4. Prophylactic efficacy of Biferon-C in pigs received from crossbred sows not treated with the preparation (M ±SEM, Zolotaya Niva LLC, Tambov Province, 2017)

Indicator	Group I (control, n = 10)	Group II (test, n = 10)
The number of piglets	332	333
Piglet weight at birth, kg	1.38±0.98	1.28±0.64
Piglets weight at weaning, kg	7.46±0.48	7.39±0.52
Average daily weight gain, g/day	243.30±8.19	244.50±9.32
Gastrointestinal infections, total/%	48/14.5	35/10.5
Deaths rate of pigs, total/%	23/6.9	16/4.8

N o t e. Description of the groups is given in the Techniques section.

Therefore, the study results presented in general confirm the characteristics of Biferon-C, claimed by the developers. We first studied the effect of this preparation in commercial farming conditions in combination with traditional medicamentous prevention schemes. Biferon-C use in sows 24 h prior to farrowing and 2 days after it is shown (on the background of Metramag intramuscular and of Iodopen intrauterine injection) to significantly change the quantitative ratio in several blood cells populations (bands, eosinophils, and monocytes content) and significantly increased the immune status indices: serum lysozyme activity, intensity of phagocytosis, absolute and relative content of T- and B-cells with a trend of increasing activity of the complement system. The death rate of piglets from such sows decreased twice, with practically the same other piglets' output and development indices (number of piglets per sow, weight at birth and at weaning, daily BW gain). The use of the preparation in sucking pigs from mothers not treated with Biferon-C also showed a downward trend in the incidence of gastro-intestinal infections and deaths (4%) with minor differences in the characteristics of growth and development.

REFERENCES

1. Cho J.G., Dee S.A., Dee J., Guedes A., Trincado C., Fano E., Faaberd K., Collins J.E., Joo H.S. Evaluation of the effect of animals age, concurrent bacterial infection, and pathogenicity of porcine reproductive and respiratory syndrome virus concentration in pigs. *Vet. Res.*, 2006, 67(3): 489-493 (doi: 10.2460/ajvr.67.3.489).
2. Gerjets I., Traulsen I., Reiners K., Kemper N. Comparison of virulence gene profiles of isolates from sows with Coliform mastitis and healthy sows. *Vet. Microbiol.*, 2011, 152(3-4): 361-367 (doi: 10.1016/j.vetmic.2011.05.002).
3. Donnik I.M., Bezborodova N.A. *Agrarnyi vestnik Urala*, 2009, 8: 84-89 (in Russ.).
4. Iheshiulor O.O.M., Esonu B.O., Chuwuka O.K., Omede A.A., Okoli I.C., Ogbuewu I.P. Effects of mycotoxins in animal nutrition: A review. *Asian J. Anim. Sci.*, 2011, 5(1): 19-33 (doi: 10.3923/ajas.2011.19.33).
5. Mirzoev E.B., Kobyalko V.O., Aleksakhin R.M. Scientific approaches to ensuring of stable development of livestock sector in ecologically unfavorable regions. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2011, 6: 11-18(in Russ.).
6. Popov V.S., Samburov N.V., Zorikova A.A. *Vestnik Kurskoi gosudarstvennoi sel'skokhozyaistvennoi akademii*, 2016, 4: 63-67 (in Russ.).
7. Peisak Z. *Zashchita zdorov'ya svinei* [Health protection in pigs]. Brest, 2012 (in Russ.).
8. Kovalenko A.B., Mironova O.A. *Zootekhniya*, 2009, 3: 29-30 (in Russ.).
9. Kotsarev V.N. *Veterinariya*, 2005, 3: 39-43 (in Russ.).
10. Jenny B., Vidondo B., Pendl W., Kümmerlen D., Sidler X. Erhebung von Risikofaktoren für Mastitis-Metritis-Agalaktie in Schweine-betrieben in der Schweiz. *Schweizer Archiv für Tierheilkunde*, 2015, 157(12): 689-696 (doi: 10.17236/sat00047).
11. Geetha T., Tensingh Gnanaraj P. A case report of mastitis-metritis-agalactia syndrome (MMA) in a sow. *Indian Vet. J.*, 2017, 94(12): 56-57.
12. Makarov V.V. Faktornye bolezni. *Rossiiskii veterinarnyi zhurnal*, 2017, 4: 22-27 (in Russ.).
13. Shakhov A.G., Anufriev A., Anufriev P.A. *Zhivotnovodstvo Rossii*, 2005, spets. vyp. po svinovod-

stvu: 24-27 (in Russ.).
14. Krasochko P.A. *Immunokorrektsiya v klinicheskoi veterinarnoi meditsine: Monografiya* [Immunocorrection in clinical veterinary medicine: monograph]. Minsk, 2008 (in Russ.).
15. Kovalenko V.L., Shved V.V. *Veterinarna biotekhnologiya*, 2013, 23(23): 184-186.
16. Biron C.A. Interferons α and β as immune regulators — a new look. *Immunity*, 2001, 14: 662-664 (doi: 10.1016/S1074-7613(01)00154-6).
17. Vilcek J. Fifty years of interferon research: aiming at a moving target. *Immunity*, 2006, 25(3): 343-348 (doi: 10.1016/j.immuni.2006.08.008).
18. Beekhuizen H., van de Gevel J.S. Gamma interferon confers resistance to infection with *Staphylococcus aureus* in human vascular endothelial cells by cooperative proinflammatory and enhanced intrinsic antibacterial activities. *Infection and Immunity*, 2007, 75(12): 5615-5626 (doi: 10.1128/IAI.00530-07).
19. Eshleman E.M., Lenz L.L. Type I interferons in bacterial infections: taming of myeloid cells and possible implications for autoimmunity. *Front Immunol.*, 2014, 5: 431 (doi: 10.3389/fimmu.2014.00431).
20. Johnson H.M. Gamma interferon: from antimicrobial activity to immune regulation. *Front. Immunol.*, 2015, 5: 667 (doi: 10.3389/fimmu.2014.00667).
21. Boxx G.M., Cheng G. The roles of type I interferon in bacterial infection. *Cell Host & Microbe*, 2016, 19(6): 760-769 (doi: 10.1016/j.chom.)
22. Baron S., Weigent D., Stanton G.J., Peterson J. The protective role of endogenous interferon in viral, bacterial and protozoal infection. *Antiviral Res.*, 1985, 5(Suppl. 1): 173-183 (doi: 10.1016/S0166-3542(85)80026-7).
23. Prokulevich V.A., Potapovich M.I. *Vestnik BGU, Ser. 2*, 2011, 3: 51-55 (in Russ.).
24. Vasil'ev A.N., Deryabin P.G., Galegov G.A. *Antibiotiki i khimioterapiya*, 2011, 56 (9-10): 27-32 (in Russ.).
25. Razaghi A., Owens L., Heimann K. Review of the recombinant human interferon gamma as an immunotherapeutic: impacts of production platforms and glycosylation. *J. Biotechnol.*, 2016, 240: 48-60 (doi: 10.1016/j.jbiotec.2016.10.022)
26. Parkhomenko S.A., Zeinalov O.A. *Rossiiskii veterinarnyi zhurnal*, 2017, 5: 26-29 (in Russ.).
27. Kozlova O.A., Medvedev G.F., Potapovich M.I., Prokulevich V.A. V sbornike: *Aktual'nye problemy intensivnogo razvitiya zhivotnovodstva* [In: Challenges of intensive livestock development. Iss. 21, Part 2]. Gorki, 2018, vyp. 21, ch. 2: 3-10 (in Russ.).
28. Pakhmutov I.A., Ul'yanova I.A. *Veterinariya*, 1984, 3: 68-69 (in Russ.).
29. *Entsiklopediya immunologii. Tom 1* /Pod redaktsiei A.M. Zemskova, V.M. Zemskova, V.A. Chereshneva [Encyclopedia of immunology. V. 1. A.M. Zemskov, V.M. Zemskov, V.A. Chereshnev (eds.)]. Moscow, 2013 (in Russ.).
30. Tarasova G.N., Fedotova E.N., Chumakova E.A., Il'yashenko M.G. *Prakticheskaya meditsina. Pediatriya*, 2012, 7(62): 170-173 (Russ.).
31. Rönnblom L. The importance of the type I interferon system in autoimmunity. *Clin. Exp. Rheumatol.*, 2016, 34(4 Supple. 98): 21-24.
32. González-Navajas J.M., Lee J., David M., Raz E. Immunomodulatory functions of type I interferons. *Nature Reviews Immunology*, 2012, 12: 125-135 (doi: 10.1038/nri3133).
33. Boasso A. Type I interferon at the interface of antiviral immunity and immune regulation: the curious case of HIV-1. *Scientifica*, 2013, 2013: Article ID 580968 (doi: 10.1155/2013/580968).
34. Inada T., Kubo K., Shingu K. Promotion of interferon-gamma production by natural killer cells via suppression of murine peritoneal macrophage prostaglandin E_2 production using intravenous anesthetic propofol. *International Immunopharmacology*, 2010, 10(10): 1200-1208 (doi: 10.1016/j.intimp.2010.06.027).
35. Sysoeva G.M., Danilenko E.D., Medvedeva T.B., Korovina O.S., Levagina G.M, Masycheva V.I. *Vestnik Ural'skoi meditsinskoi akademicheskoi nauki*, 2011, 2/2(35): 68-69 (in Russ.).
36. Su X., Yu Y., Zhong Y., Giannopoulou E.G., Hu X., Liu H., Cross J.R., Rätsch G., Rice C.M., Ivashkiv L.B. Interferon-γ regulates cellular metabolism and mRNA translation to potentiate macrophage activation. *Nature Immunology*, 2015, 16: 838-849 (doi: 10.1038/ni.3205).
37. Avau A., Matthys P. Therapeutic potential of interferon-γ and its antagonists in autoinflammation: lessons from murine models of systemic juvenile idiopathic arthritis and macrophage activation syndrome. *Pharmaceuticals*, 2015, 8(4): 793-815 (doi: 10.3390/ph8040793).
38. Korovina O.S., Gamalei S.G., Batenev A.V, Korneev D.V., Medikova L.D., Levagina G.M., Danilenko E.D., Bagryantseva M.P. *Vestnik Ural'skoi meditsinskoi akademicheskoi nauki*, 2011, 2/1(29): 40 (in Russ.).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 860-867
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

UDC 636.4:619:578:[577.2.08+51-76

doi: 10.15389/agrobiology.2018.4.860eng
doi: 10.15389/agrobiology.2018.4.860rus

THE STUDY OF ANTIGENICITY, IMMUNOGENICITY AND PROTECTIVE POTENTIAL OF DNA CONSTRUCTS CONTAINING FRAGMENTS OF GENES CP204L, E183L OR EP402R OF AFRICAN SWINE FEVER VIRUS STRAIN MK-200

A.R. IMATDINOV, O.A. DUBROVSKAYA, D.Yu. MOROZOVA, V.M. LYSKA, A.D. SEREDA

Federal Research Center for Virology and Microbiology, Federal Agency of Scientific Organizations, 1, ul. Akademika Bakuleva, pos. Vol'ginskii, Petushinskii Region, Vladimir Province, 601125 Russia, e-mail almazlcf@yandex.ru, olgadubrovskaya@list.ru, lady_d.morozova@mail.ru, diagnoz3@yandex.ru, sereda-56@mail.ru (✉ corresponding author)
ORCID:
Imatdinov A.R. orcid.org/0000-0003-2889-6112
Dubrovskaya O.A. orcid.org/0000-0002-3168-7947
Morozova D.Yu. orcid.org/0000-0001-5486-9981
Lyska V.M. orcid.org/0000-0001-5302-3108
Sereda A.D. orcid.org/0000-0001-8300-5234
The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation (the research project "Design of African swine fever virus candidate vaccine based on chimeric viruses" No 16-16-00090)
Received April 16, 2018

Abstract

African swine fever (ASF) is a viral, contagious and septic disease affecting wild and domestic pigs of all breeds and age groups. In both domestic pigs and wild European boars affected, some hyperacute or acute forms of the infection are observed which are characterized by fever, signs of toxicosis, hemorrhagic diathesis with mortality rates of up to 100 %. In endemic regions (e.g., some countries of East Africa), a subacute form of the disease with a mortality of 30 to 70 %, as well as a chronic one with very low mortality levels are reported (S. Blome et al., 2013; C. Gabriel et al., 2011; JM Sánchez -Vizcaíno et al., 2015). No vaccine against African swine fever is currently available, and the research works aimed at the development of live, inactivated and subunit vaccines have not achieved the intended result yet (P.J. Sánchez-Cordón et al., 2017; V. O'Donnell et al., 2016; S. Blome et al., 2014). Nevertheless, the opportunity of obtaining a DNA vaccine using the genes of potentially protective ASFV proteins p30, p54 and/or CD2v is considered to be a promising option in a number of laboratories worldwide. The proteins p30 and p54 are functionally important in attaching the ASF virus to the target cell. The antibody to p54 blocks the virion binding to the macrophage, whereas the antibody to p30 inhibits the virion penetration into the cell. The protein CD2v determines the hemadsorbing properties of the virus (S.D. Kollnberger et al., 2002; M.G. Barderas et al., 2001; J.G. Neilan et al., 2004). This work has been performed to study effects of the translation products of recombinant plasmids pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v induced in adhesive cells (A-cells) after transfection. The antigenic activity of the recombinant proteins produced with these DNA constructs was compared in permanent cell line HEK-293T and swine leukocyte (SL) autologous primary cultures using a direct fluorescence technique. The highest expression of the antigen-active translation products in HEK-293T and SL cells transfected with pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v was observed on day 2. The peculiarity of the pig immunization schedule applied was that the animals were thrice immunized at a 14-day interval with autologous LS A cells transfected in vitro with the above recombinant plasmids. For this, as much as 90 cm^3 of LC cell culture was produced using blood samples from each of the animals No.No. 1-4. On day 2 of the culturing, 90 μg of pCI-neo/ASFV/p30 (No. 1), pCI-neo/ASFV/p54 (No. 2) or pCI-neo/ASFV/CD2v (No. 3) was added thereto. The LS culture obtained from the pig No. 4 in a volume of 90 cm^3 was divided into three portions of 30 cm^3, and each one was transfected with one of the three constructs (i.e., pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v) by adding 30 μg of the plasmid DNA. Two days later, the pigs No. 1 to No. 4 were inoculated into the central auricular vein with about 10^7 autologous transfected A-cells of LS cultures. On days 14, 28 and 42, no antibody against ASFV proteins was detected in the blood of the immunized pigs using indirect solid-phase ELISA and immunoblotting. After the pigs were infected into the neck with 10^2 HAU_{50} of an ASFV strain Mozambique-78 on day 42, the four pre-immunized pigs (No.No. 1-4) died on day 6 to 8 and the control one died (No. 5) on day 13. Thus, the immunization of pigs with the autologous and antigenically active

LS cells transfected with the recombinant plasmids pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v failed to provide antibody response or protection against the challenge.

Keywords: African swine fever, ASFV, recombinant plasmids, ASFV proteins p30, p54 and CD2v, antigenicity, immunogenicity

African swine fever (ASF) is a viral contagious septic disease affecting wild and domestic pigs of all breeds and age groups. In domestic pigs and wild European boars affected, hyperacute or acute course of disease with fever, toxicosis, hemorrhagic diathesis and mortality rates of up to 100% are observed. In endemic regions (some countries of East Africa), a subacute form of the disease with the mortality from 30 to 70%, as well as a chronic form with very low mortality [1-3] are registered. The researches on the development of live inactivated and subunit vaccines have not yet yielded the desired results (4-6). Certain hopes are placed on the construction of recombinant DNA vaccines (7, 8).

It is likely that in case of ASF infection the formation of specific protection is ensured by the set of proteins inducing the immunological protection which is ensured by both cytotoxic T-lymphocytes and antibody-dependent cellular cytotoxicity [9-11]. Based on the data on the localization, structure and functional properties of viral proteins, polypeptide specificity of antibodies in the pigs' blood serum of after the administration of attenuated or virulent strains of ASF virus (ASFV), and on the effects of pigs immunization with the proteins extracted from infected cells or with recombinant proteins, p30, p54 and CD2v proteins are considered as potentially protective ones. The p30 and p54 proteins are functionally important for attaching ASFV to the target cell. The antibodies to p54 block the binding of the virion to the macrophage, while the antibodies to p30 inhibit the virion's penetration into the cell. CD2v protein ensures hemadsorbing activity of the virus [12-14].

Study of the immunogenic and protective properties of DNA constructs containing the genes of the viral proteins p30, p54 and CD2v confirmed the important role of cellular immunity in the formation of specific protection against ASF that opens up the prospects for developing new-generation drugs [15-17].

We previously reported on the obtaining of pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v recombinant plasmids. The calculated molecular weights of unmodified recombinant proteins amounted to 21.6 kDa (rp30), 18.7 kDa (rp54) and 28.6 kDa (rCD2v). According to the results of immunoblotting, in the continuous cell culture HEK-293T the molecular weight of the antigenically active products of translation of pCI-neo/ASFV/p30 amounted to 21.6 kDa, pCI-neo/ASFV/p54 – 20.9 kDa and 36.3 kDa [18]. According to the data of P. Gymez-Puertas et al. [19] and F. Rodriguez et al. [20], the weight of monomer of full-size p54 is 24-28 kDa. In the HEK-293T cells transfected by pCI-neo/ASFV/CD2v the translated virus-specific polypeptides had a molecular weight of 28.8; 39.8; 63.1 and 104.7 kDa. The first of them by its size corresponded to the calculated unmodified molecule of rCD2v. The others, apparently, were the forms modified at different degrees in the process of glycosylation and trimming. These results mainly correspond to the data of L.C. Goatley and L.K. Dixon [21], who identified in Vero cells transfected by the SV5CD2vHA plasmid the polypeptides of recombinant CD2v with the molecular weights of 26; 63; 89 and 104 kDa.

In the presented work, we immunized the animals with the A-cells of autologous LC culture transfected in vitro by recombinant plasmids for increasing the efficiency of induction of anti-cellular mechanisms of protection against ASF, however, as it turned out, we couldn't achieve the protective effect.

The objective of our researches is to determine the antigenicity, immu-

nogenicity and protectivity of the products of translation of pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v recombinant plasmids.

Techniques. The domestic pigs of the Large White breed with the weight of 20-25 kg were from the experimental sector of the Federal Research Center of Virology and Microbiology (FRCVM). We used the continuous cell line NEK-293T (Human Embryonic Kidney 293) from the Museum of Cells Culture of the FRCVM and the obtained primary culture of pig leukocytes. The virulent strain ASFV Mozambique-78 (M-78) and its attenuated derivative MK-200 strain were taken from the State Collection of Microorganisms of the FRCVM [22].

In order to prepare the LC culture, the blood sample (30-40 ml) was taken from the vena cava cranialis of each animal; heparin (20 units/cm^3) was used to stabilize the blood. The blood was kept for 50-60 min at 37 °C and then the upper fraction consisting of plasma and leukocytes was collected and centrifuged at 2000 g for 15 min, the supernatant was removed, and the sediment was resuspended to obtain the inoculum of leukocytes (4 million per cm^3) in Eagle's MEM medium with 10% homologous blood serum inactivated for 30 min at 56 °C, penicillin (100-200 U/cm^3) and streptomycin (100-200 mg/cm^3). The cells were cultured in 6-well plates in CO_2-incubator at 37 °C. One hour prior to the transfection, 2-day LC cell culture was transferred to serum-free medium.

The cells were transfected with DNA constructs for 6-8 hours with the use of Lipofectamine® (Thermo Fisher Scientific, United States) according to the standard protocol. Then, the culture medium was replaced with the Eagle's MEM medium with 10% homologous swine serum and incubated for 2-3 days.

The anti-ASFV positive sera were obtained according to the following scheme. The ASFV strain MK-200 at a dose of 6.5 lg HAU_{50} (day 0) was injected intramuscularly to domestic pigs. On day 17, these pigs were infected intramuscularly with ASFV strain M-78 at a dose of 10^3 HAU_{50}. During the periods of clinical signs of disease (hyperthermia, refusal of food, hemorrhages on the ears and belly) the sick animals were treated with daily intramuscular injection of phosphonoacetic acid (100 mg/kg) until the restoration of normal body temperature (3-5 days). The total exsanguination of pigs was performed on day 38 after the start of the experiment.

The obtaining of the immunoglobulins marked with fluorescein isothiocyanate (FITC) from the sera of ASF-resistant pigs (anti-ASFV FITC immunoglobulins) and conducting the reaction of direct immunofluorescence were performed according to State Standard of the Russian Federation GOST 28573-90 "Pigs. Methods of Laboratory Diagnosis of African Swine Fever". The results were documented by fluorescence microscopy (Eclipse E200 fluorescence microscope, Nikon Corp., Japan). The pig blood sera were examined for the presence of antibodies to ASFV proteins using indirect enzyme-linked immunosorbent assay (ELISA) and immunoblotting [23, 24] methods.

Results. In our work, we used previously obtained expressing DNA constructs (pCI-neo/ASFV/p30, pCI-neo/ASFV/p54, pCI-neo/ASFV/CD2v) with fragments of the CP204L, E183L and EP402R ASFV genes of the MK-200 strain of seroimmunotype III [18].

The antigenic activity of recombinant proteins produced by these DNA constructs was studied in HEK-293T and LC cell cultures. The coverslips with transfected cells were taken daily and the production of antigenically active recombinant proteins was determined by direct immunofluorescence method. The maximum expression in HEK-293T and LC cells transfected by pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v was observed on days 2-3 (Fig. 1).

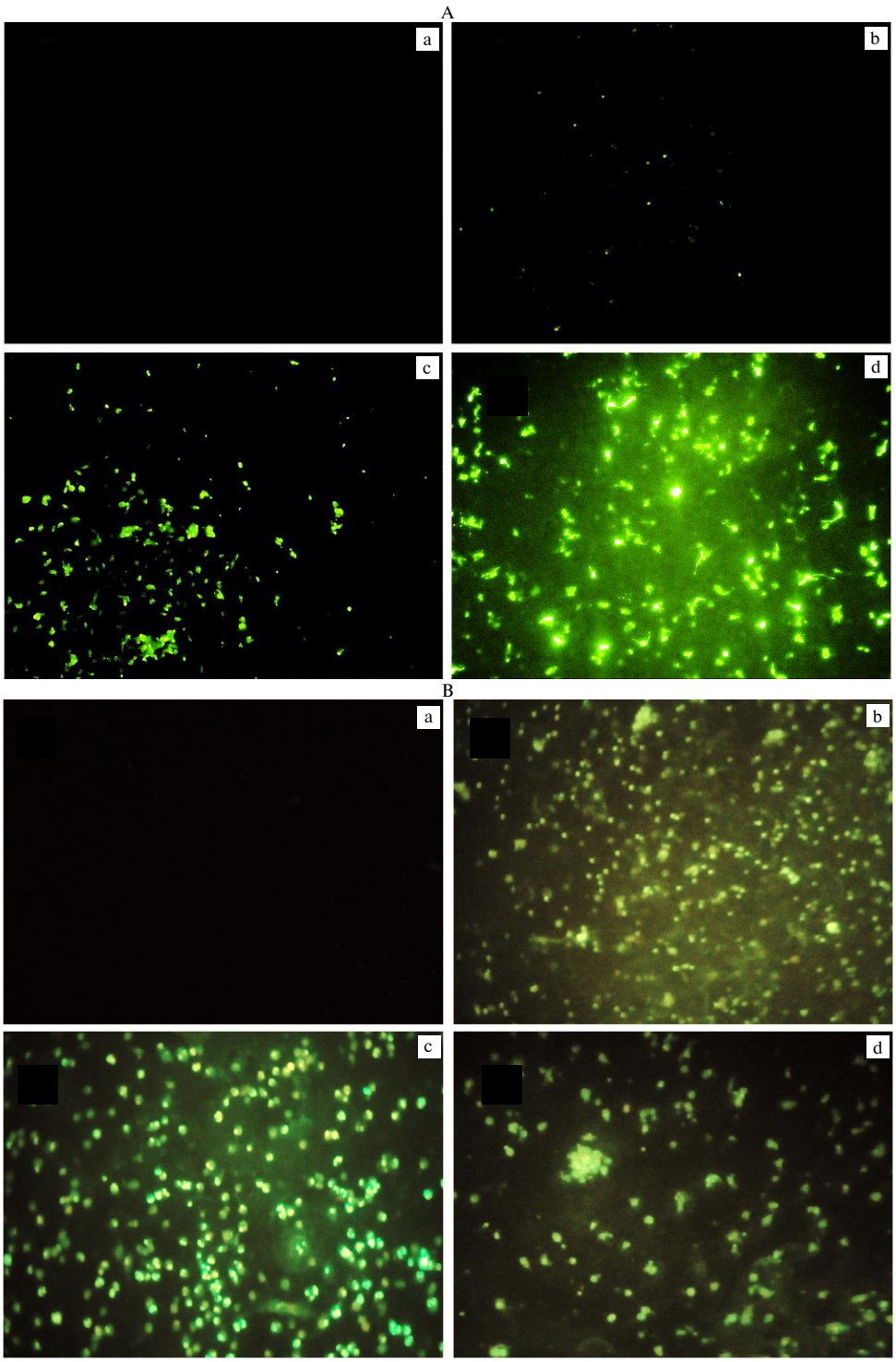


Fig. 1. Expression of antigenically active products in HEK-293T continuous cell line (A) **and in primary culture of pig leukocytes** (B) **transfected by pCI-neo/ASFV/p30 recombinant plasmid**: a, b, c, d — respectively 0, 1, 2, 3 days after the transfection (fluorescent microscopy, Eclipse E200, Nikon Corp., Japan, magnification ×100).

The peculiarity of the pig immunization scheme was that it was performed on days 0, 14 and 28 with using A cells of the autologous LC culture transfected in vitro with the recombinant plasmids. It was supposed that the im-

munization with antigen-presenting cells (macrophages) will ensure the effective induction of anti-cellular mechanisms of protection against ASF. For this, 4 days prior the immunization, LC cell cultures (90 cm^3) of pigs No. 1-4 was obtained. On day 2 of culture, 90 μg of pCI-neo/ASFV/p30 (No. 1), pCI-neo/ASFV/p54 (No. 2) or pCI-neo/ASFV/CD2v (No. 3) were introduced; 90 cm^3 LC culture of pig No. 4 was divided into three parts by 30 cm^3 and each portion was transfected with one of three constructs (30 μg pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v). After 2 days, the culture media with non-attached LC cells were removed, and the monolayer of transfected cells (about 10^7 A-cells) was mechanically taken off the substrate, washed at 2000 g for 10 min, resuspended in 3 ml phosphate buffer (pH 7.2) and injected into central ear vein to the relevant autologous pigs. The animals were clinically observed with daily measurement of body temperature. Using indirect ELISA and immunoblotting, we have not detected in the blood of immunized pigs the antibodies to ASFV proteins on days 0, 14, 28 and 42. After the control infection with M-78 ASFV strain (10^2 HAU_{50}) in the neck on day 42, four immunized pigs died in 6-8 days, and non-immunized (control) pig died in 13 days (Fig. 2).

The pig which was immunized with A-cells of LC culture transfected with the pCI-neo/ASFV/p30 construct (No. 1) died after 6 days without any clinical and pathoanatomical signs. Other immunized animals showed the increase in body temperature and died on days 7-8 with the characteristic pathoanatomical signs of ASF [25]. The control animal (No. 5) showed loss of appetite, depressed state, fever (body temperature up to 41.2-41.3 °C), paresis and paralysis of the hind limbs. Cyanosis occurred on skin of ears, belly, limbs and perineum. The pathoanatomical presentation was typical for ASF.

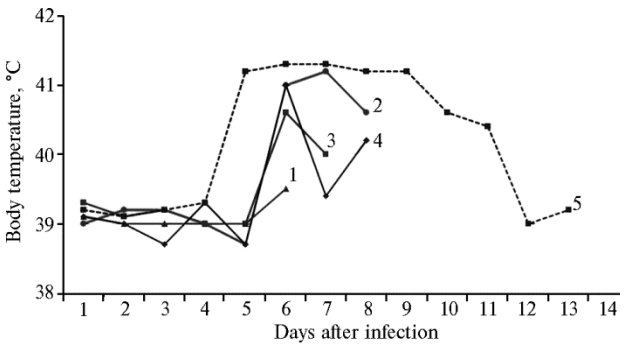


Fig. 2. Body temperature of pigs immunized with various recombinant ASFV proteins (1-4) **for the period from infection to death, and of the control animal** (5) **after the experimental infection with the virulent ASFV Mozambique-78 strain**: 1 — p30, 2 — p54, 3 — CD2v, 4 — p30, p54 and CD2v.

The significant shortcoming of candidate DNA vaccines is the relatively low induction of immune responses, especially in large mammals. In order to overcome this problem for ASF, several approaches have been tested: targeting, ubiquitination, immunization with expression libraries, s well as BacMam viruses [26]. The first attempts to induce the protective immune response against ASFV using the DNA construct encoding two viral proteins p54 and p30 as a chimeric protein (PQ) were unsuccessful. The DNA constructs encoding only PQ ensured high production of antibodies in immunized mice, but not in pigs [8].

The immunization with the pCMV-sHAPQ DNA construct supplemented with the gene for CD2v protein (HA) induced in pigs the humoral immune response, but the pigs were not protected from the control introduction of infection and showed the clinical signs of ASF and viremia kinetics which were indistinguishable from those in control animals. In order to avoid the unwanted induction of antibodies and to enhance the specific $CD8^+$-T-cell responses, the pCMV-UbsHAPQ construct encoding p30, p54 and CD2v antigenic determinants joined with cell ubiquitin (Ubs) has been developed. The twice done immunization with pCMV-UbsHAPQ has not induced the humoral response in

pigs, but ensured the partial protection against control infection with ASFV, thereby confirming the importance of the T-cell response in protection against this virus. The four-time immunization with pCMV-UbsHAPQ stimulated the formation of antibodies to p30 and p54 that apparently resulted in the decrease of the protective effect of $CD8^+$-T-cells [16]. It is possible that the earlier, as compared to the control animal, death of the immunized pigs in our experiment was due to the induction of virus-specific antibodies in titers insufficient for detection by indirect ELISA and immunoblotting methods.

Thus, expression of ASFV-specific products in HEK-293T cells and leukocytes of pigs transfected with the pCI-neo/ASFV/p30, pCI-neo/ASFV/p5 and pCI-neo/ASFV/CD2v recombinant plasmids has been proved by immunofluorescence method. The immunization of pigs with the autologous porcine leukocytes transfected with the pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v constructs has not induced the virus-specific antibodies and protection against the control infection.

REFERENCES

1. Blome S., Gabriel C., Beer M. Pathogenesis of African swine fever in domestic pigs and European wild boar. *Virus Res.*, 2013, 173(1): 122-130 (doi: 10.1016/j.virusres.2012.10.026).
2. Gabriel C., Blome S., Malogolovkin A., Parilov S., Kolbasov D., Teifke J.P., Beer M. Characterization of African swine fever virus Caucasus isolate in European wild boars. *Emerg. Infect. Dis.*, 2011, 17(12): 2342-2345 (doi: 10.3201/eid1712.110430).
3. Sánchez-Vizcaíno J.M., Mur L., Gomez-Villamandos J.C., Carrasco L. An update on the epidemiology and pathology of African swine fever. *J. Comp. Pathol.*, 2015, 152(1): 9-21 (doi: 10.1016/j.jcpa.2014.09.003).
4. Sánchez-Cordón P.J., Chapman D., Jabbar T., Reis A.L., Goatley L., Netherton C.L., Taylor G., Montoya M., Dixon L. Different routes and doses influence protection in pigs immunised with the naturally attenuated African swine fever virus isolate OURT88/3. *Antiviral Res.*, 2017, 138: 1-8 (doi: 10.1016/j.antiviral.2016.11.021).
5. O'Donnell V., Holinka L.G., Sanford B., Krug P.W., Carlson J., Pacheco J.M., Reese B., Risatti G.R., Gladue D.P., Borca M.V. African swine fever virus Georgia isolate harboring deletions of 9GL and MGF360/505 genes is highly attenuated in swine but does not confer protection against parental virus challenge. *Virus Res.*, 2016, 221: 8-14 (doi: 10.1016/j.virusres.2016.05.014).
6. Blome S., Gabriel C., Beer M. Modern adjuvants do not enhance the efficacy of an inactivated African swine fever virus vaccine preparation. *Vaccine*, 2014, 32(31): 3879-3882 (doi: 10.1016/j.vaccine.2014.05.051).
7. Lacasta A., Ballester M., Monteagudo P.L., Rodríguez J.M., Salas M.L., Accensi F., Pina-Pedrero S., Bensaid A., Argilaguet J., Lypez-Soria S., Hutet E., Le Potier M.F., Rodríguez F. Expression library immunization can confer protection against lethal challenge with African swine fever virus. *J. Virol.*, 2014, 88(22): 13322-13332 (doi: 10.1128/JVI.01893-14).
8. Argilaguet J.M., Pérez-Martín E., Nofrarías M., Gallardo C., Accensi F., Lacasta A., Mora M., Ballester M., Galindo-Cardiel I., Lypez-Soria S., Escribano J.M., Reche P.A., Rodríguez F. DNA vaccination partially protects against African swine fever virus lethal challenge in the absence of antibodies. *PLoS ONE*, 2012, 7(9): e40942 (doi: 10.1371/journal.pone.0040942).
9. Sereda A.D., Kazakova A.S., Imatdinov A.R., Kolbasov D.V. Humoral and cell immune mechanisms under African swine fever (review). *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2015, 50(6): 709-718 (doi: 10.15389/agrobiology.2015.6.709eng).
10. Lacasta A., Monteagudo P.L., Jiménez-Marín Б., Accensi F., Ballester M., Argilaguet J., Galindo-Cardiel I., Segalés J., Salas M.L., Domínguez J., Moreno Б., Garrido J.J., Rodríguez F. Live attenuated African swine fever viruses as ideal tools to dissect the mechanisms involved in viral pathogenesis and immune protection. *Vet. Res.*, 2015, 46: 135 (doi: 10.1186/s13567-015-0275-z).
11. Takamatsu H.H., Denyer M.S., Lacasta A., Stirling C.M., Argilaguet J.M., Netherton C.L., Oura C.A., Martins C., Rodríguez F. Cellular immunity in ASFV responses. *Virus Res.*, 2013, 173(1): 110-121 (doi: 10.1016/j.virusres.2012.11.009).
12. Kollnberger S.D., Gutierrez-Castaceda B., Foster-Cuevas M., Corteyn A., Parkhouses R.M.E. Identification of the principal serological immunodeterminants of African swine fever virus by screening a virus cDNA library with antibody. *J. Gen. Virol.*, 2002, 83: 1331-1342 (doi: 10.1099/0022-1317-83-6-1331).
13. Barderas M.G., Rodriguez F., Gomez-Puertas P., Aviles M., Beitia F., Alonso C., Escribano J.M.

Antigenic and immunogenic properties of a chimera of two immunodominant African swine fever virus proteins. *Arch. Virol.*, 2001, 146: 1681-1691 (doi: 10.1007/s007050170056).
14. Neilan J.G., Zsak L., Lu Z., Burrage T.G., Kutish G.F., Rock D.L. Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. *Virology*, 2004, 319: 337-342 (doi: 10.1016/j.virol.2003.11.011).
15. Ruiz-Gonzalvo F., Rodríguez F., Escribano J.M. Functional and immunological properties of the baculovirus-expressed hemagglutinin of African swine fever virus. *Virology*, 1996, 218: 285-289 (doi: 10.1006/viro.1996.0193).
16. Takamatsu H.H., Denyer M.S., Lacasta A., Stirling C.M., Argilaguet J.M., Netherton C.L., Oura C.A., Martins C., Rodríguez F. Cellular immunity in ASFV responses. *Virus Res.*, 2013, 173(1): 110-121 (doi: 10.1016/j.virusres.2012.11.009).
17. Argilaguet J.M., Pérez-Martín E., López S., Goethe M., Escribano J.M., Giesow K., Keil G.M., Rodríguez F. BacMam immunization partially protects pigs against sublethal challenge with African swine fever virus. *Antiviral Res.*, 2013, 98(1): 61-65 (doi: 10.1016/j.antiviral.2013.02.005).
18. Imatdinov A.R., Sereda A.D., Imatdinov I.R., Kazakova A.S., Dubrovskaya O.A., Kolbasov D.V. Expression of recombinant genes encoding fragments of the protective important proteins of African swine fever virus in eucaryotic cells. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2016, 51(6): 837-844 (doi: 10.15389/agrobiology.2016.6.837eng).
19. Gómez-Puertas P., Rodriguez F., Oviedo J.M., Brun A., Alonso C., Escribano J.M. The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. *Virology*, 1998, 243: 461-471 (doi: 10.1006/viro.1998.9068).
20. Rodriguez F., Alcaraz S., Yanez R.J., Rodriguez J.M., Alonso C., Rodriguez J.F., Escribano J.M. Characterization and molecular basis of heterogeneity of the African swine fever virus envelope protein p54. *J. Virol.*, 1994, 68(11): 7244-7252.
21. Goatley L.C., Dixon L.K. Processing and Localization of the African swine fever virus CD2v transmembrane protein. *J. Virol.*, 2011, 85(7): 3294-3305 (doi: 10.1128/jvi.01994-10).
22. Malogolovkin A., Burmakina G., Titov I., Sereda A., Gogin A., Baryshnikova E., Kolbasov D. Comparative analysis of African swine fever virus genotypes and serogroups. *Emerg. Infect. Dis.*, 2015, 21(2): 312-315 (doi: 10.3201/eid2102.140649).
23. Strizhakova O.M., Lyska V.M., Malogolovkin A.S., Novikova M.B., Sidlik M.V., Nogina I.V., Shkaev A.E., Balashova E.A., Kurinnov V.V., Vasil'ev A.P. Validation of an ELISA kit for detection of antibodies against ASF virus in blood or spleen of domestic pigs and wild boars. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2016, 51(6): 845-852 (doi: 10.15389/agrobiology.2016.6.845eng).
24. *Immunoblotting OIE for serological diagnosis of African swine fever (SOP/CISA/ASF/IB/1/2008).* Available http://asf-referencelab.info/asf/images/files/SOPs/SOP-AFSIB12008.pdf. No date.
25. Ryzhova E.V., Belyanin S.A., Kolbasov D.V., Balyshev V.M., Kurinnov V.V., Pronin V.V., Korneva G.V. *Rossiiskii veterinarnyi zhurnal*, 2012, 1: 10-14 (in Russ.).
26. Jancovich J.K., Chapman D., Hansen D.T., Robida M.D., Loskutov A., Craciunescu F., Borovkov A., Kibler K., Goatley L., King K., Netherton C,L., Taylor G., Jacobs B., Sykes K., Dixon L.K. Immunization of pigs by DNA prime and recombinant vaccinia virus boost to identify and rank African swine fever virus immunogenic and protective proteins. *J. Virol.*, 2018, 92(8): e02219-17 (doi: 10.1128/JVI.02219-17).

AGRICULTURAL BIOLOGY,
2018, V. 53, № 4, pp. 868-875
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

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

Cell cultures

UDC 619+616.5]:57.085.23

doi: 10.15389/agrobiology.2018.4.868eng
doi: 10.15389/agrobiology.2018.4.868rus

MULTIPOTENT MESENCHYMAL STROMAL CELLS ISOLATED FROM SUBCUTANEOUS FAT OF MAMMALS FOR THE STUDY OF *Sarcoptes Scabiei/mange* in vitro

I.P. SAVCHENKOVA

Federal Science Center Kovalenko All-Russian Research Institute of Experimental Veterinary RAS, Federal Agency of Scientific Organizations, 24/1, Ryazanskii pr., Moscow, 109428 Russia, e-mail s-ip@mail.ru (✉ corresponding author)
ORCID:
Savchenkova I.P. orcid.org/0000-0003-3560-5045
The author declares no conflict of interests
Received October 19, 2017

Abstract

Sarcoptes scabiei/mange is a small, roundish, pale gray mite that lives in the epidermis of mammalian skin and causes scabies. Despite the fact that the mite biology is well studied and its interaction with the host is intensively investigated, the data analysis demonstrates the absence of a cell culture on which the *Sarcoptes scabiei/mange* could multiply in vitro. This hinders the development of modern effective methods of diagnosis of this disease, the study of the immune response after infection in order to create vaccines and evaluate the effectiveness of the use of drugs for the clinical treatment of mite-borne disease. In this regard, the search for a cellular system that would maintain the viability of *Sarcoptes scabiei/mange* in vitro is particularly relevant. We describe cellular systems represented by multipotent mesenchymal stromal cells (MMSCs) that can be used for these purposes. MMSCs were isolated from subcutaneous adipose tissue (SAT) of cattle and humans. Biopsies were taken from healthy donors without clinical form of scabies. In MMSC of cattle and human, isolated from SAT scabies mite (*Sarcoptes scabiei/mange*) was found. The MMSCs isolated from cattle were the most contaminated by mites: of 13 MMSC cultures on the 2nd passage 9 cultures (or 69 %) were contaminated. For human MMSCs, contamination with mites was only found in one culture among 6 cultures obtained (16 %). The mite was found in the form of small dark spherical extracellular components, not characteristic for MMSCs, the number of which increased during culture. A characteristic feature of scabies mite reproduction in MMSC culture isolated from both cattle and human SAT was the formation of nests in the form of clusters. In the culture, we identified presumably larvae and nymphs of the mite. During subculturing within 3 passages, the contaminated cattle and human MMSCs retained the same vitality as not contaminated, but the monolayer formed more slowly, i.e. on days 9-10 vs. day 7 in the control groups. After freezing the samples in which the mite was found, and storage in a Dewar vessel in liquid nitrogen for a week, followed by defrosting, it was found that the mite retained viability in all samples and well tolerated freezing-thawing. After thawing, the mite was detected on the 2nd passage in 7 of 7 analyzed MMSC cultures from cattle SAT and in the only MSCS culture derived from human SAT in which the mite contamination was found (100 %). Thus, it is shown that the MMSCs isolated by us from cattle and humans SAT, may represent a promising cellular system for studying in vitro *Scabiei scabieis/mange*. Possible contamination of MMSCs from SAT with scabies makes testing of these cultures mandatory in case of their use in cellular technologies.

Keywords: multipotent mesenchymal stromal cells, subcutaneous adipose tissue, cell culture, *Sarcoptes scabiei/mange*, culturing, freezing

Itch mite (human itch mite *Sarcoptes scabiei*/animals itch mite *S. mange*) is an intradermal parasite of many mammals including domestic and farm animals. *Sarcoptes scabiei/mange* is an obligate ectoparasite which lives and reproduces itself inside the host epidermis [1, 2]. The mite destroys the host's cells both mechanically and by secreting the cytolytic components. These cytolytic components and the antigenic substances, feces or eggs of mites cause the immunopathogenic reactions [3]. The primary clinical sign is intense itching; later,

the cellular lesions in the skin may appear. Depending on the host immunopathological status, the symptoms, as well as the intensity, dissemination and course of the disease can vary greatly. The mange is widespread throughout the world and causes the significant decline of animals' life and in some cases leads to their death [4]. *S. mange* of farm animals causes the significant economic damage to the livestock sector, especially in pig breeding.

Despite the significant progress in understanding the mite biology and its interaction with the host, much is still unknown [5, 6]. For a long time, there was a discussion whether *S. scabiei* is a single species or it should be divided into several species specific for each host organism, but there are still no genetic evidences of the existence of several species or subspecies of this mite.

The analysis of literature data indicates the lack of in vitro studies of the mite. Saliva, molting enzymes and hormones, feces and nitrogenous substrates secreted by the mite into the extracellular fluid surrounding the epidermis and dermis cells can influence these cells including keratinocytes, fibroblasts, macrophages, mast cells, lymphocytes, Langerhans cells, dendritic cells and endothelial cells. The results of the series of studies carried out, in particular, on mononuclear and dendritic cells [7, 8], keratinocytes and fibroblasts [9, 10], and endothelial cells [11, 12] showed that the extract made of the bodies of the *S. scabiei* mites modulates the secretion of cytokines by human keratinocytes and fibroblasts in cell culture. Thus, the addition of the extract when cultivation of human epidermal keratinocytes leads to the significant intensification of the secretion of interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF). In addition, the substances contained in this extract stimulated the increase in the secretion of interleukins 6 and 8, as well as of VEGF in culture of human skin fibroblasts. The attempts to study the influence of cellular interactions between keratinocytes and fibroblasts when the cells are affected by scabies mites and their extracts in vitro with using a three-dimensional model equivalent to the skin are being made [13, 14]. The results show that cellular interactions play an important role in the host's response to the mites.

The non-existence of the cell culture in which the mite could reproduce itself in vitro restrains many studies including those on the development of modern effective methods for diagnosing this disease, studying the immune response after the contamination by the parasite and creation of vaccines [15-18]. Currently, acaricides are used [19] to treat the *S. mange* infection but these are expensive drugs, and besides they may pose the risk to the environment, food products, animal trainers, etc. The systematic use of acaricides leads to gaining by the mites of high resistance to them. The mite is considered to be resistant to a range of drugs [20-24]. In this regard, the particularly pressing matter is to find the cellular system which would make it possible to assess the effectiveness of anti-mange drugs in vitro.

In our study, for the first time ever, we discovered the fact of contamination by the itch mite of the multipotent mesenchymal stromal cells (MMSCs) extracted from the subcutaneous adipose tissue of mammals and described the cellular systems of MMSCs which can be used for maintaining the viability of *Sarcoptes scabiei/mange* in vitro.

The aim of the work is to show the possibility of using multipotent mesenchymal stromal cells for maintaining *Sarcoptes scabiei/mange* in vitro.

Techniques. The MMSCs were extracted from subcutaneous adipose tissue of bovine cattle (beef cattle) and humans. The biopsy samples were taken from healthy donors (without any clinical form of mange) from the accidentally found contamination by mite.

The cells were extracted according to the methods described by us previ-

ously [25)] and cultured in DMEM (Dulbecco modified Eagle's medium) with low (1 g/l) content of glucose (PanEco, Russia), 10% fetal bovine serum (FBS) (HyClone, Perbio Scientific, Belgium) and solutions (1×) of nonessential amino acids and antibiotics (PanEco, Russia). The final concentration of streptomycin in the medium was 50 μg/ml, penicillin concentration was 50 U/ml. The medium was changed every 4 days; upon reaching 90% of the monolayer, it was treated with 0.25% trypsin solution (PanEco, Russia) and the subculturing was continued at the cells density of $5\times10^3/cm^2$.

The morphology of cells and mites in culture was assessed visually using the inverted phase-contrast microscope (Carl Zeiss, Germany) with AxioVision Rel. 4.8 software (Carl Zeiss, Germany) for measurements.

The mite impact on the MMSCs contaminated by them was evaluated by the viability of cells and their rate of monolayer formation when subculturing (3 passages). The cell cultures of the same density relevant to each group but not contaminated with the mites were control samples. The cells viability was assessed by trypan blue staining (0.1% solution, PanEco, Russia).

The MMSCs was frozen according to the standard method in the cryoprotective medium with 10% dimethyl sulfoxide (DMSO) in gradual mode of cooling up to −70 °C at the rate of 1 °C per minute; then the ampoules were transferred into liquid nitrogen (−196 °C) and stored for 1 week. The cells were quickly defrozen in a water bath (37 °C) and the DMSO was immediately separated by low-speed centrifugation (1000 rpm, 5 min). The presence of mites in the samples subjected to freezing and defreezing procedures was evaluated after the formation of the cells monolayer.

Results. It is difficult to visualize *S. scabiei* in primary culture just after the extraction of cells from the tissue. We accidentally found a mite in the MMSCs extracted from subcutaneous adipose tissue (SAT) of cattle after the first subculturing. It was noted that in some samples of cell cultures extracted from SAT there are visible presence of extracellular components in the form of small dark spherical structures, which are not characteristic for MMSCs (Fig. 1, A) and the number of which had been increasing as time passes (see Fig. 1, B). When more detail viewing, both mites themselves (see Fig. 1, D) and their niduses formed by them in culture (see Fig. 1, C), with the accumulation of cells in these places have been found.

When subculturing, the MMSCs which have been contaminated by mites, for 3 passages maintained the same viability just like non-contaminated ones, but the monolayer was formed more slowly. Thus, in the control groups of cattle and human MMSCs, the monolayer was formed on day 7, and in the contaminated groups on day 9-day 10.

The cells extracted from cattle SAT samples turned out to be most contaminated by mites: altogether, 13 cattle MMSCs cultures were analyzed at the 2nd passage, of which 9 cultures (69%) were contaminated.

When analyzing the MMSCs from human SAT, only one culture of 6 obtained (16%) was found to be contaminated by mites. The finding out of *S. scabiei* in the culture of multipotent mesenchymal stromal cells extracted from human subcutaneous fat tissue is illustrated in Figure 2. The features we observed during the cultivation of mites in MMSCs of cattle SAT were noted also in the MMSCs of human SAT. The presence of mites was indicated by dark formations of spherical shape, which are not characteristic for MMSCs (see Fig. 2, A), which number had been increasing as time passes (see Fig. 2, B). When cultivation, the niduses (conglomerations) formed by mites in culture were seen (see Fig. 2, C, D). Separate mites have been found in the culture's medium (see Fig. 2, D).

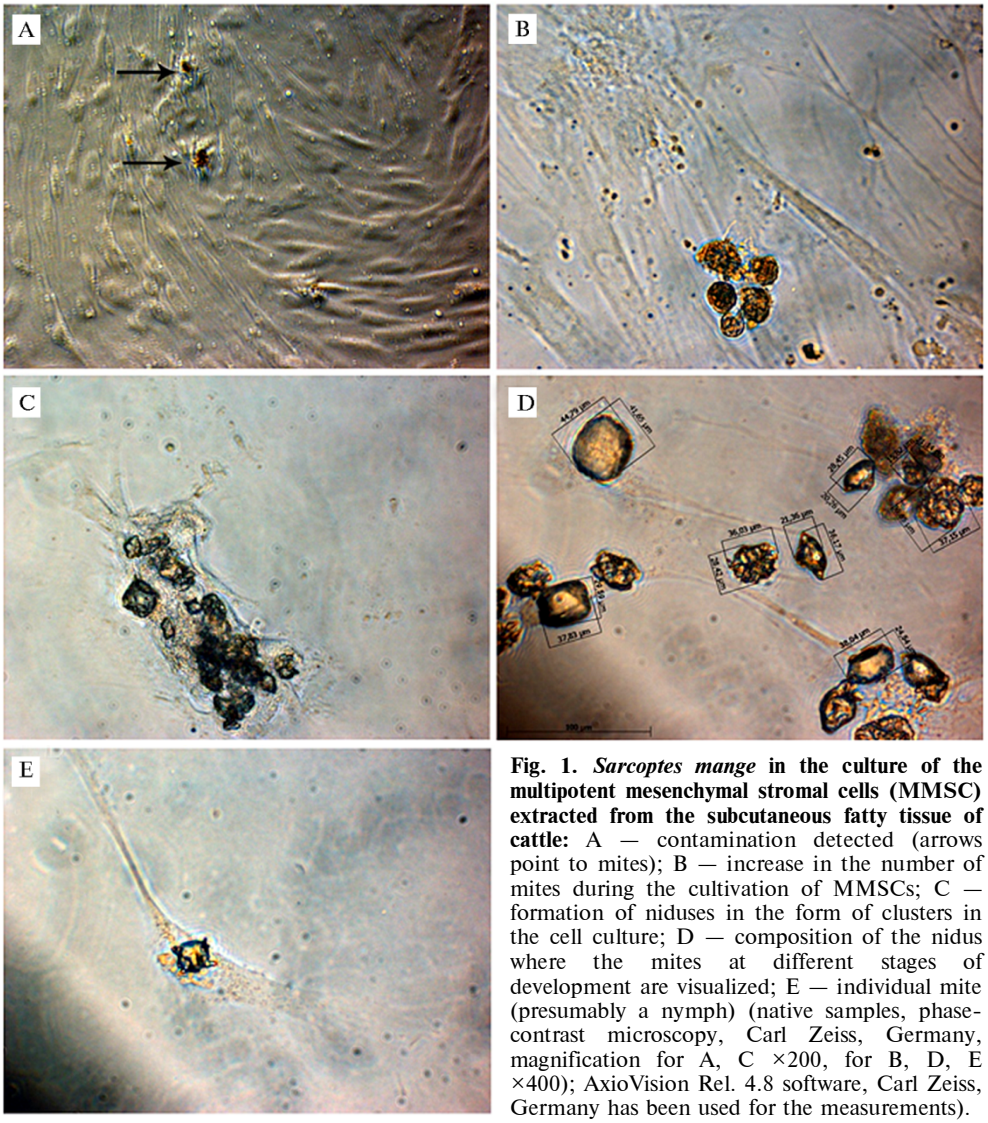


Fig. 1. ***Sarcoptes mange*** **in the culture of the multipotent mesenchymal stromal cells (MMSC) extracted from the subcutaneous fatty tissue of cattle:** A — contamination detected (arrows point to mites); B — increase in the number of mites during the cultivation of MMSCs; C — formation of niduses in the form of clusters in the cell culture; D — composition of the nidus where the mites at different stages of development are visualized; E — individual mite (presumably a nymph) (native samples, phase-contrast microscopy, Carl Zeiss, Germany, magnification for A, C ×200, for B, D, E ×400); AxioVision Rel. 4.8 software, Carl Zeiss, Germany has been used for the measurements).

When subculturing the MMSCs contaminated by mites, for 3 passages the cells maintained the same viability like non-contaminated ones, but they had been forming the monolayer more slowly. Thus, in the control groups of cattle and human MMSCs the monolayer was formed on day 7, and in the contaminated groups on day 9-day 10.

The causative agent of sarcoptosis (scabies), well known in veterinary medicine, are small, round-shaped mites of pale gray color. It is known that a male mite (length 0.23 mm, width 0.19 mm) is smaller than female mite (length 0.45 mm, width 0.35 mm). The eggs are large, oval, length 0.15-0.25 mm, have a two-layer shell. The mite ontogenesis includes the larvae and nymph stages[1-3]. In all investigated samples we have not find any eggs and adult mites. It can be assumed that they do not attach to the cells, but are in suspension. In the culture, presumably larvae and nymph of mites have been found (see Fig. 1, D).

The analysis of data presented in the scientific literature earlier demonstrates the absence of the fact of contamination of cell cultures by mites *in vitro*. The multipotent mesenchymal stromal cells (MMSCs) extracted from the stromal-vascularized fraction of subcutaneous fat of mammals have the properties similar to the properties of the MMSCs extracted from bone marrow. They are

considered to exhibit immunomodulatory properties and produce a number of key cytokines for maintaining the hematopoiesis in vitro [26, 27] that may influence on the maintenance of the mite viability in MMSCs culture. In this respect, the work [28] in which the reaction of *S. scabiei* var. *canis* to the fats (mixture of 21 lipids) being typical for the human skin epidermis has been assessed and 13 fatty acids and their derivatives attracting this mites at all stages of their development have been identified, is of interest. MMSCs in culture can be subjected to the spontaneous differentiation toward the adipogenesis [25] that also can explain our data.

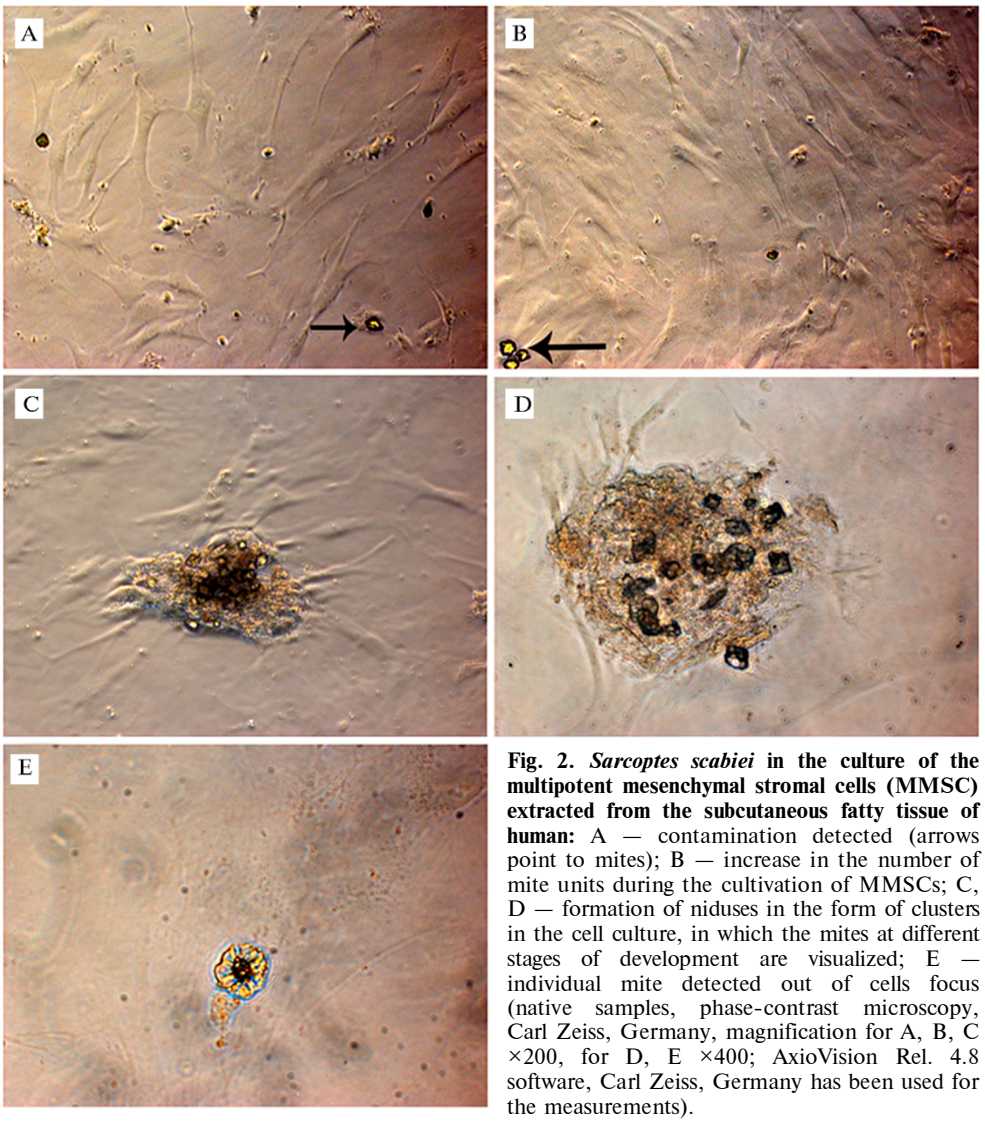


Fig. 2. *Sarcoptes scabiei* in the culture of the multipotent mesenchymal stromal cells (MMSC) extracted from the subcutaneous fatty tissue of human: A — contamination detected (arrows point to mites); B — increase in the number of mite units during the cultivation of MMSCs; C, D — formation of niduses in the form of clusters in the cell culture, in which the mites at different stages of development are visualized; E — individual mite detected out of cells focus (native samples, phase-contrast microscopy, Carl Zeiss, Germany, magnification for A, B, C ×200, for D, E ×400; AxioVision Rel. 4.8 software, Carl Zeiss, Germany has been used for the measurements).

It was of interest to assess whether mites retain their viability after the cryo-freezing of the cells culture. After freezing the samples in which mites have been detected, and storing them in a Dewar vessel in liquid nitrogen for 1 week followed by defreezing, the cells were cultured until the monolayer formation and the culture was assessed for the presence of *S. scabiei/mange*. After the defreezing, mites have been found in 7 of 7 MMSC cultures extracted from cattle SAT analyzed at the 2nd passage and in the single found MMSC culture from human SAT contaminated by mites (100%). Consequently, the mites retained the viability in all samples and tolerated freezing-defreezing well.

Due to the possibility to use the MMSC cultures extracted from human SAT in cell technologies, specialists should pay attention to the fact that, according to our data, such cultures can be contaminated by subcutaneous mites which are able to retain the viability while freezing and defreezing that indicates to the necessity of mandatory checking them for the presence of *S. scabiei*. The cultures contaminated by mites shall be discarded at the early stages after the extraction.

Thus, it has been shown that the multipotent mammalian mesenchymal stromal cells (MMSCs) extracted from the subcutaneous adipose tissue (SAT) of cattle and humans, can be the promising cellular system for studying *Sarcoptes scabieis/mange* in vitro. The discovery of the fact that MMSCs extracted from human SAT may be contaminated by mites makes the testing of such cultures mandatory in case of using in cell technologies.

REFERENCES

1. Chosidow O. Scabies. *N. Engl. J. Med.*, 2006, 354: 1718-1727 (doi: 10.1056/NEJMcp052784).
2. Currier R.W., Walton S.F., Currie B.J. Scabies in animals and humans: history, evolutionary perspectives, and modern clinical management. *Annals of the New York Academy of Sciences*, 2011, 1230: E50-E60 (doi: 10.1111/j.1749-6632.2011.06364.x).
3. Elwood H., Berry R.S., Gardner J.M., Shalin S.C. Superficial fibrin thrombi and other findings: a review of the histopathology of human scabietic infections. *J. Cutan. Pathol.*, 2015, 42(5): 346-352 (doi: 10.1111/cup.12482).
4. Arlian L.G., Morgan M.S., Rider J.S.D. *Sarcoptes scabiei*: genomics to proteomics to biology. *Parasit. Vectors,* 2016, 9(1): 380 (doi: 10.1186/s13071-016-1663-6).
5. Walton S.F., Currie B.J. Problems in diagnosing scabies, a global disease in human and animal populations. *Clin. Microbiol. Rev.*, 2007, 20(2): 268-279 (doi: 10.1128/CMR.00042-06).
6. Arlian L.G., Morgan M.S. A review of *Sarcoptes scabiei*: past, present and future. *Parasit. Vectors*, 2017, 10(1): 297 (doi: 10.1186/s13071-017-2234-1).
7. Arlian L.G., Morgan M.S., Neal J.S. Extracts of scabies mites (*Sarcoptidae*: *Sarcoptes scabiei*) modulate cytokine expression by human peripheral blood mononuclear cells and dendritic cells. *J. Med. Entomol.*, 2004, 41(1): 69-73 (doi: 10.1603/0022-2585-41.1.69).
8. Singh S.K., Dimri U., Sharma B., Saxena M., Kumari P. Assessment of the cytokine profile in peripheral blood mononuclear cells of naturally *Sarcoptes scabiei* var. *canis* infested dogs. *Vet. Parasitol.*, 2014, 206(3-4): 253-257 (doi: 10.1016/j.vetpar.2014.10.024).
9. Mullins J.S., Arlian L.G., Morgan M.S. Extracts of *Sarcoptes scabiei* de Geer downmodulate secretion of IL-8 by skin keratinocytes and fibroblasts and of GM-CSF by fibroblasts in the presence of proinflammatory cytokines. *J. Med. Entomol.*, 2009, 46(4): 845-851 (doi: 10.1603/033.046.0415).
10. Arlian L.G., Morgan M.S., Neal J.S. Modulation of cytokine expression in human keratinocytes and fibroblasts by extracts of scabies mites. *Am. J. Trop. Med. Hyg.*, 2003, 69(6): 652-656 (doi: 10.4269/ajtmh.2003.69.652).
11. Elder B.L., Arlian L.G., Morgan M.S. *Sarcoptes scabiei* (*Acari*: *Sarcoptidae*) mite extract modulates expression of cytokines and adhesion molecules by human dermal microvascular endothelial cells. *J. Med. Entomol.*, 2006, 43(5): 910-915 (doi: 10.1093/jmedent/43.5.910).
12. Elder B.L., Arlian L.G., Morgan M.S. Modulation of human dermal microvascular endothelial cells by *Sarcoptes scabiei* in combination with proinflammatory cytokines, histamine, and lipid-derived biologic mediators. *Cytokine*, 2009, 47(2): 103-111 (doi: 10.1016/j.cyto.2009.05.008).
13. Morgan M.S., Arlian L.G. Response of human skin equivalents to *Sarcoptes scabiei*. *J. Med. Entomol.*, 2010, 47(5): 877-883 (doi: 10.1093/jmedent/47.5.877).
14. Morgan M.S., Arlian L.G., Markey M.P. *Sarcoptes scabiei* mites modulate gene expression in human skin equivalents. *PLoS ONE*, 2013, 8(8): e71143 (doi: 10.1371/journal.pone.0071143).
15. Mounsey K., Ho M.F., Kelly A., Willis C., Pasay C., Kemp D.J., McCarthy J.S., Fischer K. A tractable experimental model for study of human and animal scabies. *PLoS Negl. Trop. Dis.*, 2010, 4(7): e756 (doi: 10.1371/journal.pntd.0000756).
16. Casais R., Granda V., Balseiro A., Del Cerro A., Dalton K.P., Gonzőlez R., Bravo P., Prieto J.M., Montoya M. Vaccination of rabbits with immunodominant antigens from *Sarcoptes scabiei* induced high levels of humoral responses and pro-inflammatory cytokines but confers limited protection. *Parasit. Vectors*, 2016, 9(1): 435 (doi: 10.1186/s13071-016-1717-9).
17. Casais R., Millán J., Rosell J.M., Dalton K.P., Prieto J.M. Evaluation of an ELISA using recombinant Ssλ20ΔB3 antigen for the serological diagnosis of *Sarcoptes scabiei* infestation in domestic and wild rabbits. *Vet. Parasitol.*, 2015, 214(3-4): 315-321 (doi: 10.1016/j.vetpar.2015.07.011).

18. Bhat S.A., Mounsey K.E., Liu X., Walton S.F. Host immune responses to the itch mite, *Sarcoptes scabiei*, in humans. *Parasit. Vectors*, 2017, 10(1): 385 (doi: 10.1186/s13071-017-2320-4).
19. Walton S.F., Myerscough M.R., Currie B.J. Studies in vitro on the relative efficacy of current acaricides for *Sarcoptes scabiei* var. *hominis*. *Trans. R. Soc. Trop. Med. Hyg.*, 2000, 94(1): 92-96 (doi: 10.1016/S0035-9203(00)90454-1).
20. Currie B.J., Harumal P., McKinnon M., Walton S.F. First documentation of in vivo and in vitro ivermectin resistance in *Sarcoptes scabiei*. *Clinical Infectious Diseases*, 2004, 39(1): e8-12 (doi: 10.1086/421776).
21. Mumcuoglu K.Y., Gilead L. Treatment of scabies infestations. *Parasite*, 2008, 15(3): 248-251 (doi: 10.1051/parasite/2008153248).
22. Buffet M., Dupin N. Current treatments for scabies. *Fundamental & Clinical Pharmacology*, 2003, 17(2): 217-225 (doi: 10.1046/j.1472-8206.2003.00173.x).
23. McCarthy J.S, Kemp D.J, Walton S.F, Currie B.J. Scabies: more than just an irritation. *Postgraduate Medical Journal*, 2004, 80(945): 382-387 (doi: 10.1136/pgmj.2003.014563).
24. Luo B., Liao F., Hu Y., Liu X., He Y., Wu L., Tan H., Luo L., Zhou Y., Mo Q., Dengl J., Wei Y. Acaricidal activity of extracts from *Ligularia virgaurea* against the *Sarcoptes scabiei* mite in vitro. *Exp. Ther. Med.,* 2015, 10(1): 247-250 (doi: 10.3892/etm.2015.2503).
25. Volkova I.M., Viktorova E.V., Savchenkova I.P., Gulyukin M.I. Characteristic of mesenchymal stem cells, isolated from bone marrow and fatty tissue of cattle. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2012, 2: 32-38 (doi: 10.15389/agrobiology.2012.2.32eng).
26. Gornostaeva A., Andreeva E., Buravkova L. Factors governing the immunosuppressive effects of multipotent mesenchymal stromal cells in vitro. *Cytotechnology*, 2016, 68(4): 565-577 (doi: 10.1007/s10616-015-9906-5).
27. Andreeva E., Bobyleva P., Gornostaeva A., Buravkova L. Interaction of multipotent mesenchymal stromal and immune cells: bidirectional effects. *Cytotherapy,* 2017, 19(10): 1152-1166 (doi: 10.1016/j.jcyt.2017.07.001).
28. Arlian L.G., Vyszenski-Moher D.L. Response of *Sarcoptes scabiei* var. *canis* (*Acari*: *Sarcoptidae*) to lipids of mammalian skin. *J. Med. Entomol.*, 1995, 32(1): 34-41 (doi: 10.1093/jmedent/32.1.34).