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CANDIDATE GENES PROMISING FOR MARKER-ASSISTED SELECTION IN AQUACULTURE

(review)

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Abstract

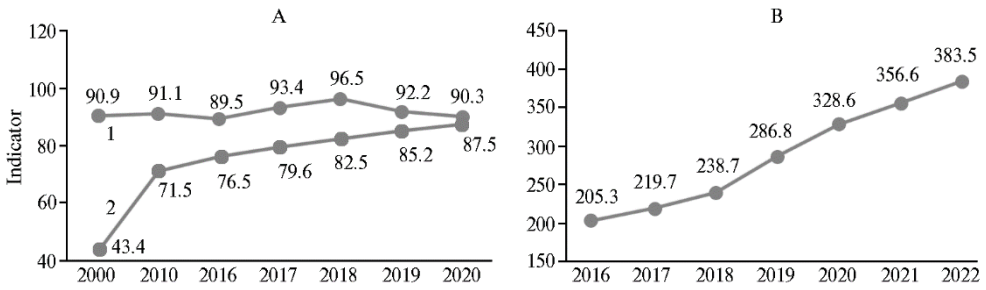
Modern aquaculture is a rapidly developing sector of food production that serves as a source of animal protein, essential amino acids, fats, vitamins, minerals, enzymes and is important for food security. In Russia, commercial fish farming is still significantly inferior in volume to industrial fish farming. A promising approach in the scientific support of commercial aquaculture is the search for polymorphic loci in candidate genes and the identification of reliable associations between various genotypes and productivity indicators for subsequent marker-assisted selection (MAS) of commercial aquaculture objects. The purpose of this review was to summarize and analyze publications concerning single nucleotide polymorphism (SNP) in genes affecting size and weight in fish. Body weight is one of the economically important characteristics for which selection is carried out in fish farms. It depends on the growth of skeletal muscle, so genes that influence the growth and development of muscle tissue are considered as potential candidate genes. The most important of them include the genes for myostatin (*MSTN*), insulin-like growth factors I and II (*IGF-I*, *IGF-II*), growth hormone (*GH*) and growth hormone receptor (*GHR*) (X.Y. Dai et al., 2015; D.L. Li et al., 2014). When assessing the effect of candidate genes on a particular trait, polymorphisms in those genes are first examined, and then the relationship between specific alleles/genotypes and phenotypic expression of the trait of interest is statistically assessed. If significant associations are found, this is considered evidence that the gene is either directly involved in the genetic control of the trait, or the functional polymorphism is located sufficiently close to the marker and the two loci are in linkage disequilibrium (M. Lynch and B. Walsh, 1997; D.L. Yowe and R. J. Epping, 1995). Myostatin plays an important role in inhibiting muscle growth and development. In most mammals, the loss or inactivation of myostatin (*MSTN*–/–) causes an increase in the size and number of myofibers, which leads to an increase in muscle mass (A. Clop et al., 2006; L. Grobet et al., 1997; D.S. Mosher et al. al., 2007; S. Rao et al., 2016). The genes for insulin-like growth factors I and II encode the corresponding polypeptide hormones which have a molecular structure similar to proinsulin and play an important role in regulation of growth, development and differentiation of cells and tissues in vertebrates (J.I. Jones et al., 1995; M Codina et al., 2008). Insulin-like growth factors I and II are the most important endocrine mediators of the action of growth hormone; they are synthesized in the liver, skeletal muscles and other tissues (W.J. Tao and E.G. Boulding, 2003; K.M. Reindl et al., 2011). Growth hormone, or somatotropin, is a polypeptide hormone that is synthesized in the somatotropic cells of the pituitary gland and participates in the regulation of somatic growth in fish (J.I. Johnsson and B.T. Bjцrnsson, 1994; B. Cavari et al., 1993). The growth hormone receptor is a transmembrane protein that belongs to the class 1 cytokine receptor superfamily and serves as an important regulator of growth and metabolism (T. Zhu et al., 2001). GHR as a receptor mediates the biological effects of growth hormone on target cells by transmitting a stimulatory signal across the cell membrane with subsequent induction of transcription of many genes, including *IGF-I* (Y. Kobayashi et al., 1999). SNPs in the genes *MSTN*, *IGF-I*, *IGF-II*, *GH*, *RGH* can affect the size and weight in various fish species and can be an auxiliary tool in breeding programs (D. Gencheva and S. Stoyanova, 2018; C. De-Santis and D.R. Jerry, 2007; Y. Sun et al., 2012). The functional characterization and associations of growth and development indicators with genetic polymorphisms in the genes of myostatin, insulin-like growth factors I and II, growth hormone and growth hormone receptor considered in the review allow us to recommend these genes as the most promising candidates for searching polymorphic loci with subsequent statistical assessment of the genotype—trait

relationship. The reliable associations can be used in marker selection to replace broodstocks and improve the efficiency of commercial aquaculture.

Keywords: candidate genes, aquaculture, body weight, polymorphic locus, marker-assisted selection, MSTN, myostatin, IGF-I, IGF-II, insulin-like growth factors I and II, GH, growth hormone, RGH, growth hormone receptor

Currently, aquaculture is one of the most promising and growing food industries. It has enormous potential to improve food security and meet consumer demand for fish products. According to FAO, over the past 20 years, from 2000 to 2020, global aquaculture production has increased from 43.4 to 87.5 million tons (Fig.) and in 2020 accounted for 49.2% of all fishery production. The growth trend continues and the aquaculture is expected to reach 52% by 2025, exceeding commercial fisheries [1].

Production of aquaculture in the Russian Federation (see Fig.) is constantly growing, from 205.3 thousand tons in 2016 to 383.5 thousand tons in 2022 [2, 3], that is, an average by 11% annually. In 2016, commercial fish farming made 4.3% of total fish production, in 2022, 7.8%, but this is significantly lower than global trends. Nevertheless, Russia has a significant fishing fund and a wide range of artificial breeding facilities that, together with growing demand, create significant potential for domestic aquaculture [4].



World fisheries products (1) **and aquaculture production** (2) (A, million tons**) [1] as compared fo commercial aquaculture** (thousand tons) **in Russia** (B) **[2, 3].**

Improving the efficiency of fish farming requires a thorough understanding of various aspects from breeding practices to molecular technologies. Global experience shows that fish breeding programs in countries with developed aquaculture (China, Korea, Norway, India, Indonesia, and Chile) involve DNA technologies to identify polymorphisms of genes for productivity traits. In Russia, it is also necessary to intensify genetic analysis of fish growth and productivity performance at the levels of genes or gene linkage groups. This will provide gene pool identification in breeding broodstocks and more precise and effective selection based on the polymorphisms of candidate genes that affect economically useful traits.

The purpose of the review is to summarize and analyze data on polymorphisms in genes affecting productivity traits in fish and to identify the most promising candidate genes for use in marker-assisted selection in domestic aquaculture.

Fish growth is economically significant and affects the efficiency of the industry. Body weight and growth rate are the indicators used for selection in fish farms. These indicators depend on the growth of skeletal muscles which account for up to 70% of the fish body weight [5]. Therefore, candidate genes may include genes for growth and development of muscle tissue [6, 7]. Skeletal muscle growth is controlled by a group of genes, the most important are myostatin (*MSTN*), insulin-like growth factors I and II (*IGF-I*, *IGF-II*), growth hormone (GH), and growth hormone receptor (*GHR*). There are reports about associations between polymorphisms of these genes and growth of some fish species in aquaculture, and

on the use of genomic data in marker-assisted selection [8-12].

To reveal the effect of candidate genes on a particular trait, polymorphisms in these genes are first examined, and the relationship between specific alleles/genotypes and phenotypic expression of the trait of interest is statistically assessed. Significant associations evidence that the gene is either directly involved in the genetic control of the trait, or the functional polymorphism is located sufficiently close to the marker and the two loci are in linkage disequilibrium [13, 14].

Let us consider some candidate genes and associations of their polymorphisms with growth and productive performce in fish.

Myostatin (*MSTN* gene). Myostatin, or growth differentiation factor 8 (*GDF-8*), is a member of the transforming growth factor-β (*TGF-β*) family which is critical for inhibiting muscle growth [15, 16]. In most mammals, loss or inactivation of myostatin (MSTN–/–) increases the size and number of myofibers, and, therefore, muscle mass [17-20].

In fish, the *MSTN* gene includes 3 exons and 2 introns; *MSTN* was revealed and characterized in *Salmo salar*, *Oreochromis mossambicus*, *Morone chrysops*, *Danio rerio*, *Lateolabrax japonicus* [21-24]. The *MSTN* gene has different expression profiles in vertebrates. In fish, unlike mammals, *MSTN* is expressed in different tissues and organs in addition to muscles. Thus, a number of studies carried out on various fish species reporte about the myostatin expression in brain, muscles, eyes, liver, ovaries, gills, kidneys, intestines, spleen, and skin [25-30].

Due to broader expression profile, it has been suggested that myostatin may also be involved in the regulation of other physiological processes unrelated to muscle growth [31]. Studies on *Danio rerio* and *Oryzias latipes* showed myostatin effects not only on growth, but also on the immune system [32, 33]. Myostatin is involved in osmoregulation and coordination of neuronal growth and development [34, 35].

Two myostatin isoforms were first identified by real-time PCR in Atlantic salmon (*Salmo salar*) as a non-mammalian species [21]. Four myostatin genes with the same genetic structure, the *MSTN1a*, *MSTN1b*, *MSTN2a*, and *MSTN2b* were foud in *Cyprinus carpio*. The *MSTN1a* and *MSTN1b* paralogs are 96% similar. *MSTN2a*, *MSTN2b* 94% similar. Differences were due to the length and sequence of introns. Two introns in the *MSTN2a* gene were longer than in the *MSTN2b* gene, 1384 bp and 1763 bp vs. 879 bp and 835 bp [36]. L. Liu et al. [37] cloned and characterized the *MSTN* gene of *Aristichthys nobilis* (abbreviated AnMSTN). The *MSTN* genomic sequence is 3769 bp long and consists of three exons and two introns, and the full length cDNA (2141 bp) of the gene had an open reading frame encoding a polypeptide of 375 amino acids. The resulting amino acid sequence of MSTN was 67.1-98.7% homologous to MSTN sequences of birds, mammals and teleosts. Sequence comparison and phylogenetic analysis showed that AnMSTN is the MSNT-1 isoform.

Phylogenetic analysis of the entire myostatin gene subfamily revealed the presence of several *MSTN* forms in teleosts. Genome duplication in the common ancestor of ray-finned fishes resulted in two distinct myostatin clades, *MSTN-1* and *MSTN-2* [38]. The second duplication event in salmonids occurred through tetraploidization and led to two subsequent divisions, one in each clade. This finding indicates that salmonids possess four different myostatin genes, two in the first clade (*MSTN-1a* and *MSTN-1b*) and two in the second clade (*MSTN-2a* and *MSTN-2b*) [39, 40]. Whole-genome duplication in ancient ray-finned fishes followed by tetraploidization in the ancestor of salmonids has complicated genomic studies of candidate genes in these fish because their genomes contain many genes with multiple copies [41].

MSTN is a candidate gene in selection for fish growth parameters that is

confirmed for different fish species (Table 1).

1. Associations of myostatin gene polymorphisms with indicators of fish growth performace

Species	Trait	Position	Authors
Cyprinus carpio	Feed conversion ratio, body weight	c.42A > G c.72C > T	Sun Y. et al., 2012 (44)
Cyprinus carpio	Feed conversion ratio, protein consumption efficiency	T2230C	Al-Khshali M.S. et al., 2020 (46)
Oreochromis niloticus	Body weight	Exon 2	Elkatatny N.A. et al., 2016 (43)
Aristichthys nobilis	Total length, body length, body weight	g.2770C > A	Liu L. et al., 2012 (50)
Salmo salar	Body weight, gutted carcass weight, headless carcass weight, fillet weight	g.1086C > T	Pecaloza C. et al., 2013 (48)
Oncorhynchus mykiss	Body weight, total length	g.1904T > C	Nazari S. et al., 2016 [49]
Verasper variegatus	Body weight, body length, body thickness	T355C	Li H. et al., 2012 [42]
Гибрид *Culter alburnus* (♀) × *Ancherythroculter nigrocauda* (♂)	Body weight, total length, body length, body height, head length	c.6T > C	Cheng L. et al., 2015 [47]
Cyprinus carpio	Average daily growth	C1031T	Yu J.H. et al., 2010 [45]
Ancherythroculter nigrocauda	Body weight, total length, body length, body height	g.1129T > C	Sun Y. et al., 2017 [51]
	Body weight, body height	g.1289G > A	

Single nucleotide polymorphisms (SNPs) in the *MSTN* gene can affect the body weight of fish. For example, SNP T355C in the promoter region of the myostatin gene is associated with growth traits in *Verasper variegatus*. Individuals with the CC genotype were superior in growth rates ($p < 0.01$) to the TC and TT genotypes. Mutations in the promoter may be involved in the control of *MSTN* gene expression, suggesting the possible existence of a regulatory mechanism to alter phenotypes [42].

Association analysis showed that SNPs c.42A > G and c.72C > T in the third exon were significantly associated with body weight ($p < 0.01$) and body condition coefficient ($p < 0.05$) in common carp (*Cyprinus carpio*), and haplotype analysis confirmed this relationship, showing an advantage ($p < 0.01$; $p < 0.05$) of the H7H8 haplotype in terms of growth [44]. In *Cyprinus carpio*, the average daily gains differed significant ($p < 0.05$) between fish with different genotypes for SNP at position C1031T of the *MSTN2a* gene. Correlation analysis showed that individuals with the TT genotype, on average, gain weight faster than carriers of the CT and CC genotypes, 112% vs. 67.3% [45]. For another SNP found in *Cyprinus carpio* at position T2230C, association analysis showed a significant effect ($p < 0.05$) of the polymorphism on feed conversion rate, protein intake, and protein efficiency ratio [46]. L. Cheng and Y.H. Sun [47] identified four new SNPs in the *MSTN* gene in the hybrid *C. alburnus* (♀) × *A. nigrocauda* (♂). One nonsynonymous SNP (c.6T > C) in exon 2 was significantly ($p < 0.01$) associated with body weight, total length, Smith body length, greatest body height, and head length. Fish with the H1H3TGGG/CAGG haplotype combination demonstrated the best growth performance ($p < 0.01$, $p < 0.05$) [47].

Three new SNPs were discovered in the *MSTN-1b* gene of *Salmo salar*. One of them (g.1086C > T) located within the 5′-flanking region had a significant relationship ($p < 0.05$) with body weight, eviscerated carcass weight, headless weight and fillet weight. Analysis of associations based on haplotypes confirmed this findings, since two haplotypes that had a significant association with body weight indicators, the hap4 and hap5 ($p < 0.05$ and $p < 0.01$, respectively) differed by a single substitution g.1086C > T. Alleles at this locus act additively thus providing a small percentage of the genetic variation in these phenotypes [48]. S. Nazari et al. [49] found an association between polymorphism at the g.1904T > C locus of the *MSTN-1* gene and growth performance (body weight and total length) in

domesticated *Oncorhynchus mykiss*. The results showed that rainbow trout with CC and TC genotypes had greater ($p < 0.05$) body weight and total length than those with the TT genotype.

The g.2770C > A polymorphism in the *MSTN-1* gene of *Aristichthys nobilis* is significantly associated ($p < 0.01$) with total length, Smith body length, and body weight [50]. Y. Sun et al. [51], in a sample of 300 *Ancherythroculter nigrocauda* individuals, revelaed a significant relationship ($p < 0.05$, $p < 0.01$) of SNP g.1129T > C with total length, Smith body length, body height and weight whereas SNP g.1289G > A was associated ($p < 0.05$) only with body weight and greatest body height. Fish with TC/TC or TC/GA genotype combinations showed better growth performance. Studies on *Danio rerio* compared the average length and body weight of individuals mutant for the *MSTNa* and *MSTNb* genes with the wild type fish during 1 to 6 months after fertilization. It was found that the body weight and length of fish with the *MSTNa–/–* genotype increased only slightly compared to the wild type, while males and females with *MSTNb–/–* genotypes at the age of 6 months had a significantly higher and wider body (by 62.36%) and greater body weight (by 51.97%).

Insulin-like growth factors I and II (*IGF-I*, *IGF-II* genes). In fish, the insulin-like growth factor (IGF) family includes three IGF peptides (IGF-I, IGF-II, IGF-III), two insulin-like growth factor receptors, and six IGF-binding proteins [52-54]. The genes for insulin-like growth factors I and II encode the corresponding polypeptide hormones which have a molecular structure similar to proinsulin and play a signifint role in regulating growth, development and differentiation of cells and tissues in vertebrates [55, 56]. Insulin-like growth factors I and II are the most important endocrine mediators of the growth hormone action; IGF-I and IGF-II are synthesized in liver, in skeletal muscles and in other tissues [57, 58].

In addition to growth, the *IGF-I* gene in fish is also associated with metabolism, regeneration [59], osmoregulation in seawater [60-62], and regulation of feed intake [63]. The distinct localization of *IGF-I* in the gonads of male and female fish indicates the role of the IGF system in differentiation of the gonads [64-67]. *IGF-I* is also involved in spermogonia proliferation and oocyte maturation [68, 69]. To study the effect of *IGF-I* on fish growth, a transgenic *Oryzias latipes* containing the promoter of the carp β-actin gene fused to the *rtIGF-I* cDNA was produced. The transgenic *Oryzias latipes* not only grew significantly faster than non-transgenic controls, but also hatched 2 days earlier than the control group. These results support the fact that *IGF-I* is involved in the regulation of fish growth and development [70]. Another study found that the expression of *IGF-I* and *IGF-II* in muscle increases dramatically in response to repeated feeding. As a reuslt, *IGF-I* and *FGF-II* are identified as promising candidate genes involved in the cellular signaling system that regulates myotomal muscle fiber growth in fish [71].

Many studies have identified *IGF-1* gene expression in a variety of salmonid tissues, including muscle, spleen, fat, intestine, liver, heart, testes, ovaries, kidneys, pituitary gland, and brain [72-74]. In juvenile carp and tilapia, the *IGF-I* and *IGF-II* genes are similarly widely expressed in different organs and tissues, with the highest levels of expression in the liver [75, 76]. Studies on sturgeon revealed an increase in the expression of the *IGF-II* gene in the spleen, stomach and kidneys compared to the *IGF-I* gene, the *IGF-I* mRNA level was higher in the intestines and muscles, and only in the liver the highest expression of two genes occurred simultaneously [77].

There are distinct differences between the gene structures that determine the synthesis of insulin-like growth factor I in mammals and fish. For example, in humans and rats, IGF-I is encoded by a single gene consisting of six exons spanning

more than 80 Kb of genomic DNA [78, 79], whereas the *IGF-I* genes of fish *Danio rerio*, *Salmo salar* and *Pleuronectes platessa* consist of five exons with the length of approximately 15, 22 and 17.5 thousand bp, respectively [80-82].

At an early stage of evolution of teleosts (approximately 320-350 million years ago), duplication of the entire genome occurred, so their IGF system is complicated by the presence of paralogous genes [83]. Additional forms of this gene arose in salmonids because the whole genome duplication of teleosts was followed by an additional duplication event in the salmon family 25-100 million years ago [84, 85] and also in the cyprinid subfamily [86]. It is estimated that 50% of duplicated genes have been further lost from the genome [87]. The remaining paralogs are undergoing subfunctionalization that modulates their expression [88].

Studies have shown the presence of multiple *IGF-I* mRNA transcripts encoding various IGF-I prohormones in salmonids. These mRNAs were designated Ea-1, Ea-2, Ea-3, and Ea-4 [89]. M.J. Shamblott et al. [90] in research with rainbow trout also detected all four types of *IGF-I* mRNA and four transcripts encoding the four proIGF-Is in salmonids [90, 70]. In *Epinephelus lanceolatus*, two *IGF-I* cDNA precursors were cloned, the *IGF-Ia* and *IGF-Ib* determining sequences of 159 and 186 amino acids, respectively, which are 98.4% and 98.7% identical to *IGF-I* found in *Epinephelus lanceolatus* [91]. M.H. Chen et al. [80] obtained data indicating the presence of two forms of the *IGF-I* gene, the *Ea-1* and *Ea-2* in *Danio rerio*; in another publication, paralogues were also found in the same fish species, the *IGF-1a* and *IGF-1b* for *IGF-I*, and the *IGF-2a* and *IGF-2b* for *IGF-II* [92].

Many studies have been carried out to search for polymorphisms in the *IGF-I*, *IGF-II* genes and assess their relationship with productivity performance of aquaculture objects. Table 2 shows the associations of polymorphisms of genes for insulin-like growth factors I and II with growth and development indicators of some fish species.

2. Associations of *IGF-I* and *IGF-II* gene polymorphisms with indicators of fish growth performace

Species	Trait	Position	Authors
Micropterus salmoides	Body weight, body thickness	5′ flanking region	Li X.H.et al., 2009 [94]
Cyprinus carpio	Body weight, body length	g.7627T > A	Feng X. et al., 2014 [97]
Pseudobagrus fulvidraco × *Pseudobagrus vachellii*	Body weight, fatness, body length, total length, head length, body height, caudal peduncle length, body thickness	97T > C	Chu M.X. et al., 2022 [54]
Oreochromis niloticus	Body weight	G161A	Yu J. et al., 2010 [99]
Salmo salar	Body weight, gutted carcass weight, headless carcass weight, fillet weight	g.5763G > T g.4671A > C	Tsai H.Y. et al., 2014 [93]
Dicentrarchus labrax	Body weight, total length	g.5127731G > T	Gokcek O.E. et al., 2020 [102]
Dicentrarchus labrax	Body weight, total length Total length	g.46749C > T g.46672A > G	Gokcek O.E. and Isık R., 2020 [103]
Sander lucioperca	Body weight	c.544+1111_544+1112 delAAinsTC	Teng T. et al.,2020 [98]
Lateolabrax maculatus	Head length, body thickness Total length Head length, body thickness Standard length	g2907C > T g3230A > C g3294C > T g5064C > T	Fan S. et al., 2023 [101]

In *Salmo salar*, three SNPs were identified in the *IGF-I* gene, namely, in the promoter (SNP1, g.5763G > T), in intron 1 (SNP2, g.7292C > T) and in intron 3 (SNP3, g.4671A > C). It was found that SNP1 and SNP3 were significantly associated with several weight traits ($p < 0.05$). Haplotype analysis confirmed the association ($p < 0.05$) between genetic variations in the *IGF-I* gene and total body weight, as well as fillet characteristics [93]. X.H. Li et al. [94] found that polymorphisms in the *IGF-I* gene promoter influence body weight and thickness

in a population of *Micropterus salmoides*. Fish with the AA genotype had significantly greater body weight and size than fish with the AB or BB genotypes. Polymorphisms in the promoter region and missense mutations in coding regions, unlike intronic polymorphisms or silent mutations in the coding regionare, seem to be related directly to the parameters affected by the candidate gene [95]. In another study [96], four SNPs (C127T, T1012G, C1836T, and C1861T) were found in the *IGF-II* gene in *Micropterus salmoides*. Association analysis showed that SNPs were not significantly associated with growth characteristics. However, significant associations ($p < 0.05$) were identified between diplotypes. Diplotypes H1H3 (CDCC/CDCC CDCC) and H1H5 (CTCC/TTTT) produced greater body weight than diplotypes H1H1 (CTCC/CTCCC), H1H2 (CTC/TGT) and H4H4 (TGC/TGC).

In the common carp (*Cyprinus carpio*), SNP g.7627T > A was identified in intron 2 of the *IGF-I* gene, which was significantly associated ($p < 0.05$) with body weight and length. The AA genotypes had a 5.9% higher average body weight than the TT genotypes [97]. In a cultivated population of *Sander lucioperca*, a SNP was found in intron 3 of the *IGF-II* gene, which has a significant correlation ($p < 0.05$) with body weight [98].

In a study on 264 *Pseudobagrus fulvidraco* × *Pseudobagrus vachellii* hybrids, one non-synonymous mutation (SNP 97T > C) was identified in the *IGF-II* gene, which was significantly associated ($p < 0.05$) with growth traits (Smith body length, total length, head length, maximum body height, caudal peduncle length, body thickness, body weight and fatness). This relationship was confirmed ($p < 0.05$) in the second population of 183 individuals [54]. In *Oreochromis niloticus* males of the GIFT breed, two G161A polymorphisms in exon 3 and a microsatellite locus in intron 3 identified in the *IGF-II* gene were significantly associated with growth. Different genotypes affected the growth rate in males ($p < 0.01$), the weight of males with the GG genotype (532 g) was 15.7% greater than that of carriers of the AG genotype (454 g). No differences were found in the growth rate of females [99]. These data are supported by another study which also found an association between a polymorphism in exon 3 of the *IGF-II* gene and body size in the GIFT population of *Oreochromis niloticus* [100].

Sequencing the *IGF-II* gene of *Lateolabrax maculatus* revealed four SNPs that significantly correlated with growth traits ($p < 0.05$). SNP g2907C > T was associated with head length and body thickness, SNP g3230A > C with total length, and SNP g3294C > T with body thickness and head length. Genotypes with SNP g5064C > T significantly differed in Smith body length [101]. Several single nucleotide polymorphisms in the *IGF-I* and *IGF-II* genes have been identified in the *Dicentrarchus labrax* population. In the 5′UTR region of the *IGF-I* gene, a relationship was found ($p < 0.05$) between SNP g.46749C > T and body weight, total length, as well as between SNP g.46672A > G and total length ($p < 0.05$). Fish with the GG genotype (*IGF-II-NdeI* locus) had greater body weight and total length ($p < 0.05$) than fish with the TG genotype [102, 103]. Overall, the research highlight the importance of the IGF system in indirectly affecting the growth and development of fish and show the possibility of using the *IGF-I* and *IGF-II* genes as genetic markers in aquaculture breeding.

Growth hormone (*GH* gene). Growth hormone, or somatotropin, is a polypeptide synthesized in the somatotropic cells of the pituitary gland. GH plays an important role in the regulation of fish somatic growth [104-107], osmoregulation [108-110], reproduction [111, 112], regulation of lipid and protein metabolism, carbohydrate metabolism through complex interactions with insulin and insulin-like growth factor 1 [113-115], in immunity responces [116, 117]. Moreover, investigations have shown that growth hormone also affects behavioral

responses such as appetite and foraging in rainbow trout and transgenic *Atlantic salmon* [118]. In salmonids, as in mammals, it is clearly evidenced that growth hormone is the major activator of the IGF system, since GH stimulates the expression of *IGF-I* and *IGF-II* genes in both the liver and other tissues [119-121].

Variability is a characteristic feature of the growth hormone gene in fish, distinguishes it from conservative growth hormone gene in mammals [122]. The growth hormone gene identified in *Ctenopharyngodon idellus*, *Hypophthalmichthys molitrix*, *Cyprinus carpio*, *Labeo rohita*, *Ictalurus punctatus*, and *Sarcocheilichthys sinensis* has five exons and four introns [123-128] that is similar to the *GH* structure in mammals [129]. However, among other teleost fish there are species in which the growth hormone gene consists of six exons and five introns, for example, *Salmo salar*, *Oncorhynch nerka*, *Oncorhynchus mykiss*, *Tilapia nilotica*, *Fugu rubripe*s, *Sparus aurata* [105, 130-134]. In many fish species, the growth hormone gene has a higher level of variation in non-coding regions than in other vertebrates, which is due to the presence of two functional copies of the gene, *GH1* and *GH2*. Two paralogues of the growth hormone gene were identified in *Oncorhynch nerka*, *Tilapia nilotica*, *Carassius auratus*, *Oncorhynchus tshawytscha*, *Cyprinus carpio*, *Salmo salar*, and *Oncorhynchus mykiss* [132, 135-140]. Expression of the hormone gene occurs in many tissues and organs, including the brain, liver, muscle, heart, spleen, kidneys, and ovaries, but the highest expression is found in the pituitary gland [91, 128, 141].

Complete or partial sequencing of the growth hormone gene in different fish species has revealed single-nucleotide polymorphisms and microsatellites which are proposed for marker-assissted selection. Table 3 presents associations of polymorphisms of the growth hormone gene with indicators of fish growth and development.

In the common carp (*Cyprinus carpio*), SNP A1132T was identified in intron 3 of the *GH* gene. Fish with the AA genotype had a significant superiority ($p < 0.05$) in body weight at the end of the growing period, average daily gain, relative growth rate, and specific growth rate over carriers of the AT and TT genotypes [142]. Correlation analysis (marker–trait) based on a general linear model (GLM) also showed a significant association between *GH-1* gene genotypes in *Cyprinus carpio* and body weight. The body weight of fish with genotype D was significantly ($p < 0.05$) greater compared to other genotypes [143].

3. Associations of the growth hormone gene polymorphisms with indicators of fish growth performace

Species	Trait	Position	Authors
Cyprinus carpio	Body weight, average daily gain, relative growth rate, specific growth rate	A1132T	Al-Azzawy M.A. et al., 2018 [142]
Cyprinus carpio	Body weight		Berenjkar N. et al., 2018 [143]
Sarcocheilichthys sinensis	Body length, total length, body weight, body height, body thickness, body condition factor	g.1541A > G	Zhu T. et al., 2020 [128]
	Body condition factor	g.242InDel	
Paralichthys olivaceus	Body weight, head length	1763(C > T)	Ni J. et al., 2006 [148]
Siniperca chuatsi	Body weight, total length, body length, body height	g.4940A > C	Tian C. et al., 2014 [144]
	Total length, body height	g.4948A > T	
	Body length	g.5045T > C	
Larimichthys crocea	Body weight, body length	(T > C) 692	Ni J. et al., 2012 [147]
Oreochromis niloticus	Body weight, gutted carcass weight, fillet weight, fillet length		Tanamati F. et al., 2015 [150]
Oreochromis niloticus	Total length, body length, body height, body thickness	Intron 1	Blanck D.V. et al., 2009 [151]
Oreochromis niloticus	Body weight	Promoter	Dias M.A. et al., 2019 [153]
Siniperca chuatsi	Body weight, body length, body thickness	g.5234T > G	Wang H. et al., 2016 [145]
	Body thickness	g.5045T > C	

Continued Table 3

Sparus aurata	Body weight	Promoter	Almuly R. et al., 2005 [149]
Siniperca chuatsi	Body weight, total length, body length, body height, head length	G1g.197C > A	Sun C.-F., et al., 2019 [146]
	Head length	G2g.2558C > G	
	Body weight, total length, body length, head length	G3 .2643C > G	

Four SNPs were identified in the *GH* gene of *Siniperca chuatsi* three of which have a significant correlation ($p < 0.05$) with growth parameters. Individuals with the CC genotype (g.4940A > C) had greater body weight, total length, Smith body length and body height than fish with the AA or AC genotypes. The carriers of the TT genotype (g.4948A > T) were superior to the genotypes AA or AT in height and the total body length, and fish with the genotype CC (g.5045T > C) had significant differences only in Smith body length [144]. In another study, two SNPs in exon 5 (g.5045T > C) and intron 5 (g.5234T > G) identified in *Siniperca chuatsi* were significantly associated with growth performance. Fish with the GG genotype (g.5234T > G) were significantly superior to carriers of the TT and TG genotypes in body weight ($p < 0.01$), Smith body length ($p < 0.05$) and body thickness ($p < 0.01$). The g.5045T > C locus significantly influenced ($p < 0.05$) only body thickness [145]. Analyzing the results of two previous studies, it should be noted that SNP g.5045T > C had a significant effect on productive indicators in two different populations of *Siniperca chuatsi*, which once again confirms the promise of this polymorphism in marker-assisted selection of this fish species. C. Sun et al. [146] identified four loci that significantly correlated with growth traits in *Siniperca chuatsi*. Loci G1 (g.197C > A), G3 (G3 g.2643C > G) and GH-AG are associated ($p < 0.01$) with body weight, total length, Smith body length, greatest body height and head length.

J. Ni et al. [147] in a *Larimichthys crocea* population from Zhejiang, identified a SNP (G→A) at position 196 of intron 1 of the *GH* gene associated with the highest body height ($p \leq 0.05$). In the population from Fujian, a SNP at position 692 (T→C) was identified in intron 2. The CD genotype had a positive correlation with body weight and total body length ($p \leq 0.01$). In P*aralichthys olivaceus*, in exon 4 of the growth hormone gene, one nonsynonymous mutation was identified at position 1763 (C > T), which positively correlates ($p < 0.05$) in the AB genotype with body weight and long head [148]. It was shown that in *Sarcocheilichthys sinensis* the polymorphic locus g.242InDel is associated ($p < 0.05$) with the fatness coefficient, and the polymorphism g.1541A > G ($p < 0.01$; $p < 0.05$) is associated with Smith body length, total length, greatest body height, body weight, body thickness and body condition index [128]. In the *Sparus aurata* population from the hatchery, a dinucleotide microsatellite polymorphism was detected in the promoter region of the growth hormone gene. Alleles 250 and 254 were found to be associated with the weight of the studied fish [149].

F. Tanamati et al. [150] identified polymorphisms in the *GH* promoter region in *Oreochromis niloticus*. Carriers of the *GH*db genotype had significantly heavier ($p < 0.05$) body weight, eviscerated carcass weight, as well as fillet weight and length, which indicates a correlation between *GH* variations and productivity traits in *Oreochromis niloticus*. D.V. Blanck et al. [151] described a polymorphism in intron 1 of the *GH1* gene in *Tilapia nilotica* that has a significant correlation with total length, standard length, and body height and thickness. It was found that the PstI+/– genotype is associated with better performance regardless of the fish breed. The authors believe that this association may be due to a direct effect of the *GH* gene's own regulation. S.K. Jaser et al. [152] and M.A. Dias et al. [153] also found SNPs in the promoter region of the growth hormone gene associated with fish growth performance in *Oreochromis niloticus*.

Growth hormone receptor (*GHR* gene). The growth hormone receptor, a transmembrane protein of the class 1 cytokine receptor superfamily [154] is a critical regulator of growth and metabolism. GHR, as a receptor, mediates the biological effects of growth hormone on target cells by transmitting a stimulatory signal across the cell membrane, followed by induction of transcription of many genes, including *IGF-I* [155].

In fish, the growth hormone receptor gene contains ten exons and is present as a double copy, the *GHR-I* and *GHR-II*, in *Paralichtys olivaceus*, *Salmo salar*, *Oncorhynchus mykiss*, *Sparus aurata*, and *Anguilla japonica* [156-159]. The *GHR-I* and *GHR-II* genes are highly transcribed, but their expression is unevenly localized showing some tissue specificity, i.e., the *GHR-I* expression in the liver and adipose tissue exceeds the *GHR-II* expression [158].

4. Associations of the growth hormone receptor gene *GHR* polymorphisms with indicators of fish growth performace

Species	Trait	Position	Authors
Pangasianodon hypophthalmus	Body length, body height, caudal peduncle length	SNP1 A > G	Jiang L.-S. et al., 2022 [160]
	Body length, body height	SNP2 T > G	
	Body weight, total length	SNP3 G > C	
	Body height	SNP4 A > G	
Oreochromis niloticus	Body weigh	2116C > A 2117A > G	Aboukila R.S. et al., 2021 [5]
Cynoglossus semilaevis	Body weight, gonad weight	c.G1357A	Zhao J.L, 2015 [163]
Oreochromis niloticus	Body weight, total length, head length, body height, body thickness, caudal peduncle length	Exon6_G121A Exon7_G72A Exon10_T66A Exon10_T129G Exon10_C153A	Chen B-L. et al., 2020 [162]
Culter alburnus	Body weight, body length, body height	Cal-GHR2-1	Liu Z.J. et al., 2020 [161]
	Body weight, body length	Cal-GHR2-3	
	Body mass	Cal-GHR2-4	

The growth hormone receptor is an important regulatory factor of the growth axis with great potential for use in fish marker-assisted selection. *GHR* genetic polymorphisms may interfere with normal GH function, thereby influencing growth traits. Therefore, an underlying mutation in the growth hormone receptor gene can affect its expression level [141, 160]. Table 4 presents associations of the *GHR* gene polymorphisms with indicators of fish growth performance.

L.-S. Jiang et al. [160] identified five SNPs in the 3′UTR of the growth hormone receptor gene in *Pangasianodon hypophthalmus*. It was found that fish with the GG genotype (SNP1 A > G) had greater Smith body length, the greatest body height and the length of the caudal peduncle ($p < 0.05$) compared to the AA genotype. In SNP2 T > G, the GG genotype was superior to the TT genotype in Smith body length, greatest body height and caudal peduncle length ($p < 0.05$). Fish with the GG genotype (SNP3 G > C) were significantly superior ($p < 0.05$) to carriers of the GC genotype in body weight and total length. The GG genotype (SNP4 A > G) had superiority in body height ($p < 0.05$). In another study, correlation analysis showed that four polymorphic microsatellite loci were significantly associated ($p < 0.05$) with growth traits in *Culter alburnus*. The Cal-GHR2-1 locus was associated with Smith body length and body weight, the Cal-GHR2-3 locus with body length and body weight, and the Cal-GHR2-4 locus with only body weight [161].

In *Oreochromis niloticus*, a significant relationship was revealed between polymorphisms of the Exon6_G121A, Exon7_G72A, Exon10_T66A, Ex-on10_T129G, Exon10_C153A, G214C loci of the *GHR1* gene and body weight, total length, head length, maximum body height, body thickness, and caudal peduncle length in Nile tilapia [162]. Two more weight-related SNPs at positions 2116C > A and

2117A > G of the growth hormone receptor gene were identified in *Oreochromis niloticus* [5]. In *Cynoglossus semilaevis*, an association ($p < 0.01$) of the c.G1357A locus of the *GHR* gene with body weight and gonad weight was detected [163].

Summarizing the review materials, we can conclude that in countries with developed aquaculture, genetic research is an integral part of programs to reduce long and labor-intensive periods of fish farming, and to increase the yield of marketable products. In the Russian Federation, genetic technologies has ensured identification of desirable genotypes in livestock, which has improved animal breeding programs [164-169].

Thus, many publications confirm functional parameters and associations of fish growth and productivity performance with genetic polymorphisms in the genes of myostatin, insulin-like growth factors I and II, growth hormone, and growth hormone receptor. Therefore, we can conclude that the *MSTN*, *IGF-I*, *IGF-II*, *GH*, and *GHR* are candidate genes the most promising for searching polymorphic loci and genotype–trait associations. The reliable associations can be involved in marker-assisted selection and replacement of broodstocks to improve the efficiency of commercial aquaculture.

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THE INFLUENCE OF VARIOUS KISSPEPTINS ON THE REPRODUCTIVE FUNCTION OF *Bos taurus*
(review)

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Abstract

Kisspeptins (KP) are a family of peptides of various lengths with a receptor (KISS1R). Kisspeptins with gonadotropin hormone-releasing hormone (GnRH), gonadotropins (luteinizing hormone and follicle-stimulating hormone) and sexual steroids are important regulators of reproductions of various animals (S. Ohkura et al., 2009; K.-L. Hu et al., 2018). Active study of KP began in 2003. However, at present, there is not enough information about the possibilities to purposefully and effectively control the sexual cycle of *Bos taurus*, especially in dairy cows, with the help of KP (B.R. Alves et al., 2015; T. Songraphasuk et al., 2021). The KP is produced mainly in neurons of various nuclei of the hypothalamus (V. Prashar et al., 2023). Considering that the location of neurons producing the KP is specific, the approaches to control with their help the reproductive function may vary (A. Gunn et al., 2020). Kisspeptin is synthesized using the gene *kiss1*. Initially, the KP is hydrolyzed to the KP-53, which later breaks up to shorter peptides (KP-14, KP-13 and KP-10) with various biological activity (A.E. Oakley et al., 2009; J. Tomikawa et al., 2010). Neurons producing KP are also coexption of peptide neurokinin B (NKB) and dinorfin, which determined the name of this population of nerve cells (KNDy-neurons, kisspeptin/neurokinin B/dinorphin) (R.L. Goodman et al., 2013; Q. Xie et al., 2022). In cattle KNDy-neurons are mainly fixed in the arcuate core of the hypothalamus, which is considered important for both positive and negative reverse regulation by sex steroids of the synthesis of GnRH (A. Hassaneen et al., 2016; Y. Uenoyama et al., 2021). Using the histochemical method, it was demonstrated that the activation of KNDy-neurons in cattle depends on the phase of the sexual cycle (A. Hassaneen et al., 2016). Kisspeptin-, neurokinin B-, and dinorfin-immunoreactive cellular bodies and fibers detecting throughout the arcuathed core of the hypothalamus in all phases. Unlike the arcuate core, numerous kisspeptin-immunoreactive cellular bodies were found in the reservoir region of the hypothalamus in the follicular phase, while only a few immunoreactive cellular bodies are recorded in the luteal phase. As for neurokinin, in the reservoir region a small amount of neurokinin of B-immunoreactive cellular bodies and fibers in both the follicular and lutein phase is naked. Dinorfin-immunoreactive cellular bodies and fibers in the follicular phase were larger than in luteal phase. In this regard, cattle are closer to sheep and primates, including human (V.M. Tanco et al., 2016). Since the initial identification of KNDy-neurons producing KP, there are a large number of unresolved issues relating to the function of various populations of these nerve cells, depending on the location, as well as the possibilities of new technologies for their study, including in relation to *Bos taurus*. There is a need to study various concentrations of kisspeptins and their influence on the ovulation of cows. This review discusses the basic information about the location and structural-functional characteristics of the *Bos taurus* KP, the distribution and functions of the KP neurons in the brain, the content of the KP in the blood and their effect on the organs of the reproductive system. Separately data

on the exogenous regulation of KP functioning of the reproductive system *Bos taurus* are discussed. The emphasis is on scientific research data, the main object of which was *Bos taurus* animals.

Keywords: *Bos taurus*, cows, hormone, estrus cycle, kisspeptin, gonadotropin-releasing hormone, neurons

Anatomical and functional development of animal reproductive system continues from early embryogenesis until puberty. Regulation of the reproductive system functions is performed mainly through the hypothalamic-pituitary-gonadal (HPG) axis. In cattle, as in other mammals, gonadotropin-releasing hormone (GnRH), the master hormone regulating reproduction is of special importance [1].

GnRH stimulates the secretion of luteinizing (LH) and follicle-stimulating (FSH) hormones in the anterior pituitary gland, which, acting on the gonads through the peripheral circulation, regulate steroidogenesis and gametogenesis [2]. The feedback action of sex steroids produced during these physiological processes regulates the GnRH secretion. This pattern of the HPG axis hormonal regulation inherent in almost all animal species has long raised several important questions. First, there was no explanation of how the sex steroid feedback regulates the GnRH release by the hypothalamic neurons which lack estrogen receptors α (ERα), the main receptors that provide both negative and positive feedback [3]. Secondly, it was not clear how tonic and pulsatile GnRH secretion is controlled.

The opportunity to answer these questions arose with the discovery of kisspeptins (KP), a family of peptides that originated from the prohormone encoded by the *kiss1* gene. In 1996, the first identified protein of this gene was named metastin due to its ability to inhibit the metastasis of cancer cells. After 3 years, the receptor for this hormone, KISS1R (previously designated GPR54, AXOR12 or Hot7T7T175), was identified. In 2003, the influence of KP on the reproductive function was discovered.

For more than 12 years, the role of the KP has been demonstrated as the main factor in the initiation of puberty and the regulation of tonic and pulsatile release of GnRH, which has a significant impact on the fertility of females, including secretion of gonadotropins, the onset of puberty, sexual differentiation of the brain, the onset of ovulation and metabolic regulation of fertility [4, 5]. There are many publications revealing the neurohumoral and physiological role of the KP in various species of wild and domestic animals [6-10].

The purpose of this review is to systematize the accumulated data on the effect of kisspeptins on reproductive function in *Bos taurus*.

Localization and structural and functional characteristics of *Bos taurus* kisspeptins. Based on data for other species, it is possible to assume a similar localization of the *kiss-1*/KISS1R system in the central nervous system (mainly in the hypothalamus) and the placenta. Expression has also been reported in the gonads, pancreas, liver, small intestine, spleen, adipose tissue, and lymph nodes [7, 9, 10].

In cattle, the *kiss1* gene on chromosome 16 encodes a prohormone, the hydrophobic protein consisting of 135 amino acid residues (aa). This prohormone is hydrolyzed into kisspeptin 53 protein (KP-53). There are data indicating further hydrolysis of KP-53 into short peptides (KP-14, KP-13 and KP-10). Similar information has not been obtained for cattle, but a similar pattern can be assumed (Fig. 1). All four forms of the peptide have affinity, bind efficiently to receptors, and are highly stable in all vertebrate species [11].

The most functionally active kisspeptin consisnts of 52-54 amino acids depending on the animal species [6]. The amino acid sequence of kisspeptin is structurally conserved among various mammalian species (Fig. 2) [11, 13]. For example, the amino acid sequence of the goat KP is 98% similar to the sheep KP, 91% similar to the cow KP, and 77% similar to the pig KP. In particular, the 10

amino acid C-terminal domain is the minimal sequence for maximum receptor activation and remains identical in most of the species mentioned (see Fig. 2). [14, 15].

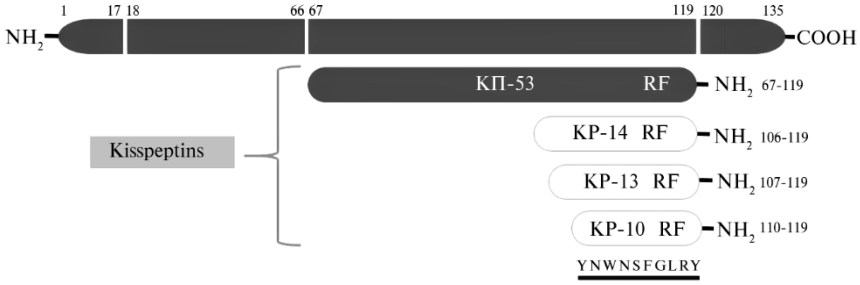

Fig. 1. Main structural features of *Bos taurus* kisspeptins resulted from post-translational modification of the prohormone. Kisspeptins presumably produced by hydrolysis of KP-53 are highlighted in white. All kisspeptins contain an RF amide that can bind and activate KISS1R (adapted from reference 12).

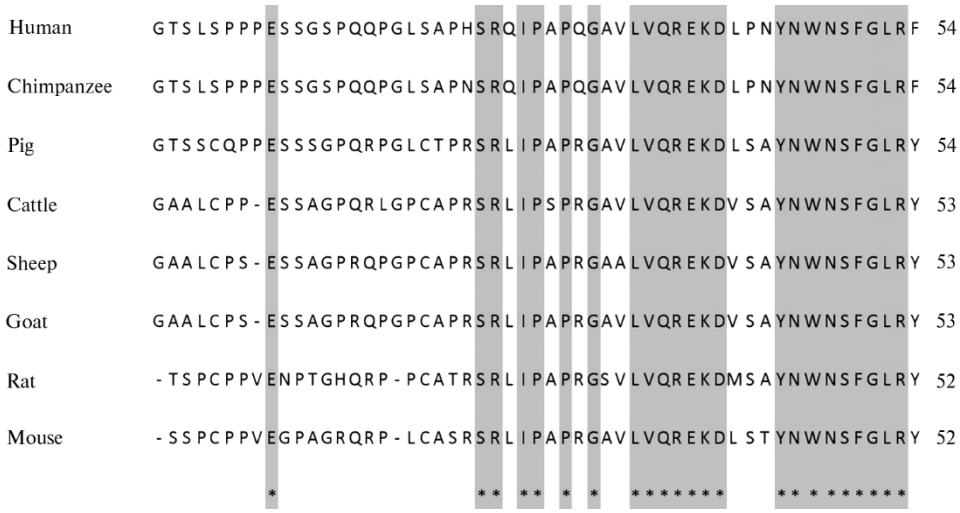

Fig. 2. Comparison of kisspeptin amino acid sequence in different mammalian species [11]. The last column indicates the number of amino acid residues. Identical amino acid residues are marked with an asterisk (*). The highlighted sequence YNWNSFGLR shows the KP-10 region which is similar in many mammalian species.

Distribution and function of kisspeptin neurons in the *Bos taurus* brain. The existence of a tonic system of Gn-RH secretion in both sexes, and a cyclic system only in females is consistent with the idea that the regulatory centers for the tonic and pulsatile systems are located in different areas of the hypothalamus. This fully applies to the areas of the hypothalamus in which neurons producing KPs have been identified. Kisspeptin neurons coexpress the peptide neurotransmitters neurokinin B (NKB) and dynorphin [16-20]. Due to co-localization of three neuropeptides, this cell population is called KNDy neurons (kisspeptin/neurokinin B/dynorphin) [21, 22].

The localization of KNDy neurons in the hypothalamus is species specific. In cattle, KNDy neurons are mainly located in the arcuate core of the hypothalamus (arcuate, ARC), which is considered important for both positive and negative feedback regulation of GnRH synthesis by sex steroids [23-25]. Most KNDy neurons express estrogen receptors α (ERα) and progesterone receptors [25-28], so the synthesis and secretion of all products of these neurons depend on the level of sex steroids. Note, there are areas of neuronal populations that produce KPs with a small amount of dynorphin and NKB [22]. However, it is important to consider

data that calls into question only the direct effect of kisspeptins on neurons producing GnRH. According to A. Gunn et al. [25], GnRH neurons in small numbers are located adjacent to kisspeptin neurons. The authors suggested that the effects of estrogen may be transmitted through kisspeptin neurons, but in cattle, unlike most other mammalian species, this is unlikely to be the main stimulatory factor.

Using a histochemical method, it was demonstrated that the activation of KNDy neurons in cattle depends on the phase of the reproductive cycle [22]. Kisspeptin-, neurokinin B-, and dynorphin-immunoreactive cell bodies and fibers are found throughout the ARC in all phases. In contrast to the ARC, numerous kisspeptin-immunoreactive cell bodies are found in the preoptic area of the hypothalamus (POA) during the follicular phase, whereas only a few immunoreactive cell bodies are recorded for the luteal phase. As for NKB, a small number of neurokinin B-immunoreactive cell bodies and fibers were found in the POA in both the follicular and luteal phases. At the same time, there were more dynorphin-immunoreactive cell bodies and fibers in the follicular phase than in the luteal phase. In this regard, as reported by V.M. Tanco et al. [29], cattle are closer to sheep and primates, including humans. For example, in rodents, cells expressing *kiss1* mRNA are located in both the ARC and POA [30]. In sheep, goats, and deer, most KNDy neurons were located in the ARC and only a small portion in the POA [26, 31, 32].

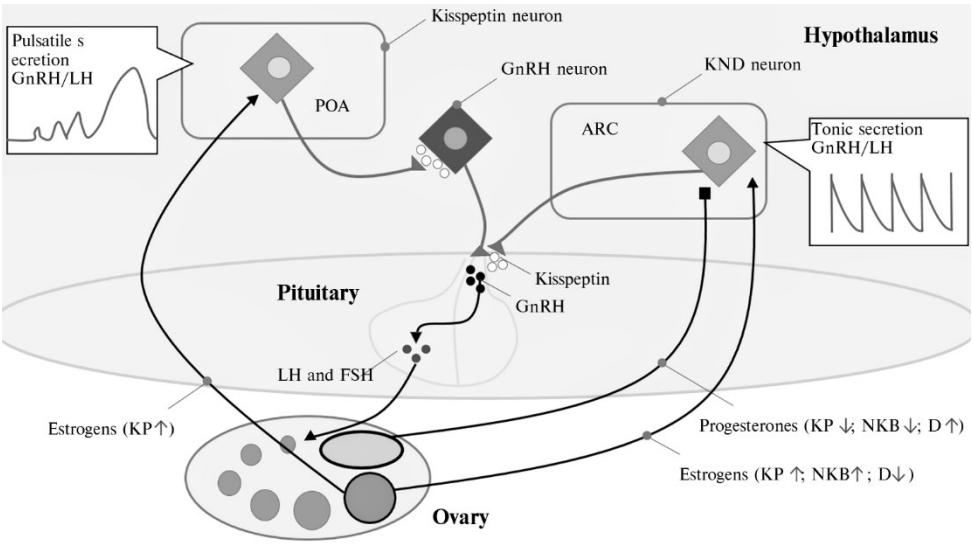

Fig. 3. Regulation of reproductive function in *Bos taurus* by kisspeptin: GnRH — gonadotropin-releasing hormone, LH — luteinizing hormone, FSH — follicle-stimulating hormone, POA — preoptic area of the hypothalamus, ARC — arcuate core of the hypothalamus, KP — kisspeptin, NKB - neurokinin B, D — dynorphin.

Secretion of the three substances by KNDy neurons in the ARC during all phases of the sexual cycle provides tonic release of GnRH (Fig. 3). This is largely possible due to the fact that KNDy neurons in the ARC, due to connections through axons and/or dendrites, form a population of neurons that mutually influence each other. Moreover, in the ARC, KNDy neurons form a dense network, which probably contributes to the synchronization of their activity [3, 22]. Synchronous activity of KNDy neurons is considered necessary for pulsatile GnRH secretion. Some authors assign the main role in such synchrony to the NKB system with its receptor NK3R [33, 34]. Kisspeptin neurons do not express KISS1R but do express receptors for dynorphin and neurokinin B. That is, KNDy neurons intercommunicate through neurokinin B and dynorphin, but not through kisspeptin [35]. NKB, secreted by a KNDy neuron, binds to its receptor NK3R

on another KNDy neuron, causing an influx of Ca^{2+} into it. An increase in intracellular Ca^{2+} concentration can promote its movement into neighboring KNDy neurons and glial cells through intercellular GAP junctions, even if these KNDy neurons do not express NK3R. This forms the synchronized activity of KNDy neurons which is induced by NKB-NK3R signaling.

Dynorphin synthesized by a KNDy neuron binds to κ-opioid receptor (KOR) on a neighboring KNDy neuron and can cause its inhibition. Each oscillation mediated by NKB/NK3R and dynorphin/KOR is thought to induce a pulsatile release of kisspeptin [33]. It is worth noting that the dynorphin/KOR system in KNDy neurons ensures negative communication through sex steroids estrogens, progesterone and testosterone [36]. These findings suggest that in *Bos taurus*, KNDy neurons in the ARC function throughout the estrous cycle and regulate follicular development and corpus luteum function through tonic release of GnRH, whereas the second large population of kisspeptin neurons found in the POA during follicular phase may be involved in the preovulatory rise in GnRH/LH concentrations [22].

Data from I.J. Clarke et al. [37] provide indirect evidence that kisspeptin-producing neurons are more active in the ARC. In cows with spontaneous ovarian cycles, *kiss1* expression in the arcuate core is almost 2 times higher compared to cows with various problems in cyclicity [37].

A. Hassaneen et al. [22] indicated kisspeptin-immunoreactive cell bodies and nerve fibers in the supraoptic ridge of the OVLT (organum vasculosum laminae terminalis). This is of interest because a dense cluster of GnRH-producing neurons and fibers has previously been found in the OVLT [29]. Ihis work also documented immunoreactive fibers in the PVN (paraventricular nucleus), DMH (dorsomedial hypo-thalamic nucleus), VMH (ventromedial nucleus), and LaH (lateral hypothalamus) [22]. Notable is the accumulation of immunoreactive fibers in the outer zone of the median eminence, indicating that kisspeptin is secreted into the portal vasculature beyond the blood-brain barrier. The inner zone contains numerous fibers that allow interaction with neuronal fibers also secreting into the portal vessels [38].

Similar data on OVLT were obtained by C.E. Leonardi et al. [39]. They studied the distribution of GnRH and KP neurons in the hypothalamus during the reproductive cycle in cows. The main clusters of KP neurons were founs in the POA, ARC, and OVLT [39]. Noteworthy is the fact that the largest number of KP neurons was recorded in the POA in the luteal phase (metestrus), and the smallest in diestrus. This is consistent with data that increased progesterone, which is characteristic of the diestrus phase, causes the synthesis and secretion of dynorphin that reduces Gn-RH production [40].

Increased levels of dynorphin in response to stress and exercise may suppress GnRH/LH secretion [36] (see Fig. 2). Stress factors (heat, transportation, veterinary manipulations) disrupt reproductive function by reducing the secretion of GnRH not only through dynorphin. This occurs in part due to cortisol acting through the type II glucocorticoid receptor. In this case, GnRH neurons do not express the receptor, but KNDy neurons are capable of the expression.

The examples with dynorphin and cortisol further prove that the hypothalamic control of fertility depends on various external factors. In cattle, one of the most important factors is the energy status of the animals. GnRH neurons lack many nutrient-dependent metabolic hormone receptors, implying the existence of neurons presynaptic to GnRH neurons. KP neurons express receptors not only for steroids, but also for metabolic hormones such as insulin, leptin, and ghrelin [41]. It can be assumed that KP neurons may be key for coordinating the energy state of animals with their reproductive function [23, 38, 42, 43].

Blood kisspeptin content in *Bos taurus*. Currently, there are very few research works on the content of kisspeptins in the body of cattle. This is explained by the complexity of experiments and the lack of kits necessary for assay. There are practically no enzyme immunoassay kits for various kisspeptins (KP-53, KP-14, KP-13, KP-10), whereas radioimmunoassay, due to its specificity, is not always available. An alternative is to quantify the kisspeptin prohormone for which commercial kits are available.

One of the few studies assessed the content of KP-10, progesterone and estradiol in dairy cows on days 10, 12, 14 and 16 after calving [44]. The KP-10 content was 116.99±35.29, 114.86±27.34, 149.5±36.67 and 124.69±42.76 pg/ml, respectively, whereas progesterone levels remained practically unchanged, approximately 0.35 ng/ml, and the estrogen concentration was 0.92±0.34, 1.33±0.4, 1.81±0.37 and 1.78±0.33 ng/ml, respectively. It can be noted that the maximum amounts of KP-10 coincided with an increase in estradiol concentrations. The authors concluded that an increase in estrogen levels may, by a positive feedback, enhance the synthesis and secretion of hypothalamic KP-10 [44]. This conclusion is confirmed by A. Rizzo et al. [45]. They recorded a significant increase in KP-10 duering post-calving period (80±15 days) in cows with follicular cysts compered to healthy cows, 125.06±34.47 vs. 97.72±21.34 pg/ml. In addition, estradiol and progesterone concentrations also increased almost 2-fold [45].

The effect of kisspeptin on the reproductive organs. A large number of publications postulate that the main function of the KP is realized in the hypothalamic regulation of the HPG axis. However, data have emerged proving their regulatory role in the functions of the ovaries, embryonic trophoblast and placenta [46], which requires more in-depth study.

H. Liu et al. [47] found expression of KP-10 in the cow's preantral follicles, i.e., in the oocytes, granulosa and theca cells, with maximum expression in the latter. This is of interest because in cattle, most follicles growing in vivo gradually become atretic during the growth phase that begins with the formation of preantral follicles. There was a dose-dependent negative effect of KP-10 on follicular growth, up to and including atresia, and it has been suggested that KP suppresses the expression of the FSH receptor [47].

During the follicular growth stage, the development and maturation of oocytes depends primarily on their connection with granulosa cells. H. Liu et al. [48] demonstrated that KP-10, by inhibiting proliferation, promotes the initiation of apoptosis in ovarian granulosa cells in cows. KP-10 may also have a slightly different effect that is not associated with cell apoptosis. Some authors note the ability of KP-10 to reduce the expression of one type of microRNA, the miR-1246 which inhibits synthesis of progesterone by granulosa cells in follicles [49].

Perhaps the inhibitory effect of KP is associated with the need to stop the appearance of new preovulatory follicles. In the early stages of embryogenesis, the influence of kisspeptin can be stimulating. M.M. Soares et al. [50] studied the effect of KP in in vitro culture of embryos and revealed that the KP-10 added to the medium increased the rate of blastocyst formation.

The expression of *kiss1*/KISS1R during placentation which has species differences is also of interest. In cows, implantation is a less invasive process. During synepithelial-chorionic placentation in ruminants, the trophoblast does not penetrate the basement membrane and stroma of the uterus, but forms villous outgrowths in the endometrial epithelium. Based on this, M.J. D'Occhio et al. [46] suggested an influence of fetal trophoblast *kiss1* expression on uterine KISS1R. The gene may influence the initial attachment of the trophoblast to the endometrial epithelium of the uterus through interaction with intercellular adhesion molecules and extracellular matrix proteins. Therefore, in livestock, the *kiss1*/KISS1R

system may play a lesser role.

The *kiss1*/KISS1R system is also involved in the regulation of placenta development in cows. N.A. Martino et al. [51] demonstrated that bovine epithelial cell cultures derived from first-trimester pregnant cows expressed KISS1R [51]. The KP-10 added to cultures both stimulated and inhibited epithelial cell proliferation in two separate cell lines. Stimulation occurred in a cell line with overregulation of KISS1R mRNA. This is especially important since the failure of ruminant embryos to attach and implant is one of the main causes of declining reproductive capacity [46, 52].

Exogenous kisspeptin regulation of reproductive functions in *Bos taurus*. Since blood KP in cattle is difficult to of measure, researchers almost immediately drew attention to testing KP by exogenous administration and analysis of its effects. However, studying these effects in cattle is still problematic due to the lack of drugs intended for the *Bos taurus* species. Most studies have used human or mouse KP-10. Considering that KP-10 is the most similar in its amino acid sequence in almost all mammalian species, this peptide is most often used.

However, note that different KP-10 may make adjustments to the data obtained. This was shown by C.E. Leonardi [53]. A single bolus or multiple intravenous injections of human KP-10 more effectively increased blood plasms level of LH compared to similar treatment with murine KP-10 [53]. Because of small number of reports on the effect of exogenous KP on *Bos taurus*, in the review we combined data from publications where various kisspeptins were used.

In this regard, the first experiments with cell cultures of the adenohypophysis of calves and adult animals are of interest in order to detect the effect of KP-10 on the content of various hormones [54-56]. A dose-dependent effect of exogenous KP-10 on increasing the amount of LH and growth hormone in the cells of the adenohypophysis, regardless of age, has been revealed [54, 55]. A.A. Ezzat et al. [56] studied the effect of KP-10 on the secretion of LH, FSH and prolactin by cells of the anterior pituitary gland of cattle, assessing the ability of sex steroids to enhance the sensitivity of gonadotropic and lactotropic cells to KP-10. KP-10 significantly stimulated LH secretion in cells treated with estradiol and testosterone, but not in cells treated with progesterone. In contrast, KP-10 did not affect FSH secretion regardless of steroid use. KP-10 significantly stimulated prolactin secretion, but no effect of sex steroids was detected. These results suggest that estradiol and testosterone increase the LH secretion by gonadotropic cells in response to KP-10. KP-10 directly stimulates the secretion of prolactin by pituitary cells, and sex steroids do not increase the sensitivity of lactotropic cells to KP-10 [56].

The results of the described experiments allow us to conclude that the stimulating effect of kisspeptin on LH secretion in cattle is expressed at least in two ways, via GnRH neurons with its secretion in the POA or ARC (cell bodies, dendrites or dendrons) and by direct stimulation of LH secretion in the pituitary gland. This may influence the generation of GnRH/LH pulses as well as the preovulatory GnRH/LH surge. In other animal species, a third way is found, that is, kisspeptin from KNDy neurons located in the ARC can act on the terminals of GnRH neurons in the median eminence, but similar experiments have not been performed in cattle [57, 58].

Stimulation activity of kisspeptin has been demonstrated in studies of injections of KP-10 and KP-53 in cows and heifers of different breeds and ages [59-61]. In most cases, kisspeptin injections resulted in a surge in LH concentrations as well as an increase in growth hormone concentrations [62-64]. However, the KP-10 was less effective compared to exogenous GnRH, whereas injections of KP-53 provided LH levels sufficient for ovulation [59, 63, 65].

A.E. Ahmed et al. [62] assessed the inhibitory effect of progesterone on

the ability of kisspeptin to increase LH concentrations. For 7 days, cycling cows were treated with a progesterone releasing intravaginal device (PRID). Animals received a single intravenous injection of KP-10 for 3 days after removal of the device. Blood progesterone concentration was higher on the date of PRID removal, followed by a decrease on days 1 and 2. KP-10 did not significantly alter plasma LH concentrations at the date of PRID removal. However, KP-10 significantly stimulated the release of LH in the following days. The authors concluded that KP-10 stimulates LH release in cycling postpartum cows, and high plasma progesterone concentrations may reduce the effect of kisspeptin on LH secretion [62].

In almost all works on the FSH level as influences by kisspeptins in cows of different breeds and ages, the concentration of FSH remained virtually unchanged. However, there are the opposite data. M.A. Rodríguez et al. [66] studied the effectiveness of KP-10 administration. In calves aged 4, 7 and 11 months intravenous administration of KP-10 increased FSH levels.

In male cattle, the positive effect of kisspeptins on FSH concentration is more often recorded. Several studies have shown that a single intravenous injection of KP-10 significantly stimulated the release of LH and FSH in calves and bulls [67, 68]. S.L. Northup et al. [69] studied the effect of intravenous administration of KP-10 to bulls during puberty on the blood LH and FSH concentrations. Acute intravenous infusion of KP-10 increased blood LH and did not change FSH. Chronic intravenous infusion had no effect on LH but decreased the blood FSH mean concentration and the amplitude of its pulsatility [69].

Summarizing the data on the exogenous administration of kisspeptins, we can note their stimulating effect on the LH synthesis and secretion. With regard to growth hormone, the effect of KP-10 is determined by the age of the animals, which may explain the positive effect of steroid hormones (progesterone and estradiol). On the effect of kisspeptins on FSH, the data are contradictory and require clarification, although in most reports the synthesis and secretion of FSH were less susceptible to stimulation. The explanations may be the greater resistance of FSH to pulsed GnRH secretion and the suppressive effect of inhibin A and B on FSH secretion [36, 70].

Interest in the exogenous (injection) administration of kisspeptins is obvious, since there is the possibility of controlling the sexual cycle through control of the secretion of GnRH and gonadotropins [71]. For example, M. Mondal et al. [72] showed that the kisspeptin-based synchronization protocol induced better follicular growth than the ovsynch (ovarian synchronization protocol). The frequency of ovulation was significantly higher, and luteolysis began earlier, even before the injection of prostaglandins [72].

However, in general, most researchers still have questions about the injection of kisspeptins to control ovulation, especially since endogenous peptides have clear limitations. For example, the half-life of KP-10 in the bloodstream is less than 1 min, and in most cases the desired effect can only be achieved by perfusion (intravenous or intracerebroventricular) or hourly injection. The use of intracerebroventricular or intravenous perfusion in agricultural settings is difficult to implement, and multiple intravenous injections require increased veterinary costs. Not surprisingly, researchers have become interested in using synthetic analogues of kisspeptins that have higher activity and longer half-lives [73].

For example, P.A. Parker et al. [74] studied the effect of a synthetic analogue of kisspeptin called Compound 6 (C6) on the concentrations of LH, FSH, and testosterone in prepubertal bulls. The in vitro activity of C6 was approximately 8 times higher than that of KP-10. It was shown that intramuscular administration of C6 increased the concentration of LH without changing the content of FSH

and testosterone in the blood. The work of C.R. Burke et al. [75] is another example of using TAK448, a synthetic analogue of kisspeptin. The authors concluded that kisspeptin treatment, if performed when there is a mature dominant follicle in the ovaries, can induce ovulation in postpartum dairy cows followed by estrous cycles of normal length [75].

S.M. Popa et al. [76] using genetically engineered mice that exhibited only 5% KISS1 expression, found that both males and females reached puberty and remained fertile. Y. Uenoyama et al. [3] confirmed this phenomenon in rats, finding that 20% of KNDy neurons are sufficient to support GnRH pulses and folliculogenesis, indicating functional redundancy of the KNDy neuron population. The authors pointed out that there is not yet enough information about the functional redundancy of KNDy neurons in mammalian species other than rodents [3].

Although much has been learned since the initial identification of KP-producing KND neurons, there are still many unresolved questions regarding the function of different populations of these nerve cells depending on location. Another aspect is new technologies for studying KND neurons, including KND neurons of *Bos taurus*. It is necessary to analyze the effect of different concentrations of kisspeptins on ovulation in cattle. Importantly, there is a problem that has not yet been solved even in human medicine. M.A. Hussain et al. [77] noted that commercially available methods for quantifying blood kisspeptin in various mammals are not reliable enough due to large differences in assay methods, detection ranges, and uncertainty about which forms of kisspeptin (e.g. KP-10, KP54) are detected [77]. Thereof, methods for measuring kisspeptin concentrations in biological fluids should be improved.

Thus, the effect of kisspeptin on the reproductive function of *Bos taurus* is of interest. Despite accumulated information on the issue, the data are mostly obtained upon exogenous administration (injection) of kisspeptin-containing drugs and measurements of the resulting hormonal levels in animals. There is an obvious lack of information about the natural levels of kisspeptin in young animals, the concentration of kisspeptin in the blood of cycling and pregnant cows, and the relationship between kisspeptin concentrations, reproduction and productivity. The available information is often obtained for other animal species. Kisspeptins used in experiments on cows are in most cases non-species specific or synthetic. In general, despite the fact that the role of kisspeptin in reproductive function has been established long ago, this hormone is interesting for further study, provided that the necessary tools for accurate detection and measurement of its concentrations become available.

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LIPOPHILIC PROPERTIES OF PESTICIDES: BIOACCUMULATION AND BIOMAGNIFICATION IN ANIMALS, THE TOXICITY FORECASTING

(review)

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Abstract

The problem of pesticides contamination save actuality because of the growing demand for food and multi-factorial processes of their biotransformation and bioaccumulation in living organisms. As of July 11, 2023, more than 1,200 approved insecticides, acaricides and herbicides have been registered in Russian Federation (excluding fungicides, rodenticides, repellents, desiccants, plant growth regulators, microbiological and biological pesticides, etc.), many of them are included in the list of particularly dangerous according to PAN data, for example, diazinon, chlorpyrifos, dimethoate, imidacloprid, malathion, spinosad (PAN List of HHPs, 2021). Their uncontrolled using results the accumulation of parent compounds, metabolites and degradation products in soil, water, plants, and animals and the subsequent biomagnification of persistent pollutants at higher trophic levels (V.P. Kalyabina et al., 2021; C.M. Volschenk et al., 2019; Z. Zhang et al., 2019). Pesticides have an adverse effect not only for target pests, but also on the crops, soil microbiota, natural ecosystems objects and humans. Biopesticides are safer, but at the same time, their high selectivity becomes a disadvantage in solving several agrotechnical objectives (W.-H. Leong et al., 2020; De O.H. Gomes et al., 2020). The absorption, distribution, and transport of pesticides in biological systems are determined by their lipophilicity (T. Chmiel et al., 2019; R. Beiras, 2018; S.-K. Kim et al., 2019). High lipophilicity generates conditions for high metabolic clearance of compounds. The biological activity of substances in the organism could be predicted by logP which describes their affinity for target proteins (T. Chmiel et al., 2019), where P is the distribution coefficient showing the ratio of the compound concentrations in two immiscible phases at equilibrium state. The extremely lipophilicity of pesticides (logP > 5) can result to their binding to hydrophobic targets, which provides non-selectivity and higher toxicity (C. Olisah et al., 2021). Insufficient data has been obtained on the metabolism and bioaccumulation of pesticides in farm animals and synergistic effects in real conditions by this time. The distribution of pesticides in soil, ground and surface waters depends not only on their lipophilicity, but on pH, temperature, the initial amounts of preparations, organic and inorganic substances content, solids sorption properties (S.D. Burlaka et al., 2019; S. Hintze et al., 2021; F.A.P.C. Gobas et al., 2018). The accumulation of pesticides in the soil results the decreasing of the involved in the circulation of elements and organic substances degradation soil microorganism activity and can be the biological indicator of ecosystems pollution. Generally, the levels of pesticide residues in environment are measured by gas, high-performance and ultra-high-performance liquid chromatography, enzyme immunoassay and capillary electrophoresis (A. Samsidar et al., 2018; S. Hintze et al., 2021; L. Fu et al., 2018). Gas chromatography is appropriate for volatile and thermally stable compounds, while high-performance liquid chromatography is more relevant for non-volatile and polar compounds. A combination of chromatographic separation with high-resolution mass spectrometry could be required for non-targeted analysis that allows the not detected in the target study compounds identification and determination. The search for safe plant protection substances and forecasting of their toxicity, bioaccumulation processes in environment and the transfer through food chains, is possible using a combination of two approaches.

These are «non-targeted search» and modern QSAR mathematical models. The «non-targeted search» allows both targeted and non-targeted analysis of pesticides and their metabolites, and QSAR models are based on the correlation of physicochemical, particularly lipophilic properties of molecules and their effects on living organisms (A. Speck-Planche, 2020; N.A. Ilyushina, 2019; O.G. Columbin, 2020).

Keywords: pesticides, lipophilicity, bioaccumulation, environmental pollution, toxicity, microbiome

The need for food is growing worldwide. According to UN forecasts, over the next 30 years the world's population will increase by 2 billion people, reaching 9.7 billion by 2050, and 11 billion by 2100 [1]. Despite growing organic agriculture, approximately a third of the world's crops are produced using pesticides [2].

Pesticides can have adverse effects not only on target pests, but also on crops themselves [3] and on soil microbiota [4]. Farm animals are exposed to pesticides through contaminated feed and water [5-7] and through direct contact, e.g., through inhalation of polluted air near agricultural land. Thereof, even low concentrations of chlorpyrifos, dichlorophenyl dimethylurea and their mixtures negatively affected the physiological and biochemical parameters of chickens [8]. There was a decrease in acetylcholinesterase activity, changes in the content of liver biomarkers (alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase) and renal biomarkers (total protein, creatinine, uric acid and urea). The consumption of poultry meat in the world is steadily growing, and the accumulation of toxicants in livestock products can pose a threat to human health [6, 8, 9]. Pollution of environment with pesticides also concerns plant-pollinating insects [10, 11], including honey bees (*Apis mellifera*), which provide a significant increase in crop yields [12-14].

With prolonged use of pesticides, pests are able to develop resistance to their active ingredients [15-17]. In addition to the increase in the number of resistant arthropod species, there is the development of multiple resistance to toxicants of different chemical classes [18]. Excessive use leads to the accumulation of these compounds, their metabolites and decomposition products in soil, water, plants and the body of farm animals. Possible negative effects of bioaccumulation include the development of cancer pathologies [19], reproductive dysfunction, immunological, endocrine, neurodegenerative disorders [20, 21], birth defects and respiratory disorders [22-24].

In the Russian Federation, state registration of pesticides and agrochemicals is currently governed according to the approved procedure (Order of the Ministry of Agriculture and relevant administrative regulations No. 442 dated July 31, 2020, as amended on January 19, 2022). State registration includes tests of a pesticide or agrochemical, an examination of regulations for use, state registration and issuance of the certificate, inclusion in the State Catalog of Pesticides and Agrochemicals approved for use in the Russian Federation. At the beginning of 2020, the state catalog contained 652 drugs (separate and mixed according to the active ingredients) [25]. As of July 11, 2023, more than 1,200 approved insecticides, acaricides, and herbicides (excluding fungicides, rodenticides, repellents, desiccants, plant growth regulators, microbiological and biological pesticides) have been registered [26]. Moreover, as of March 2021, some of them according to the criteria of the Pesticide Action Network were included in the PAN International List of Especially Hazardous Pesticides, e,g., diazinon, chlorpyrifos, dimethoate, imidacloprid, malathion, spinosad, etc. [27].

The purpose of this review is to analyze the properties of pesticides that determine their sorption, distribution, bioaccumulation and toxicity for biological systems, stability and biodegradation in the environment, in order to predict the biological activity of drugs developed on their basis.

The most dangerous organochlorine pesticides for living beings, e.g.,

dichloro diphenyl trichloromethyl methane (DDD), hexachloro cyclohexane (HCH), aldrin, hexachloro benzene, endrin, heptachlor, chlordane are prohibited in Russia, as in most countries of the Northern Hemisphere. However, they continue to be used in Southeast Asia and, due to their extreme persistence, are capable of migrating to other regions by water circulation and sea currents. In the work of V.Y. Tsygankov [28], HCH isomers, DDD and its metabolites were detected in all marine organisms collected in the Sea of Japan, Bering and Okhotsk from 2000 to 2017.

The two main groups of pesticides are chemicals and biopesticides [19]. Chemicals are mainly synthetic compounds. Biopesticides are produced from natural sources (animals, plants, bacteria, some minerals) [2]. Chemical pesticides, based on their origin, are divided into a large number of groups and subgroups. The most common are organochlorines (OCs), organophosphates (OPs), carbamates, pyrethroids, benzimidazoles and triazoles. Biopesticides are divided into microbiological (bacteriophages, bacteria, yeast and fungi; *Bacillus thuringenesis* is most often used); biochemical (natural non-toxic substances, for example sex pheromones of insects, plant extracts) and built-in plant protection agents produced by plants [19]. Biopesticides are safer for animals and selective to target pests, which is both their advantage and disadvantage when solving several agrotechnical problems simultaneously [19, 29].

Based on the target organism, pesticides are classified as herbicides (kill plants), insecticides (kill insects), fungicides (kill fungi), zoocides (kill warm-blooded animals), nematicides (kill nematodes), molluscicides (kill shellfish), algaecides (kill algae), bactericides (kill bacteria, although they are generally considered among fungicidal agents) [30, 31]. The first three groups are the most representative; within them, there are additional subgroups.

The World Health Organization (WHO) classifies pesticides according to the acute toxicity value (LD_{50}, peroral, mg/kg), Ia stands for extremely dangerous (< 5), Ib for very dangerous (5-50), II for moderately dangerous (50-2000), III for slightly dangerous (> 2000), IV for unlikely to pose a danger (5000 or more) [19, 32]. The United States Environmental Protection Agency (EPA) identifies four hazard classes [20]. The hygienic classification of drugs adopted in the Russian Federation includes four hazard classes by average lethal dose when administered into the stomach (mg/kg), the extremely dangerous (≤ 50), highly dangerous (51-200), moderately dangerous (201-1000) and low hazardous (>1000) [33]. In addition, not only acute toxicity are taken into account, but also allergenicity, teratogenicity, embryotoxicity, reproductive toxicity, mutagenicity and carcinogenicity, as well as persistence in soil.

It is not possible to prevent pesticides from getting into the soil, water, air and plants, so a search is underway for new, effective, safe for humans, environmentally friendly plant protection products, and responsible handling of pesticides of earlier generations is being practiced [2, 3, 16, 34].

Properties of pesticides that determine sorption, distribution, bioaccumulation and toxicity for biological systems. Metabolism, biological activity and bioaccumulation of pesticides in the organs and tissues of farm animals are primarily determined by their structure and, thereof, by physicochemical properties. Pesticides in the body bind to plasma proteins, blood cells, lipids and are distributed in various organs and peripheral tissues [35]. The absorption, distribution, and movement of chemical compounds in biological systems depend on their lipophilicity [35-38]. Lipophilicity determines the ability of xeno- and endobiotics to undergo metabolic transformations and the affinity to target proteins [36]. The biological activity and toxicity of compounds depends on their lipophilic properties [35, 39]. Accordingly, their optimization in the

new pesticide formulations can help in identification of structures that determine toxicity and biodegradation potential.

The soil is capable of retaining pesticides, as a result, the correct biochemical pathways in biogeochemical soil cycles is disrupted and the activity of microorganisms that play a key role in the cycling of elements and the decomposition of organic matter is reduced [17]. The enzymatic activity of soils, which characterizes its biological activity, is highly sensitive to external influences. A decrease in the activity of soil microorganisms can serve as a biological indicator of ecosystem pollution [4].

Soil microflora is highly sensitive to triazoles; the maximum toxic effect occurs in the initial period after their application; recovery can take up to 10 weeks [40]. A toxicity study of malathion (organophosphorus pesticide, OPP) demonstrated inhibition and reduction in the number of *Bacillus* spp., *Pseudomonas* spp., *Arthrobacter* spp., *Azotobacter* spp., *Flavobacterium* spp. and *Penicillium* spp. already at 1PC (pesticide concentration) used, at 2PC, *Pseudomonas* spp. and *Bacillus* spp. become dominant species, and at 5PC the death of the entire microenvironment occurs [41]. The negative effect of the insecticide is associated with the phosphorus and sulfur atoms, and with the morphological features and susceptibility of microorganisms.

Lipophilicity, namely the degree of hydrophobicity, determines the distribution of compounds between the aqueous and organic phases. It is characterized by the separation (distribution) coefficient P which shows the ratio of the concentrations of a compound in two immiscible phases at equilibrium. However, other solubilizing media, such as biomembranes, can also participate in this distribution [36, 37]. The logP value predicts the biological activity of substances in the body because it describes their affinity to target proteins [36].

The metabolism of pesticides, like other xenobiotics, may involve one or two stages. In the first stage, a polar group is introduced into the molecule with the participation of predominantly cytochrome P450-dependent monooxygenases and, to a lesser extent, flavin-containing monooxygenases and hydrolases [36]. In the second stage, when uridine-5-diphosphate glucuronosyltransferase, sulfotransferase and glutathione-S-transferase introduce much larger substituents (sugars, sulfates or amino acids), a significant increase in water solubility of the compound occurs [36]. Enzyme binding sites are primarily lipophilic and readily accept lipophilic pesticide molecules.

Organochlorine pesticides are highly lipophilic, persistent, toxic, and bioaccumulative [42]. The most dangerous are 4,4-dichlorodiphenyl trichloromethylmethane (DDT) and γ-hexachlorocyclohexane (γ-HCH, lindane), logP 6.91 and 3.72, respectively [43]. They can persist in soils for many years, are resistant to degradation, have estrogenic and carcinogenic properties, and are banned for use in most countries [2, 44]. Organophosphate pesticides, carbamates and pyrethroids are now more widely used due to their lower persistence than in organochlorines. However, they cannot be unambiguously classified as low-hazard compounds, since some of them form highly toxic products during transformation, such as 3,5,6-trichloro-2-pyridinol in the case of chlorpyrifos [4, 7, 45].

Through risk assessment and review of short- and long-term effects, many of the registered pesticides are classified as prohibited. For example, in 2018, it was shown that the use of sulfoxaflor (registered in 2007, a group of sulfoximines), as well as some members of the neonicotinoid group (imidacloprid, thiamethoxam and clothianidin) [13, 29, 46] leads to a decrease in the number of pollinating bees [47]. Chlorpyrifos, cypermethrin, and thiacloprid have been shown to cause endocrine disruption in honey bees [48]. Chlorpyrifos causes the strongest expressional changes, i.e., the induction of marker genes for MRJP2 (*mrjp*2), MRJP3 (*mrjp*3) (major royal jelly proteins), ILP1 (*ilp1*) (insulin-like peptide), HBG3 (*hbg3*) (alpha-glucosidase) and Sima (*sima*), and down-

regulation of *buffy*.

Many factors influence the persistence and biodegradability of pesticides in ecosystems. The distribution in soil, groundwater and surface water depends on the soil characteristics, the content of organic and inorganic substances and the sorption properties of solid particles [3, 36, 49], the structure of the compound which determines its water solubility, pH, temperature, and the amount of applied preparations (in accordance with the seasonality of agricultural activities) [35, 50, 51]. The distribution of compounds between the water environment and soil, as well as bioaccumulation, depends on lipophilicity (hydrophobicity) [35, 36, 51]. The increase in phytotoxicity of pesticides depends on the soil water regime which determines the migration of compounds deeper into soil horizons [3].

It is known that OC pesticides are more stable in an acidic environment than in an alkaline environment, and the organic compounds present in the soil bind most pesticides into water-insoluble forms, so they are not hydrolyzed and become practically inaccessible to microorganisms [3, 49, 52]. For example, a study of the prohibited γ-HCH using the C^{14}-lindane model showed that the amount of the bound form in the soil increases over time, and the bulk is localized mainly in the 0-3 cm upper layer [3]. The accumulation of pesticides that are resistant to decomposition in the reproductive organs of plants leads to their entry into beekeeping products with nectar and pollen [9]. The use of surface water for agricultural purposes (irrigation and livestock farm water supply) can also facilitate recycling of pesticides.

When pesticides are washed out of the soil and from the surface of treated plants, they enter water sources which negatively affects the condition of living organisms [11, 24, 53]. Fish can be a bioindicator of pollution [53]. Bioaccumulation of glyphosate and its metabolite aminomethylphosphonic acid (AMPA), bifenthrin, azoxystrobin and cyproconazole has been observed in fish living in water in rice fields [54]. Bifenthrin was detected in all fish after fumigation, it has the greatest bioaccumulation potential of the four compounds studied, is stable and is characterized by high persistence and bioavailability. For lipophilic OC pesticides, species-specific accumulation in fish organs and tissues was observed, correlating with lipid content [42]. However, bioaccumulation is influenced by metabolic processes. Detoxification of pyrethroids occurs through oxidation by cytochrome P450 and subsequent hydrolysis catalyzed by carboxylesterase [54]. The presence of aromatic amino acids in the active site of acetylcholinesterase creates a hydrophobic region, and due to the lipophilicity of pyrethroids, they can interact with the active site, causing inhibition of enzymatic activity. Aquatic organisms lack the carboxylesterase, so the destruction and excretion of pyrethroids in fish is reduced. Acetylcholinesterase activity in fish brain was significantly reduced, and although it is considered a specific neurotoxic biomarker of PO pesticides and carbamate exposure, several studies have reported similar effects for triazoles, pyrethroids, and glyphosate [54].

When xenobiotics enter the animal's body with feed and water, the intestinal epithelial membrane serves as the main physiological barrier to transport the toxicant into the bloodstream [36]. Passive transport by diffusion across lipid membranes in transcellular and paracellular mechanisms is the dominant route for lipophilic molecules. Penetration of xenobiotics into the central nervous system also occurs through passive diffusion, and, as a rule, higher lipophilicity allows for easier passage of the blood-brain barrier. Too high a lipophilicity ($\log P > 2.8$), on the contrary, can lead to increased nonspecific binding to plasma proteins (36). Compounds that are excessively lipophilic ($\log P > 5$) tend to bind to hydrophobic targets, increasing the risk of nonselectivity and toxicity [39].

As already noted, OC pesticides are stable and capable of bioaccumulation; they are cytotoxic to both higher animals and microorganisms. Despite the ban on their use, their circulation in agroecosystems continues. According to research by the Institute of Water and Environmental Problems of the Siberian Branch RAS (Barnaul), in areas of former storage and use of pesticides in the Altai Republic, there are pockets of intense contamination with DDD and HCH of soils and associated natural environments (surface waters, bottom sediments and plants) [55]. Moreover, in feed and food plant and animal products from contaminated areas, residual pesticide contents exceeding their maximum permissible levels (MPL) are sometimes still found. In addition, OC pesticided are capable of being transported over unlimited distances with air and water, and their transfer to higher trophic levels leads to biomagnification [56-58]. Wild animals also suffer from contamination from organochlorine compounds used in agriculture [29, 58, 59]. The use of lipid reserves leads to the redistribution of toxicants in the body of migratory birds and has a negative effect during the breeding season [29, 59]. The biomagnification of OC pesticides is due to their high lipophilicity. When additional links are included in the food chain, such as predatory fish, carnivorous mammals, or humans, the dose of pesticides entering the body can increase many times over [35, 60].

The metabolism and bioaccumulation of pesticides in the body of farm animals, as shown by an analysis of the literature for 2017-2022 (PubMed, PubChem, ScienceDirect and eLibrary), is not given enough attention, and only a few works are devoted to the effects of their combinations [61]. The results of in vivo studies on laboratory animals (mice, rats, dogs, rabbits) and in vitro on lymphocyte cell cultures (human and animal), L8824 (grass carp liver hepatocytes), Neuro-2a (mouse neuroblastoma) and C6 (rat glioma induced in vivo by N-nitrosomethylurea) [62-64]. Synergistic interactions between pesticides and the possible increase in toxicity for living organisms when combined, even if the current standards for each of them separately are not violated, are also not sufficiently studied at present. For example, for bees, existing risk assessment procedures are designed primarily for individual compounds and do not take into account real-world exposure to multiple pesticides simultaneously. Typically, this involves the use of fungicides and insecticides, most often combinations of triazoles or PO pestiides with pyrethroids [12, 47]. Data obtained from single studies have shown that the combined effects of pesticides on *Apis mellifera* in natural conditions can cause serious damage from additive, antagonistic and synergistic interactions. It has been established that activation of PO pesticides by cytochrome P450 can lead to a decrease in the ability to detoxify pyrethroids due to the inhibitory effect of PO pesticides on esterases. A similar mechanism has been noted for tetraconazole (triazole) in combination with pyrethroids [12]. There are not enough studies of synergy in real conditions yet. This requires a cumulative risk assessment rather than summing the effects of individual compounds [35].

Modern methods for determining the content of pesticides and their metabolites. Prediction of biological activity of new drugs. When choosing a method for determining the content of pesticides, their volatility, thermal stability and polarity are most important. Pesticides are mostly determined by classical analytical methods, e.g., gas chromatography (GC), high-performance and ultra-high-performance liquid chromatography (HPLC and UHPLC), enzyme-linked immunosorbent assay (ELISA) and capillary electrophoresis (CE) [2, 6, 19, 50, 65]. GC is typically used for volatile and thermally stable compounds, HPLC for nonvolatile and polar compounds [15, 19, 66]. Modern chromatographic methods serve as the gold standard in determining the content of pesticides and their metabolites. They are selective and highly sensitive due to

the combination of GC and HPLC with mass-spectrometric detection in MS/MS mode and HRMS (high resolution mass spectrometry) [5, 15, 67, 68]. Currently, UHPLC and HPLC–HR-MS have become the most effective tools for monitoring the residual content of pesticides and their metabolites in environment and food products [69].

The presence of pesticide metabolites in the environment and livestock products poses a risk due to their possible toxicity and quantities exceeding the residual content of the parent compounds, as is the case with DDT and its metabolites dichlorodiphenyldichloroethane (DDD) and dichlorodiphenyldichloroethylene (DDE). DDT is metabolized and accumulated in high-fat foods. In eggs, the total concentrations of DDD and DDE may exceed the DDT initial concentration [70]. Analytical techniques are targeted at the most relevant, frequently detected and well studied metabolites, while other presumed transformation products in food samples are underestimated. Moreover, when assessing health risks, the ability of a chemical to transform into more toxic products is one of the main criteria for identifying priority chemicals unintentionally present in foods [71).

In the environment, pesticides can undergo degradation, that is, metabolic degradation by microorganisms, plants and animals and abiotic degradation in chemical reactions such as hydrolysis and photodegradation. Thereof, thousands of new compounds with unknown toxicity, stability, or bioaccumulation susceptibility may be generated [72]. For example, in Russia, 12 drugs are registered for the treatment and prevention of varroatosis of honey bees, containing amitraz as active ingredient [73]. Such drugs are not approved for use in Switzerland. Amitraz degrades very quickly, and three major degradation products identified are N-(2,4-dimethylphenyl)-N-methylformamide, N-(2,4-dimethylphenyl)-formamide, and 2,4-dimethylaniline [74]. In particular, (+)-trans-chrysanthemum acid (the main metabolite of λ-cyhalothrin), 2-hydroxy-4-methoxybenzophenone (a metabolite of deltamethrin), methylmethoxylic acid (a metabolite of metalaxyl), and 3-phenoxybenzoic acid (metabolite of λ-cyhalothrin and deltamethrin) were detected in crops treated with pesticides [72]. 2,4-Dimethylaniline is highly toxic to aquatic organisms [75]. Some neo-nicotinoid metabolites have also been found to exhibit toxicity equivalent to or greater than that of the parent compound [76, 77].

HPLC–MS/MS is primarily suitable for targeted analysis because the spectral information is relevant only for analytes within the scope of a particular technique. Non-targeted analysis detects compounds not identified in a targeted study. Untargeted analysis by HPLC–MS/MS is only possible if the number of analytes is small. The combination of chromatographic separation with HRMS is a better tool for a non-targeted approach, as it provides a complete mass spectrum with m/z peaks for all analytes in the sample. Developed “untargeted search” techniques allow simultaneous targeted and untargeted analysis of samples to identify both pesticides and their metabolites [72, 78-81].

Sample preparation is the most important and limiting stage of analysis when quantifying pesticides and their metabolites due to the differences in physicochemical properties of the substances. The least labor-intensive procedure for complex matrices (plant and livestock products, soil) remains the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) dispersive solid-phase extraction method [19, 23, 42, 69, 82]. In addition, analytical tasks, for example, monitoring a specific object suh as water, air, soil, food raw materials or finished food products, risk assessment, control of wastewater treatment plants, toxicological examination, arbitration analysis to confirm a previous semi-quantitative result, determine the methods for preparing samples and their examination [14, 23, 66, 83, 84]. Screening methods are useful for a large set of routine samples, but detection of a pesticide will require quantitative confirmation.

Due to the growing need for novel pesticides wirh less toxicity and potential danger to farm animals and humans, various mathematical models re increasingly being used that are based on the relationship between biological activity and physicochemical properties of compounds and prediction of their toxicological parameters and effects. An example is mathematical models QSAR (quantitative structure-activity relationship, quantitative structure-property relationship). S. Hansch [36] was the first to use mathematical methods for correlation studies of the physicochemical properties of molecules and their biological activity. QSAR modeling has found widespread application in biomedical chemistry [85-87]. Models with different lipophilicity descriptors have been developed to address biological, pharmaceutical, and environmental applications [36, 88]. Most of these include lipophilicity expressed as logP. Recently, several but still very few reports were published, including in Russia, on screening and predicting the toxicity of pesticides {89-91]. A preliminary study of a non-experimental (based on previously described data) screening model for lipophilicity, water solubility, toxicity, bioavailability and mutagenicity using the example of 490 pesticides and their active ingredients revealed a satisfactory agreement (83-94%) between previously described experimental parameters and calculations using QSAR models [91].

Stability and biodegradation of chemical pesticides. The stability of pesticides in soil varies and is determined, in addition to hydrophobicity and polarity, by many factors (pH, microbial activity, humidity and temperature). The time for decomposition into non-toxic components can range from several weeks to tens of years [92]. Organochlorine pesticides are resistant to decomposition in soil, which increases their accumulation in pollen and nectar of honey plants and transfer to beekeeping products (honey, royal jelly, beebread, bee pollen), although the residual quantities may not exceed the MPL [9]. Triazole derivatives can persist in soil for one year [40]. Glyphosate (a PO pesticide) quickly loses activity, and its half-life, depending on the type of soil, is from 2 to 197 days, the typical half-life in field conditions is 47 days [93], the most up-to-date information was obtained in 2010. However, the half-life of its metabolite aminomethyl phosphonic acid (AMPA) in soil ranges from 119 to 900 days [22].

Pesticide degradation occurs through hydrolysis, photolysis, and biodegradation [24]. Biodegradation can also be affected by environmental conditions, such as soil moisture. It determines not only the ratio of bound and hydrolyzed forms of compounds, but also the diversity of soil microorganisms and their activity [17, 94, 95]. The main groups of bacteria involved in biodegradation are *Bacillus*, *Pseudomonas*, *Klebsiella*, *Actinomycetes*, *Nocardia*, *Streptomyces*, *Thermoactinomycetes*, *Micromonospora*, *Mycobacterium*, *Rhodococcus*, *Flavobacterium*, *Comamonas*, *Escherichia*, *Azotobacter* and *Alcaligenes* [95]. Generally, aerobic microbial activity increases with soil moisture until it reaches a maximum and then decreases. Low soil water content reduces microbial activity, which may promote longer sorption of pesticides and less degradation in arid regions [94].

The use of old-generation chemical pesticides which are persistent and toxic to the agroecosystem requires the effective methods for their detoxification. Among the biological methods for detoxifying pesticides in soil, the stimulation of its own microbiota is still the most accessible [4, 34]. The specialized microorganisms selected from the natural environment or bred by genetic engineering are promising in laboratory testing but can cause unpredictable environmental consequences [34, 96]. In some cases, metabolization of xenobiotics may produce more toxic intermediates [35, 36, 45].

Thus, lipophilicity determines the sorption of pesticides, their distribution and toxicity to biological systems. Excessively high lipophilicity (logP > 5) entails the binding of pesticides to hydrophobic targets, resulting in non-selectivity and

higher toxicity. Lipophilic properties are considered decisive in bioaccumulation and biomagnification of pesticides in farm animals, and in further degradation of these compounds. Based on lipophilic properties, bioactivity of drugs under development may be predicted. Currently, drugs that are effective, safe for non-target organisms and easily decompose in the environment are of inerest. It is also important to improve analytical methods for monitoring pesticide residues. The combination of non-target screening of pesticides and their metabolites in soil, water, animals and livestock products using UHPLC–HR-MS and QSAR modeling (for preliminary toxicity assessment) can become an effective tool for assessing the risk to animal and human health and ensuring food safety.

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MARINE ALGAE: EVALUATION OF THE POTENTIAL FOR USE IN FARM ANIMAL DIETS

(review)

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Abstract

Currently, in the feed industry, along with energy-saving progressive technologies, non-traditional raw materials and secondary products of food industry are widely used. Processing and use of non-traditional resources at food enterprises significantly increases their profitability and reduces grain costs in compound feeds (P. Burtin, 2003). Natural components provide high-quality feeding for animals, strengthen their health and improve production performance. Currently, studies of the biological activity of algae phlorotanins are still relevant. The variety of biological properties determines their practical use, including in the production of feed additives for animals (S.B. Wang et al., 2013). An important problem is the uncontrolled use of antibacterial drugs, which can lead to the transfer of antibiotic resistance from animal to human (I.I. Kochish et al., 2019). Probiotics, prebiotics, symbiotics, organic acids, etc., serve as an alternative to feed antibiotics. These supplements are not inferior to antibiotics in effectiveness, but exclude their negative effects (I.A. Egorov et al., 2019). Seaweeds have a prebiotic effect due to the oligo- and polysaccharides and antimicrobial, immunomodulatory, antioxidant and anti-inflammatory activity due to bioactive compounds. Depending on the purpose of application and with the optimal dosage, seaweeds can positively affect animal ontogenesis, productivity and the quality of the products obtained. In poultry farming, seaweeds strengthen the immune state, reduce the microbial load in the digestive tract and improve product quality (A.M. Abudabos et al., 2012). Green algae (*Entermorpha prolifera*) contribute to better digestibility of nutrients, increase the level of metabolized energy, lead to higher egg production and a better egg quality (an increase in weight, shell thickness, change in yolk color), as well as reduce the yolk cholesterol level (S.B. Wang et al., 2013). Dried, boiled and autoclaved brown algae (*Sargassum dentifebium*, *Turbinaria conoides*, *Dictyota dentata*, etc.) in the diet of young chickens and laying hens have no adverse effects on productivity performance and feed intake while positively influence yolk coloration and the calcium content in the shell. Dietary brown algae *Sargassum* sp. reduces levels of cholesterol and triglycerides in blood and yolk in laying hens with an increase in the yolk carotene, lutein and zeaxanthin concentrations (M.A. Al-Harthi et al., 2012). Red algae (*Asparagopsis taxiformis*) in the diet of animals can positively change the microbiome of the gastrointestinal tract, increasing the diversity and abundance of beneficial bacteria (B.M. Roque et al., 2019). Thus, due to its special biochemical composition, seaweeds is promising in feeding highly productive crosses of poultry, pigs and cattle.

Keywords: algae, antibiotic resistance, intestinal microflora, immunity, probiotics, prebiotics, *Campylobacter*, polysaccharides, biochemical analysis, feed additive

Currently, in the feed industry, along with the energy-saving advanced technologies, non-traditional raw materials and secondary food production resources have become widespread, reaching 60-80%, in some cases 95%, by weight of feed. Processing of secondary resources at food enterprises significantly increases their profitability and reduces the cost for grain in the commercial compound feed [1].

In 2050, due to continued population growth and climate change, 60-70% more animal products will be needed than currently consumed [2]. Livestock production will require more feed, which will be a significant challenge given land degradation due to past intensification methods and weather conditions [3]. Expanding the feed supply through new resources or additives that improve feed efficiency can play a key role in the development of animal farming [4].

Natural feed resources are one of the most effective ways to improve animal nutrition, health, functional performance and productivity [5].

Algae which contain large amounts of bioactives and nutrients are underutilized as a crop [6-8]). In vivo studies in ruminants, pigs, poultry and rabbits show that some seaweeds can meet protein and energy requirements, while others contain bioactive compounds that enhance animal performance and health [9]. In seaweed, the protein yield per unit area is 2.5-7.5 $t \cdot ha^{-1} \cdot year^{-1}$, for soybeans, legumes, wheat 0.6-1.2, 1-2 and 1.1 $t \cdot ha^{-1} \cdot year^{-1}$, respectively. It is also worth noting that freshwater conditions and arable land are not required to grow algae [10].

The second important problem in livestock farming is the uncontrolled use of antibiotics. Along with bacteriostatic and bactericidal effects against most gram-positive and gram-negative bacteria, antibiotics have undesirable side effects, the microbiota of the gastrointestinal tract (GIT) is inhibited, immunity is weakened, and pathogens mutate and develop resistance [11]. Uncontrolled use of antibacterial drugs can lead to the transfer of antibiotic resistance from animals to humans [12], which was the reason for the ban of most of them in the United States and Western Europe [13].

Drugs that serve as an alternative to feed antibiotics include probiotics, prebiotics, symbiotics, and organic acids [14]. These additives are not inferior to antibiotics but exclude these negative effects [8]. Mariculture products, namely seaweed is such raw materials. They contain substances, many of which are absent in terrestrial organisms, and have increased biological activity [15, 16].

In this review, we summarized published data on the properties of micro- and macroalgae that determine their potential as feed resources and/or additives for farm animals and poultry, and examined practical examples.

All algae are divided into 10 sections. Of these, brown algae (*Phaeophyta*), blue-green algae (*Cyanophyta*), and red algae (*Rhodophyta*) are the main [17, 18]. Over the millions of years of our planet's history, macrophyte algae acquired perfect morphological and physiological features and adapted to changing environment, which led to their wide distribution and diverse biotic relationships with other species [19-21].

Algal fields and marine plantations have high biological productivity [21-23]. Algae fields dampen sea wave energy, limiting destructive impact on the coastline during storms [24]. Algae serve as an additional substrate on which eggs and larvae of aquatic organisms settle, and as a refuge from predators for the young of many animals [25-27]. A favorable hydrological and hydrochemical regime is created in algal rhizoids and between the plates, promoting the development of microalgae, the accumulation of detritus and the formation of a microbial film [28, 29]. Algae are biological filters for purifying polluted coastal waters near large cities. Macrophyte populations in wastewater discharge sites reach their fullest development due to nutrition improved with additional sources of nitrogen and phosphorus [30-32]. A 1 ha kelp plantation is capable of extracting about 250 kg of nitrogen from water per day. Seaweed produces a significant proportion of the world's oxygen, 80% of oxygen is produced by macrophyte seaweeds and microalgae, and only 20% by terrestrial plants [33, 34].

Macro- and microalgae. *Macroalgae.* Macroalgae are brown (*Phaeophyceae*), red (*Rhodophyceae*) and green algae (*Chlorophyceae*) [35].

Brown algae primarily live in shallow waters or on coastal rocks and have flexible stems that allow them to withstand seewaves. Because of large size and ease of collection, brown algae are the most studied and more commonly used in animal nutrition than other types of algae. The most common genera are *Ascophyllum*, *Laminaria*, *Saccharina*, *Macrocystis*, *Nereocystis*, and *Sargassum* [35-38].

Red algae have a characteristic bright pink color caused by the pigments R-phycocyanin and R-phycoerythrin. Most marine red algae are found at depths of up to 100 m. The main genera of red algae are *Pyropia*, *Porphyra*, *Chondrus* and *Palmaria*. Dead coralline red algae, especially *Phymatolithon* and *Lithothamnion*, form calcareous deposits that are used throughout the world for calcium carbonate.

The color of green algae is due to chlorophyll in the chloroplasts. Color may vary depending on pigment balance. The main genera of green algae are *Ulva*, *Codium*, *Enteromorpha*, *Chaetomorpha* and *Cladophora*. These algae are common in well-lit areas in shallow waters (39).

The chemical composition of seaweed is variable and depends on the species, time of collection (season of the year), habitat, and external conditions, e.g., water temperature, light, concentration of nutrients in water, etc. [16, 40]. Seaweeds are characterized by a high content of macro- and microelements, that is, the concentrations may exceed those in terrestrial plants [36]. The high mineral content is due to absorption of inorganic substances from the environment. Algae also contain polysaccharides and small amounts of fats, which are mainly polyunsaturated fatty acids. C. Corino et al. [41] point out the high content of vitamins A, B_1, B_{12}, C, D, E, B_2, B_3, B_5, B_9 in algae.

Among marine organisms, algae are one of the richest sources of natural antioxidants and antimicrobial substances [42]. Due to the significant water content (70-90%), seaweed must be dried or sold quickly due to the risk of mold. To produce seaweed meal, wet seaweed is passed through hammer mills with sieves of decreasing size. Then seaweed mass is dried in a drum dryer at 700 to 800 °C and finally at 70 °C to 15% humidity and stored in sealed containers [4]. Uncontrolled use of dietary algae can negatively affect animal performance [43].

Microalgae. Of the 30,000 species of microalgae thought to exist, only a few are used and grown commercially. The most biotechnologically important microalgae are green algae *Chlorella vulgaris*, *Haematococcus pluvialis*, *Dunaliella salina* and the cyanobacterium *Spirulina maxima*. According to model calculations based on the cultivation of microalgae in open pools and experimental installations, the annual biomass yield is 15 t DM/ha. In phytobioreactors production can be doubled. These differences also depend on climate and the rate of photosynthesis.

Composition of microalgae depends on the nutrient medium, time of year, light intensity, and temperature. Bioreactors ensure regulation of culture regimes and, thereof, the composition. Vitamin content depends on environmental conditions, post-harvest processing and drying method. Microalgae are 7-14% ash and approximately 5% nucleic acids which also depends on the culture conditions. As with macroalgae, it is always worth paying attention to accumulation of heavy metals [44]. The Table indicates the main chemical components of some maro- and microalgae.

Approximate chemical composition of some macro- and microalgae [38, 44]

Name	Content, % dry matter		
	crude protein	carbohydrates	crude fat
Macroalgae			
Ulvalactuca	10-25	36-43	0.6-1.6
Chondrus crispus	11-21	55-68	1.0-3.0
Laminaria digitata	8-15	48	1.0

Continued Table

Microalgae			
Spirogyra sp.	6-20	33-64	11-21
Chlorella vulgaris	51-58	12-16	14-22
Arthrospira maxima (*Spirulina*)	60-71	13-16	6-7

Algae in poultry farming. According to many reports, 1-5% seaweed added to feed can be used in poultry farming to improve the immune status, reduce the microbial load in the digestive tract and improve the quality of the resulting product [45-47].

Calcined red seaweed is a valuable source of organic calcium for broilers due to greater availability compared to inorganic limestone. Red seaweed improved the poultry's skeletal system, reducing limb weakness and lameness [48].

Green algae *Entermorpha prolifera* fed at 2-4% of the total diet promote better utilization of nutrients and energy metabolization in broilers, which may be due to an increase in amylase content in the duodenum. There was a positive effect on feed intake, feed conversion ratio and average daily gain, with a decrease in abdominal and subcutaneous fat thickness and, therefore, improved breast quality [46].

Adding 1-3% dietary *E. plifera* improves the egg production and quality in chicken, egg weight, shell thickness and the yolk color intensity increase, and yolk cholesterol decreases. In addition, the abundance of *Escherichia coli* in feces decreased, which could indicate improved bird health. The feed conversion ratio also improves [47].

M.A. Al-Harthi et al. [49, 50] reported that up to 6% of dried, boiled and autoclaved brown algae *Sargassum dentifebium* can be added to the diet of young poultry and laying hens from week 14 to week 42 without a negative effect on productivity and feed consumption. The yolk color improved by 12.31% and the shell calcium content by 9.1% compared to the control.

M.A. Al-Harthi et al. [51] also recommend up to 6% of the brown alga *Sargassum* sp. in the diet of laying hens aged from 23 to 42 weeks to reduce the content of cholesterol and triglycerides in the blood and yolk. In addition, the amount of carotene, lutein and zeaxanthin increased, as well as yolk palmitic acid [51].

AS per A.A. El-Deek et al. [52], laying hens fed S. *dentifebium* at 1 g/kg diet in combination with green tea (1 g/kg) or vitamin E (300 mg/kg) increased productivity with better egg quality under heat stress (32±4 °C). According to M.A. AI-Harthi [49], brown algae increased egg production by 1.2% and improved feed conversion by 5.2% compared to control. When using vitamin E, egg shell was 6.6% thicker, and brown algae together with vitamin E increased the yolk color intensity by 9.1% and decreased the cholesterol content in fresh eggs by 16%.

Replacing 3% corn in the diets of broiler chickens aged 12 to 33 days with green algae *Ulva lactuca* contributed to an increase in breast muscle mass by 2.3%, a decrease in the blood concentrations of total lipids by 125.1 g/100 ml, cholesterol by 29.5 g/100 ml, and uric acid by 2.68 g/100 ml [47].

In order to find an alternative to antibiotics, G. Kulshreshtha et al. [53] tested the red algae *Chondrus crispus* and *Sarcodiotheca gaudichaudii* on 67-day-old Lohmann Brown Classic laying hens. In the intestines of birds fed 2% dietary red algae the population of beneficial bacteria *Bifidobacterium longum*, *Streptococcus salivarius* and the number of *Clostridium perfringerns* increased. There was also an increase in the concentration of short-chain fatty acids, including acetic, propionic, butyric and isobutyric acids. The authors note that the chickens had a larger villi surface area and crypt depth. When fed 1% red algae, the weight of the yolk and eggs increased [53]. In further research, it was suggested that the previously

obtained results [53] are associated with the content of biologically active compounds in algae, such as agars, carrageenans, xylans, sulfated galactins and porphyrins [54, 55].

According to Y.A. Mariey et al. [56], laying hens fed 0.2% spirulina had better feed conversion (4.54 vs. 3.46) and produced more eggs (52.3 vs. 63.3%), additionally, the egg weight increased (48.5 vs. 51.82 g) compared to the control. The microalgae increased the percentage of yolk to albumen. Due to the supplement, the cholesterol concentration decreased in the yolk (13.5 vs. 10.2 mg) and in the blood (116.25 vs. 108.91 mg/100 ml). The percentage of fertilized eggs (90.87 vs. 96.58%) and hatchability (89.81 vs. 95.75%) were higher in the chickens fed a spirulina supplement [56].

In a study of the antimicrobial properties of six red seaweed extracts against *Salmonella enteridis* using a the nematode *Caenorhabditis elegans* model of infection, only two species of macroalgae, the *Sarcodiotheca gaudichaudii* and *Chondrus crispus* had the necessary properties. Aqueous extracts of algae, from 0.4 to 2 mg/ml, significantly reduced the growth and mobility of Salmonella enteritidis and reduced the formation of biofilms. RT-PCR results showed that the extract suppressed the expression of the *sdiA* gene (quorum sensing) and the pathogenicity island (island-1) genes *sipA* and *invF* in *S. enteridis*. It was hypothesized that the algae extract could reduce the *S. enteritidis* invasion in the host by attenuating virulence factors. In addition, aqueous extracts significantly improved the survival of infected *C. elegans* by interfering with the ability of *S. enteridis* to colonize the digestive tract of nematodes and increasing the expression of *C. elegans* immune genes (*irg-1*, *irg-2*, *hsf-1*). The authors suggest that extracts may also have beneficial effects on animal and human health [54, 57].

M.L. Manor et al. [58], studying the effect of the microalgae *Nannochloropsis oceanica* on laying hens of the Shaver-White Leghorn cross, revealed an increase in the concentration of ω-3-docosahexaenoic acid in eggs, liver and muscles, which correlated with the activation of key genes for elongation (*ELOVL3*, *EKIVL4*, *ELOVL5*) and desaturation (*FADS5*, *FADS6*) of polyunsaturated fatty acids. The authors point out the need for further research to confirm their findings.

When 3-day-old chicks were infected with *Campylobacter jejuni*, it was found that laminarin or laminarin/fucoidan extracts from the brown algae *Laminaria digitata* improved feed intake, increased the expression of key genes involved in the immune response (*IL-6*, *IL-8*), and promoted an increase in villus height in the small intestine and the growth rate of chickens. Laminarin extract was effective in increasing villous height and *TLR-4* gene expression compared to the control and laminarin/fucoidan extract groups. However, these supplements did not affect *C. jejuni* colonization [59].

Algae in cattle breeding. Macroalgae, when < 2% added to cattle diets, are capable of powerful prebiotic activity, 5.5 times greater than prebiotics from fructooligosaccharide (FOS) or inulin. As a result, the pathogenic load is reduced, the condition of the gastrointestinal tract is improved, productivity increases, the immune system is strengthened, and the animals' resistance to stress increases. Positive changes in the composition of the gastrointestinal tract microbiota resulted in increased digestibility of the entire diet and a reduction in methane emissions [43]. P.S. Erickson et al. [60] state that the use of algae supplements has been practiced for quite some time on organic dairy farms in the United States.

Research into the causes of *E. coli* infection in cattle has identified feces as an important source of reinfection. The drug Tasco (Acadian Seaplants, Ltd., Canada) based on *Ascophyllum nodosum*, used at a dose of 20 g/kg of diet for 7 days, effectively reduced the duration and intensity of *E. coli* O157:H7 excretion

in feces in bulls. The results obtained were confirmed in the second experiment when feeding the supplement to lambs at a dose of 10 g/kg for 28 days [61]). C.C. Lopez et al. [62] in a study on lactating cows proved that the use of *A. nodosum* in mixed feed, 100 g/kg, increased the milk iodine content compared to the control, up to 1.96 mg/l vs. 0.92 mg/l. In addition, the supplement increased the abundance of the gram-positive bacterium *Lactococcus lactis*, which is essential in cheese production [62].

Microalgae can stimulate the growth of microorganisms in the gastrointestinal tract of cattle. Supplements containing probiotic microorganisms do not lead to persistent microflora colonization. A.V. Konovalov et al. [63] found that a chlorella suspension (500 ml) increases the average daily gain in bodyweight of young cattle by 89 g and helps reduce the period of adaptation to changes in diet at 3 months of age [63]. According to C.N. Garces et al. [64], the spirulina microalgae fed to Montbéliard cattle 21 days before calving (15 and 30 g/day) did not affect bodyweight, productivity performance or the somatic cells counts in milk. In colostrum, the highest IgG content (75.6 g/l) was observed at a spirulina dose of 15 g/day. The lactose content in milk turned out to be the highest (5.6 vs. 5.5% in the control) at 30 g fed per day [64].

According to V.V. Petryakov [65], the amount of spirulina optimal for increasing the protective function of cattle is 400 ml/day. In a study on red-motley Holstein cows, he found that feeding spirulina increased natural resistance, e,g., blood bactericidal activity by 8.5%, lysozyme activity by 6.5%, phagocytic activity of neutrophils by 2.1%.

According to E.A. Tretyakov et al. [66], feeding black-and-white heifers with a chlorella suspension (500 g/day) during milk feeding contributed to an increase in feeding activity and average daily gain. A positive effect of spirulina on the rumen microflora in young animals was revealed. No differences were observed between the groups in physiological parameters. In the experimental group, the content of total blood protein increased by 6.7%, gamma globulins by 28% (vs. 25.47% in the control). Other indicators remained within normal limits.

Red algae *Asparagopsis taxiformis* in an amount of 5% of dairy cattle diet can reduce methane emissions. 16S rRNA gene amplicon sequencing showed that algae influence the composition of the rumen microbiome. In fermentation vessels under in vitro conditions, the relative content of methanogens decreased significantly compared to the control. A rapid effect of *A. taxiformis* on the metabolic activity of rumen methanogens was revealed, while the effect on the microbiome composition (e.g., a prevalence of methanogens) was delayed [67].

Algae in pig farming. The prebiotic effect and antimicrobial activity of laminarin and fucoidan may be useful for the preventive treatment of gastrointestinal diseases and increasing the digestibility of the diet in piglets after weaning. Laminarin supplementation (600 mg/kg) significantly increases the expression of mucin genes (*MUC 2* and *MUC 4*), exerting a protective effect on epithelial cells. Anti-inflammatory effects and a decrease in cytokine response occurred at a dose of 1 mg/ml. An immunomodulatory effect was achieved using seaweed extract (1.8 g/day) in pregnant sows. Increased production of immunoglobulins A and G in pigs was observed for a 0.8% extract [68].

Alginic acid oligosaccharide is a natural polysaccharide isolated from the cell walls of seaweeds. The addition of an oligosaccharide in an amount of 100 mg to the diet of weaned piglets increased the average daily weight gain, antioxidant activity, and the blood concentrations of IL-10, IgG and IgA. The content of secretory immunoglobulin A, the height of the villi and the activity of disaccharidase (lactase and sucrase) in the small intestine increased. The population of bacteria, including *E. coli*, decreased, and the abundance of *Bifidobacterium* and *Lactobacillus* increased [69].

C. Corino et al. [41] summarized reports on using algae in pig diets. They paid particular attention to brown seaweed, emphasizing its effect on the immune system, gut health and antioxidant status of animals. The use of brown algae in the diets of weaned piglets is recommended for the prevention of gastrointestinal diseases. An increase in the production of immunoglobulin and cytokines has been noted [41].

It was established [70] that the brown seaweed *Ecklonia cava* at 0.15% of the diet increased average daily weight gain of weaned piglets and changed the intestinal microflora, the number of *Lactobacillus* spp. increased by 0.26%, while *E. coli* spp. and *Clostridium* spp. decreased by 0.26% and 0.22%, respectively. The supplement had no effect on villous development in the small intestine [70].

M. Dell'Anno et al. [71] assessed the antioxidant and antimicrobial capacity of the brown alga *A. nodosum* and the microalga *Schizochytrium* sp. against the intestinal pathogen *E. coli* O138 in vitro using the broth macrodilution method. It turned out that *A. nodosum* is able to modulate the growth of *E. coli* for a short time, and *Schizochytrium* sp. has antioxidant capacity, which can be enhanced due to a synergistic effect. Given the urgent need for innovative functional feed additives, these algae can be used as an alternative to antibiotics [71].

Seaweed powder in sow diets (30 g/day) from 85 days of pregnancy to the end of lactation improved the immune status of piglets. Based on the analysis of T-lymphocyte subsets in 40-day-old piglets, an increase in the population of CD4+ CD8+ T-cells was noted in the thymus, lymph nodes, tonsils, peripheral blood mononuclear cells, spleen and liver [72].

Spirulina platensis at 2 and 3 g/day in pig diets led to an average daily increase in live weight by 14.3% and an improvement in feed conversion by 6.1%. There was a slight effect on hematopoiesis (15 and 13% more red blood cells and hemoglobin), and there was also a tendency to reduce morbidity by 59% [73].

P. McDonnel et al. [74] suggest that laminarin may improve the intestinal health of piglets after weaning. The authors recorded a decreased counts of *E. coli* in feces and noted an increase in the average daily weight gain. The co-use of laminarin and fucoidan (a sulfated polysaccharide) after weaning can reduce the incidence of diarrhea in piglets [74]. N. Derek et al. [75] in an experiment on piglets revealed an improvement in intestinal microflora and lower *E. coli* counts. The effect was presumably due to bioactive compounds alginates, fucoidans, laminarins, phloratannins of the *A. nodosum* supplement [75].

In order to strengthen the immune system and further replace antibiotics in pig farming, M. Katayama et al. [76] recommend using seaweed (0.8%) and licorice (0.15%). The authors note an increase in the amount of IgA in the mucous membrane and blood IgG. The supplement complex was shown to enhance the anti-inflammatory effect. i.e., interleukin gene expression was detected [76].

Biologically active substances of algae against campylobacter. Lack of sensitivity to antibiotics in bacteria is one of the most important risk factors for human health, since it reduces the effectiveness of drugs used to treat diseases. This also leads to an increase in the frequency of transfer of genes encoding resistance to other microorganisms, as a result of which new pathogens with increased aggressiveness appear in the environment. According to the World Health Organization (WHO), resistance of bacteria, mainly zoonotic pathogens, to antibiotic drugs currently poses one of the greatest threats to humanity [77]. The World Organization for Animal Health, concerned about the rapid development and spread of antibiotic resistance among animal pathogens, has developed recommendations for veterinary services [78].

The active use of antibiotics as growth stimulants in animal feeding plays an important role in the emergence and spread of resistant bacteria [79]. The

intensification of agriculture, expanded range of disinfectants and antiseptics, and the uncontrolled use of antibiotics in livestock farming increasingly select foodborne pathogens that are antibiotic resistant. The most epidemiologically significant are gram-negative bacteria of the *Enterobacteriaceae* family and the genus *Campylobacter*. The main transmission factors for campylobacteriosis are poultry and poultry derived products [80]. Campylobacteriosis monitoring in Europe officially estimates approximately 200,000 cases per year [81, 82]. The causative agents of intestinal campylobacteriosis include thermophilic bacteria of the genus *Campylobacter*, species *C. jejuni*, *C. coli, C. lari*, *C. upsaliensis* and *C. helveticus* [83, 84].

To prevent and treat campylobacteriosis in poultry, it is necessary to increase the number of beneficial bifidobacteria and lactobacilli and to reduce stresses from changing diets and vaccinations in traditional regimens for the use of veterinary drugs [85].

Algae contain two important groups of substances that have a wide range of biological activities. The first group is fucoidans, which are characterized by antibacterial, antiviral, anticoagulant, immunomodulatory, anti-inflammatory, and antitumor activity [86, 89]. Another most important compounds are polyphenols, namely phlorotannins, the polymers of phloroglucinol. The content of phloroglucinol varies depending on the type of brown algae and the place where they grow, reaching 20% DM [34]. Phlorotannins have been described as antioxidant with activity 2-10 times higher than in ascorbic acid and α-tocopherol [90], antiviral [91], antitumor [92], neuroprotective [93], antithrombotic [94], antimicrobial [95], and antiallergic [96]) properties.

Various formulations containing fucoidans as bioactive components are being developed for medical use, such as dressings [87]. The lack of toxicity along with bacteriostatic properties allows their use in the food industry, increasing the shelf life of products and not suppressing beneficial microflora [88].

Thus, the marine environment is a promising source of bioactive compounds that are currently underutilized in commercial animal farming. The variety of biological properties of algae determines their various practical use, including production of feed additives for pig farming, poultry farming, and cattle breeding. Algae exhibit not only a prebiotic effect due to the oligo- and polysaccharides they contain, but also antimicrobial, immunomodulatory, antioxidant and anti-inflammatory properties due to bioactive compounds. Depending on the purpose of use and subject to the optimal dosage, algae can have a positive effect on ontogeny, animal performance and the quality of the resulting product. Algae can also module the composition of microbial communities in a positive way, suppressing pathogenic microorganisms. Reducing the risk of infectious diseases is due to healthy microbiota, which can provide high resistance to intestinal colonization by pathogens. The condition of the intestines determines the health of the animal, the efficiency of utilizing nutrients and bioactive substances. The bioactive feed additives, in particular algae, which support normal intestinal microflora and stimulate the body's defenses are considered as one of the most promising in the prevention and treatment of infections and as an alternative to feed antibiotics.

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GLUCOSINOLATES IN RAPE AND CAMELINA: COMPOSITION, CONCENTRATIONS, TOXICITY AND ANTI-NUTRITIVE EFFECTS IN POULTRY, METHODS OF NEUTRALIZATION — A MINI-REVIEW

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Abstract

During the last decades animal nutrition and feed production in the World encounter the increasingly important problem of the deficit of feed-grade protein especially urgent for the regions (including Russian Federation) where the soybeans (considered an "ideal" protein source in feeds for all animal and poultry species) cannot be effectively cultivated. To solve this problem, decrease feeding costs and dependence on the imported soybeans the local vegetable protein sources are increasingly used including *Brassicaceae* oil crops rape (*Brassica napus* L.) and camelina (*Camelina sativa* L.) which are highly tolerant to the conditions of cultivation. Though cakes and meals of these crops are rich in protein they contain certain anti-nutritive factors including glycosides called glucosinolates (GLs), a vast group of protective secondary plant metabolites, alkyl-aldoxime O-sulphates containing the residue of β-D-thioglucopyranoside bonded to the hydroximine carbon in *cis*-position to the sulphate group. At present over 120 natural GLs are identified (B.A. Halkier and J. Gershenzon, 2006). The toxic and anti-nutritive effects of the GLs in rapeseed and methods for their neutralization are relatively well studied; however, the effects of GLs in camelina are still understudied (due to its relatively short history of large-scale cultivation) and hence the data obtained on rape should be used for the assessment of possible effects of camelina. The detrimental biological effects of the GLs on poultry, their mechanisms and methods of neutralization are reviewed herein. The GLs per se are non-toxic and their protective role in the plants is related to the endogenous plant enzymes β-thioglucosidases (myrosinases): GLs and myrosinases normally (in an intact plant) localized in different tissues contact after the damage of the plant (e.g. by insects or other herbivores) resulting in the enzymatic hydrolysis of the GLs and transformation of their aglycone residues into the potentially toxic products: isothiocyanates, thiocyanates, oxazolydine-thiones, nitriles, epithionitriles (D.J. Kliebenstein et al., 2005). Similar processes could be also induced by the enzymes of intestinal microbiota in poultry. Main toxic effect of almost all these products is goitrogenicity involving disturbance of the synthesis and secretion of thyroid hormones into the bloodstream and (in cases of heavier exposure) hypertrophy of the thyroid gland and formation of the goiter. In sub-toxic doses these GL metabolites can hamper the growth in young poultry, decrease egg production and quality in adult hens, induce "fishy taint" of the eggs. Since 1960s the rape has been intensively selected for decreased GL content and a wide range of low-GL cultivars are now present in the market; similar work with the camelina is still at its start. Concentrations of the GLs in cakes and meals of these crops can be decreased by thermal treatment (at ca. 100 °C), soaking in water, treatments with solutions of alkali or copper sulphate, solid-phase microbial fermentation, micronization, extrusion, etc. (M.K. Tripathi and A.S. Mishra, 2007). Maximal permissible level of the GLs in diets for poultry is apparently 5-6 mM/kg, corresponding to dietary levels of the products of the native rape ca. 10 %, low-GL rape varieties 15-20 %, camelina products 5-10 %. The studies on the toxic and anti-nutritive effects of rape and especially camelina are necessary for the practice of poultry nutrition and important for further genetic improvement of these crops.

Keywords: poultry, nutrition, rape, camelina, cakes, meals, glucosinolates, goitrogenicity, selection

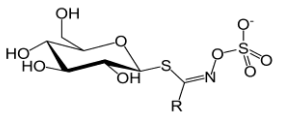
In the last two decades, due to growth of population, consumption and livestock production, feed protein deficiency has become an increasingly pressing global problem, especially in regions where soybeans, an ideal source of feed pro-

tein for animals and poultry, are not grown. In Russia, domestic soybean production is low, therefore, feed protein deficit is also relevant. To reduce the cost of feed and import dependence, domestic poultry farming uses local feed resources, in particular rapeseed (*Brassica napus* L.) and camelina (*Camelina sativa* L.) of the *Brassicaceae* family. They are unpretentious and highly productive even in the most unfavorable conditions, and are resistant to most pathogens [1, 2].

Cake and meal, the by-products after extracting oil from camelina and rapeseed seeds, are rich in protein, but contain anti-nutritional factors, primarily glycosides glucosinolates (GL) [1, 2]. This fact has been known for quite some time, but is often underestimated in diet formulation. In addition, anti-nutritional properties have been studied mainly in rapeseed, while camelina has been cultivated relatively recently compared to rapeseed and has been less studied as a fodder crop.

Antinutrients of *B. napus* have become less acute issue due to created low-glycoside canola varieties. Nevertheless, study of rapeseed remains relevant in terms of its improving as a forage crop and clarifying protocols for its use. In addition, these data partially compensate for the lack of information on camelina.

This mini-review consideres toxic and antinutritive property of glucosinolates on the example of poultry, the mechanisms of toxic action and methods for its neutralization.



Geeneral formula of glucosinolates. R is a side chain.

Biodiversity of glucosinolates and their content in rapeseed and camelina. In chemical structure, GLs are a broad class of O-sulfates of alkylaldoximes, in which a β-D-thioglucopyranoside residue is attached to the hydroxymine carbon atom in the cis position to the sulfate group (Fig.). The diversity of GLs derived from differences in the side chain (R) structure. Currently, more than 120 natural compounds of this class have been identified [3, 4].

GLs are not toxic, and their protective action is due to the endogenous enzymes β-thioglucosidases, also known as myrosinases, of the same plants. In an intact plant, they can be expressed in various tissues. When damaged, for example, by insects or animals, the enzyme and its substrate (GL) interact, the GL hydrolysis occurs with the release of glucose. Next, the aglycone residue of GL is transformed into various toxic products, e.g., isothiocyanates, thiocyanates, oxazolidinethiones, nitriles and epithionitriles. The type of substrate (GL), pH, the presence of iron ions and/or epithiospecific protein determine the ratio of these hydrolysis products [4-7]. The same process of enzymatic hydrolysis of GL by myrosinases can occur, depending on the technology conditions, during oil extraction. The resulting toxic compounds, primarily isothiocyanates, as the most thermostable products of GL hydrolysis, accumulate in cakes and meals [8]. To a certain extent, the same GL hydrolysis can occur in the digestive system of animals and poultry due to microbial transformations [9].

In the organs of the same plant, GLs with different side chains may predominate. Thus, in rapeseeds the main GLs are progoitrin (2-hydroxy-3-butenyl-glucosinolate) and gluconapine (3-butenyl-glucosinolate), and in the vegetative parts and especially in the roots glucobrassicanapine (4-pentenyl-glucosinolate), glucobrassicin (3-indolylmethyl glucosinolate) and 4-hydroxyglucobrassicin, gluconasturcine (2-phenylethyl glucosinolate) [10, 11]. Moreover, these GLs not only differ structurally, but, apparently, perform different functions. GLs of seeds are responsible for protecting the offspring, while GLs of vegetative parts are responsible for protecting the territory. Remaining in the soil, they are hydrolyzed, mainly to isothiocyanates, and have allelopathic phytotoxic effects on other plants [12]. It is precisely this effect that is associated with yield decrease in the crops followed rapeseed in crop rotation. It has been reported, for example, that soybeans

and sunflowers in fields after rapeseed were severely retarded in early growth, which did not occur in barley or when soybeans and sunflowers were planted after winter fallow [13]. In camelina seeds, GLs with long aliphatic side chains, e.g., the 9-methylsulfinylnonyl-glucosinolate and especially 10-methylsulfinyldecyl-glucosinolate (glucocamelinin), predominate accounting for apprx. 60-70% of all seed GLs [14].

The composition and concentration of GL in batches of seeds highly depend on the variety, growing area and cultivation technology [15]. For example, in the UK, in seeds of ordinary rapeseed varieties the GL content varies from 90 to 186 mmol/kg fresh weight (a moisture content of apprx. 9%), in seeds of varieties bred for low GL content from 10 to 14 mmol /kg [16]. In camelina, less GL accumulates in the seeds than in rapeseed. Thus, in 10 studied genotypes of camelina, the GL content in seeds varies from 13.2 to 36.2 mmol/kg DM and averages 24.0 mmol/kg [14]. In a more recent study of 47 camelina samples, these values were 19.6-40.3 and 30.3 mmol/kg, respectively [17].

Toxicity of glucosinolates and their effect on poultry. Goitrogenicity is the main effect of toxic products derived from GL hydrolysis and biotransformation in animals and poultry. Goitrogenic effect is a violation of the thyroid hormone synthesis and release into the bloodstream by the thyroid gland. Under more severe intoxication, the gland hypertrophies and a goiter heppens. Almost all supposed products of GL hydrolysis are involved in the goitrogenic effect, excluding (epithio)nitriles. Thiocyanate anions are direct competitors of iodine for interaction with the transmembrane protein sodium-iodine symporter (SIS) which ensures their penetration through cell membranes and binding to tyrosine residues of thyroglobulin in thyroid follicles. Oxazolidinethiones, for example goitrin derived from hydrolysis of rapeseed progoitrin inhibit the dimerization of diiodotyrosine (T2) into thyroxine (T4), the reaction of T2 with monoiodotyrosine (T1) to form triiodothyronine (T3), and the hydrolysis of thyroglobulin by the endogenous protease of the gland, followed by T3 and T4 release into the bloodstream. Although conversion of isothiocyanates to thiocyanates or oxazolidine thiones may occur, isothiocyanates are less likely to contribute to the goitrogenic effect. Together, this reduces the amount of T3 and T4 in the blood, increases the biosynthesis of thyrotropin in the anterior pituitary gland, which, in turn, activates the thyroid follicles and ultimately leads to their hypertrophy [18].

Nitriles, the hydrolysis products of GL, cause irritation of the gastrointestinal mucosa and local necrotic lesions, and exhibit hepatotoxicity and nephrotoxicity. When feeding laying hens with a 20% GL-rich rapeseed diet, alogn with a significantly lower egg production, the blood concentration of urates statistically significantly increased, and the liver enlargement occurred with reticulosis and bleeding lesions in it [19]. In turkeys fed high-GL diets for 16 weeks, fibrosis and degeneration of parenchymal cells in the central lobe of the liver occurred. From week 4 to week 12, numerous foci of necrosis appeared, and by week 16 extensive cirrhosis of the liver developed [20].

Bird kidneys and liver can quite efficiently metabolize and excret GLs and their breakdown products. In meat and even in these organs, residual amounts of these substances are practically not detected by chemical analysis. Meat quality deterioration is also not detected organoleptically even with 17-20% rapeseed in the diet [21]. However, the quatyigication of residual GL metabolites in poultry products has been carried out quite a long time ago, and the κesults may be worth reconsidering using more sensitive modern analytical methods.

The fishy smell in eggs is often due to rapeseed in the diet, especially in the more susceptible brown layers. At first, the cause was thought to be solely

rapeseed sinapin, but it was later discovered that this undesirable effect also depends on progoitrin. The fishy smell in eggs was reported to begin at 0.3 μmol/g progoitrin in a rape-free diet that corresponds to a total GL dose of 0.5 mmol/kg for brown layers and 1 μmol/g for white layers [21]. The fishy smell of eggs is due to the high content of trimethylamine (TMA) in the yolk. Bacterial fermentation of choline in the digestive tract geterats TMA that is then transferred through the bloodstream to the developing follicles in the ovary [22]. On the one hand, feeding high doses of rapeseed can lead to an excess of choline in the intestinal chyme due to the high content of sinapine in rapeseed. On the other hand, goitrin produced from rapeseed progoitrin when degraded by myrosinases or gut microflora, competitively inhibits flavin-containing monooxygenase-3 (FMO3), an enzyme that catalyzes the intestinal oxidation of TMA to the smell-less trimethylamine-N-oxide [23].

Later it was shown that the decrease in FMO3 activity is associated with a single nucleotide polymorphism (SNP) A to T at position 984 of the coding sequence of the gene for this enzyme on chromosome 8. This SNP leads to the replacement of threonine with serine at position 329 in the enzyme, The mutation can be recessive [24] or additive [25]. This mutation in the evolutionarily highly conserved sequence of FMO3 does not cause changes in the expression of the enzyme gene in genotypes that differ in the indicated single nucleotide polymorphism, the AA, TT, and AT. The fishy egg smell is most likely due to the effect of the mutation on the substrate recognition site of the enzyme [24]. Therefore, to reduce, if not eliminate this influence, laying hens with the mutation should not be used in breeding programs. In addition, it was reported that when brown laying hens of different genotypes for the specified polymorphism were fed for 4 weeks with 0, 6, 12, 18 and 24% dietary canola meal, egg yolk TMA accumulation grew linearly ($p < 0.05$) only in the homozygous mutant genotype TT, but not in AA and AT. Feeding TT laying a control diet with a higher content of synthetic choline that corresponded to the amount of sinapine in the same doses of canola meal did not increase the yolk TMA content [26]. This likely means that in canola meal, progoitrin, as an FMO3 inhibitor, makes a more significant contribution to the observed effect than sinapine, as a substrate supplier for FMO3. These data also indicate that genetic selection of laying hens can successfully address the problem of egg fishy smell at high rapeseed doses.

Subtoxic amounts of GLs in rapeseed products do not cause pronounced negative sings while relatively high doses of GLs negatively affect the efficiency of feed use and productivity performance in laying hens. In brown laying hens, 30% dietary rapeseed cake or meal, vs. no additives in control, reduced ($p < 0.01$) the egg weight, the digestibility of feed DM by 5%, crude protein by 4%, the use of gross energy by 7%, and the digestibility of all essential amino acids except for tryptophan. A 1.6% increase ($p < 0.01$) in the yolk content of monounsaturated fatty acids (MUFA) was a favorable effect [27]. The fact that these negative effects are associated specifically with rapeseed anti-nutrients, the GL and erucic acid is confirmed by another experiment. M.A. Oryschak et al. [28] assessed the efficiency of feed digestion and egg quality as affected by 7 and 14% expeller rapeseed meal in the diet vs. 30%. The four varieties used to obtain the expeller meal differ in the GL and erucic acid accumulation [28]. During 8 weeks of the experiment, the birds fed meal with the maximum GL content were inferior in digesting all main feed nutrients. The height of intestinal villi and crypt depth, egg mass and quality paramters, especially albumen height and Howe units also decreased [28]. To compare three rapeseed varieties with different seed GL contents, laying ducks were fed the meal, 10% of the diet, for 12 weeks, which significantly decreased egg weight and feed consumption [29]. The latter effect is quite common and is

traditionally explained by the sour-bitter taste of GLs and their hydrolysis products, which makes feed less attractive to animals and poultry [30]. However, the albumen height and the Hau units did not decrease compared to the control, the yolk MUFA concentration significantly decreased, which contradicted the data of another experiment [27], and the concentration of polyunsaturated fatty acids (PUFA) increased [29]. The resulting ratio of total unsaturated and saturated fatty acids remained within the control value. In all three test groups, the concentration of TMA and 5-vinyl-1,3-oxazolidine-2-thione in the yolk increased ($p < 0.01$) the more pronounced, the higher the GL content in the meal. These showed a negative effect of GL on the quality of the yolk, but not the albumen. The data obtained may indicate a certain species-specific response of birds to GLs [27-29].

In broiler chickens, moderate GL doses in diets can significantly reduce feed consumption and live weight gain without significant pathological changes due to GL toxicity, including blood concentrations of thyroid hormones and symptoms of hepatotoxicity. Interestingly, feed conversion can remain virtually unchanged [31]. The same study reveled a significant negative correlation between GL consumption and live weight gain ($r^2 = –0.74$, $p < 0.05$). It was also reported that 2-4 mmol/kg of total GL in the diet, which corresponds to approximately 20% dietary canola meal, has virtually no effect on the growth of broilers, and only doses of more than 10 mmol/kg significantly decreases average daily bodyeight gain [32]. In a recent experiment [33], 10 and 30% full-fat unmodified rapeseed in the diet led to a highly significant ($p < 0.0001$) deterioration in feed conversion, a decrease in feed consumption, a decrease in live weight and its gain compared to the control. Broilers fed 20 and 40% canola meal performed worse than controls but better than birds fed full-fat canola [33]. In another experiment [34], the addition of 20% dietary canola meal, on the contrary, did not lead to a decrease in either feed intake or growth efficiency of broilers compared to the control. The diets without rapeseed (control) and with canola meal were equal in metabolic energy, but the coarse fiber content in the control diet was noticeably less. As a result, in the cecum of chickens from the experimental group, the number of cellulolytic microorganisms increased, as well as the chyme content of short-chain fatty acids produced by these microorganisms. Short-chain fatty acids serve as energy sources for the host, which is a positive effect. However, metabolome analysis of the pancreas, liver and breast muscles of chickens showed that a diet with rapeseed, even canola, increases the risk of pancreatitis and oxidative stress in the liver. Nevertheless, the metabolic profile of the studied organs and productivity parameters indicated thay chickens can effectively counteract feed stress, at least with a moderate amount of rapeseed meal in the diet.

The differences in poultry productivity in experiments with similar amounts of rapeseed additives can be explained by both the unequal content of antinutrients, including GLs which was not determined in all cases, and the unequal availability of energy and amino acids from these additives. Indeed, in broilers, a comparative study of canola meal samples from six Canadian plants found great and significant differences in amino acid availability and metabolizable energy content [35]. In addition, differences in proteomic profiles depending on the origin of rapeseed may be important, which affects the efficiency of protein degradation by poultry enzymes and and utilization of amino acids [36].

Camelina, currently used in feed throughout the world, is much less genetically diverse than rapeseed. To our knowledge, no experiments have been conducted to evaluate the effects of purified camelina GL preparations on poultry, and the results when feeding camelina cake or meal are contradictory. This may be due to the place and conditions of the camelina cultivation and/or the oil extraction technology. Thus, 5 and 10% mechanically pressed camelina cake the

added to broiler diets from day 1 to day 37 of life linearly and significantly reduced live weight and feed consumption from day 15 to day 37 with no effect of both doses on the relative weight of the thyroid gland and the severity of symptoms of hepatotoxicity [37]. In another experiment [38], 4% camelina oil and 5 and 10% cake or full-fat seeds significantly ($p < 0.05$) reduced feed intake and live weight gain in broilers under high altitude and cold stress conditions.

Other authors reported that at a stepwise increase in the dietary camelina meal concentration from 5 to 25% during 10-37 days of life from, the feed consumption differed insignificant at 0-20% meal and decreased only at 25%. The live weight gain began to decrease significantly at doses above 15%, and the relative weight of the thyroid gland significantly increased only at 20-25% of the meal [9]. In a 42-day experiment on broilers with increasing doses of camelina cake in the diet (8-24% in 8% increments) [39], there was no significant effect of the additive on either growth rate or feed consumption. The weight of the thyroid gland also did not alter, although the blood T3 and T4 concentration grew in a dose-dependent manner. The weight of the pancreas on days 28 and 42 of life increased linearly and significantly with increasing dose of camelina. The digestibility of all main nutrients also decreased linearly ($p < 0.01$).

The advantages of camelina as a feed crop include its high content of n-3 PUFAs [40, 41]. Due to this, camelina enriches poultry eggs and meat with PUFAs and, for example, can prevent ascites in broilers at high altitudes [38]. Camelina contains from 25.9 to 36.7% α-linolenic acid ($C_{18:3}$ n-3) of the total fatty acids [41]. Feeding camelina cake (8-24%), seed (10%) or oil (2.5-6.9%) to broilers increases the content of α-linolenic acid in muscles 1.3-4.4-fold, 2.4-2.9-fold and 2.3-7.2-fold, respectively, compared to the control, and n-3 PUFAs in muscles and liver by 1.5-3.9 times [41]. Another researchers obtained similar data [42].

The results on feeding camelina cake to laying hens are contradictory. According to one report, when feeding 5, 10 and 15% camelina cake to laying hens, better egg production and the best fatty acid profile of eggs were obtained at 5%; at 15%, feed consumption significantly decreased, and shell quality deteriorated with an increase in the percentage of soft-shelled eggs [9]. In another experiment, 10 and 20% cake in the diet from week 18 to week 51 of life did not reduce either feed consumption or egg production of laying hens, and the quality of the shell, on the contrary, improved, especially with increasing age of the hens [43].

Thus, the available information on the effectiveness of camelina in poultry feeding is ambiguous, and therefore it is of interest to determine the content of GLs and, possibly, other anti-nutritional factors in camelina, as well as amino acid and fatty acid profiles.

Selection to reduce glucosinolate content. Significant accumulation of GLs, as well as erucic acid and sinapine in rapeseed seeds stimulated breeding for reduced content of these anti-nutritional factors. In 1967, an allele responsible for a significant reduction in the GL accumulation in seeds was discovered in the Polish rapeseed variety Bronowski. The introduction of this allele into varieties with a normal level of GL synthesis produced hybrids with genetically reduced GL production [44]. Since then, many low-glycoside varieties of rapeseed have been obtained, in which the amount of GLs in the seeds does not exceed 25-30 mmol/kg. Such varieties are usually called canola. However, the developed varieties turned out to be less resistant to pests, e.g., insects and wild birds, and pathogens. Since the accumulation of total GLs in seeds and leaves are traits with a high positive correlation ($r^2 = 0.79$), selection to reduce the amount of GLs in seeds led to a decrease in the GL content in leaves. Therefore, the next task was

to create low-glycoside varieties that retain effective concentrations of glycosides in the vegetative parts of the plant.

It was found that in cabbage plants, GLs synthesis occurs in three stages and involves amino acids which determine the structure of the side chain. Based on the type of these chains, GLs can be divided into three groups, These are aliphatic GLs the biosynthesis of which necessitates alanine, leucine, isoleucine, valine, methionine and its metabolites with an extended carbon chain, benzene GLs with phenylalanine and tyrosine derivatives involved, and indole GLs with tryptophan derivatives used [4, 45]. Biosynthesis of these three GL groups proceeds independently and is regulated by different sets of genes [45]. Aliphatic GLs predominate in the seeds, and benzene and indole GLs in the vegetative parts. Therefore, presumably, there could be rapeseed genotypes low in total GLs in seeds, but with a normal content of GLs in leaves. In a recent genome-wide genetic association study (GWAS) of GL biosynthesis traits in rapeseed, *BnaA03g40190D* was identified as a candidate genes responsible for this combination [46].

In addition, it turned out that the biosynthesis of GLs (including aliphatic ones) occurs predominantly in feeding tissues (leaves, walls of seed pods), and synthesized GLs are transferred to the seeds (embryos) through the phloem by GL specific transport proteins [47]. In the rhizome of *Arabidopsis thaliana* (L.) Heynh., a popular model species for studying plants biochemistry and genetics in the *Brassicaceae* family, two such proteins were found, the GTR1 and GTR2. In plants mutant for the genes of both proteins, there is no accumulation of GLs in the seeds, while the level of GLs in the leaves and pod walls is more than 10 times higher [48]. Therefore, it is possible to create rapeseed variety with reduced seed GL content by influencing the GL biosynthesis (i.e. reducing the aliphatic GL production of while maintaining indole synthesis) and expression of GL transporters [49].

Unlike rapeseed, commercial cultivation of camelina began relatively recently in world practice [2], and, as far as we know, the creation of low-glycoside commercial varieties has not yet been reported. However, there is evidence of genetic differences between varieties of camelina (*C. sativa*) and related species (*C. microcarpa*, *C. alyssum*, *C. rumelica*, *C. hispida*) in the content of some GLs [50], which indicates the fundamental basis of such selection .

Other methods for reducing the content of glucosinolates in feed products. The content of GLs and their hydrolysis products in rapeseed cakes and meals can be reduced by heat treatment at ~ 100 °C), soaking in water, treatment with aqueous solutions of alkalis or copper sulfate, solid-phase microbial fermentation, micronization, extrusion [15]. In particular, heat treatment at temperatures below 70 °C is enough to neutralize myrosinase [51]. These methods differ in their effectiveness in neutralizing GLs (from 40-45 to 90-95%). However, physical methods are very energy-intensive and therefore expensive. In addition, many of them significantly reduce the quality of the feed, in particular the solubility, ileal digestibility and absorption of crude protein and some amino acids. Rapeseed breeding is now considered the most effective strategy for reducing the content of GLs and toxic products of their biotransformation in rapeseed feed products [52, 53].

When extracting oil with solvents, subsequent heating (toasting) of the meal to desolventize it ensures fairly effective neutralization of GLs, myrosinases and associated toxins. It should be remembered that the temperature and duration of toasting have a positive effect on the efficiency of GL neutralization, but a negative effect on the feed quality of the meal. It has been shown that increasing the duration of toasting from 48 to 93 min significantly reduces the digestibility

of crude protein and most amino acids fed to pigs, and also increases the content of neutral and acidic fiber fractions in the meal [54]. Analysis of 40 samples of meal and 40 samples of cake produced in Poland from rapeseed harvest in 2003 showed that the average GL content in meal was 14.6 mmol/kg DM, in cake 17.4 mmol/kg, being within acceptable limits. It has also been shown that increasing the toasting time of both cakes and meals from 20 to 30 min reduces the coefficient of lysine availability [55]. It makes sense to toast cold (mechanically) pressed rapeseed cakes if the GL content exceeds the permissible threshold of 20 mmol/kg. For example, in a study [8], the content of total GLs in cold-pressed cake was 9.9±0.7 mmol/kg. Such cakes can be used in feed without pre-treatment, as well as for the production of protein concentrates, and during their production the GL content decreases even more.

Fermentation is among the most effective methods for reducing the GL content in rapeseed products, which maintain the amount and availability of amino acids and increase the metabolic energy content. In fermentation, specially selected compositions of microorganisms are usually used. Recently, solid-phase fermentation technology for rapeseed meal has been developed using strains of *Lactobacillus acidophilus*, *Bacillus subtilis* and *Saccharomyces cerevisiae*. It was reported that the amount of metabolizable energy increases from 7.44 MJ/kg in unfermented meal to 8.51 MJ/kg in fermented meal. Ileal digestibility (availability) of alanine, valine, isoleucine, leucine, tyrosine, lysine, arginine and phenylalanine increased, and the content of aspartic and glutamic acids, histidine, threonine, serine, proline, glycine, methionine and cystine remained at the level of unfermented meal [56]. Fermented meal not only significantly ($p < 0.05$) increased feed consumption and live weight gain in broilers compared to unfermented meal, but also significantly reduced oxidative stress. It was evidenced by increased blood concentrations of superoxide dismutase and total antioxidant capacity and decreased malondialdehyde concentration [57]. All this indirectly indicates the effectiveness of neutralization of GLs during the fermentation process. Fermentation of rapeseed meal using *Bacillus subtilis* and *Aspergillus niger* significantly reduced the GL content in it [58]. When feeding such meal to laying hens instead of 33, 66 and 100% soybean meal, significantly ($p < 0.05$) higher rates of egg production and egg weight were noted than when adding unfermented rapeseed meal. However, when soybean meal was completely replaced with both types of rapeseed meal, these parameters significantly decreased compared to the control [58].

It is also effective to simultaneously add exogenous enzymes β-glucanase, xylanase, pectinase, and cellulase to diets, which increase the digestibility of coarse fiber in rapeseed meal. It was reported [59] that 20% fermented canola meal combined with a multi-enzyme preparation containing, among others, the above enzymes, improves ($p < 0.05$) productivity, antioxidant status and immune status of chickens, i.e., bursa Fabricius weight and antibody titer against Newcastle disease, compared to groups fed 20% unfermented or fermented meal, or 20% unfermented meal with the same enzyme preparation.

For camelina cakes and meals with a high GL content, their decontamination using the desciribed methods is still quite relevant. Recently, micronization of full-fat camelina grains was shown to be effective in broiler chickens [60, 61]. However, since camelina has become widely used in feeding relatively recently, there are still few such reports, and the problem of neutralizing camelina GL requires further study. At the present stage in practical feed production and feeding, one can rely on the results obtained earlier in studies on rapeseed (with the necessary adjustments).

Recommended doses of rapeseed and camelina products in poultry diets. According to M.K. Tripathi and A.S. Mishra [15], the maximum permissible

level of GL in poultry diets can be considered 5-6 mmol/kg. For laying hens, low-glycoside varieties of rapeseed (00, canola), up to 10% of the diet, are recommended with virtually no damage to health and productivity, for broilers up to 20%. Additives from cakes and meals of conventional varieties of rapeseed should make up no more than 10-15% of the diet for broilers and 7-8% for laying hens. Preliminary toasting of these products is mandatory. When rapeseed products are used as the main source of protein in poultry diets, they can be supplemented with alternative protein sources, such as sunflower meal [62]. This also reduces the cost of the diet and the final GL content.

The amount of camelina products in the diets of broiler and laying hens is determined by the GL content and can usually be up to 5-10% [40]. The same amounts of camelina supplementation are recommended for growing meat quails [63]. There is every reason to believe that selection of camelina to reduce the GL content in seeds will open up broader prospects for the use of this crop in poultry feeding.

Finally, it is worth noting that in recent years, the beneficial effects of moderate GL doses on human have been repeatedly reported. Population studies have found that consuming a significant amount of cabbage crops reduces the risk of cancer and cardiovascular diseases. It is believed that isothiocyanates (hydrolysis products of GLs) prevent carcinogenesis, tumor growth and metastases, and also exhibit anti-inflammatory and antioxidant properties [64, 65]. Theretore, low feed doses of GLs can have similar positive effects in poultry. In particular, it was reported that allyl isothiocyanate (500 and 1000 g/t) added to the diet of broilers infested with *Eimeria maxima* improved the morphofunctional state of the intestine [33].

So, data on the toxicity and antinutritional properties of glucosinolates (GLs) and their hydrolysis products show that their high doses cause hypertrophy of the thyroid gland, hepato- and nephrotoxicity. When the dose of GLs is reduced, the function of the digestive system, feed consumption, digestibility and utilization of nutrients decrease without pronounced toxic effects. These anti-nutritional properties adversely affect the growth of young birds, decrease egg production and quality in adults and can cause fishy smell of eggs. Rapeseed and camelina and their products obtained during oil extraction contain GLs, but are valuable sources of protein necessary for feeding animals and poultry. The ability to use these products is especially important for regions where climatic conditions do not allow effective cultivation of soybeans, but more unpretentious rapeseed and camelina can be grown. To reduce the GL content in such feed additives, various methods have been developed (micronization or extrusion, microbial fermentation, in particular solid-phase). The most effective approach seems to be plant breeding to reduce the content of GLs and other antinutrients (erucic acid, sinapine). With the introduction of low-glycoside canola varieties, the problem of antinutrients in rapeseed has become less acute. However, the study of this species remains relevant for improving its quality as a fodder crop and clarifying regulations for use. In addition, these data partially compensate for the lack of such information on camelina.

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DETERMINATION OF CONSENSUS GENOTYPES BY MICROSATELLITES FOR MUSEUM ACCESSIONS OF CATTLE (*Bos taurus*)

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Abstract

DNA analysis of ancient and historical samples, including specimens stored in museum and craniological collections, is an invaluable source of genetic information for reconstructing the origin of local breeds of livestock. Given the high degree of DNA degradation in most of these samples, studies are usually conducted on the mitochondrial genome, since it is present in hundreds or even thousands of copies in a single cell. However, in some cases, the study of mitochondrial DNA (mtDNA) does not allow us to fully trace the demographic history of animal species and breeds, especially when crossbreeding is used in breeding work. An informative tool for analyzing these types of demographic events is the study of microsatellites, or short tandem repeats (STRs). However, in microsatellite genotyping for DNA extracted from museum specimens imposes an increased risk of amplification errors. The aim of our work was to improve the algorithm for determining consensus STR marker genotypes for samples containing highly degraded DNA and to evaluate the effectiveness of the algorithm suggested for cattle craniological museum samples. The material were museum exhibits of cattle skulls dated from the end of the 19th to the first half of the 20th century and stored in the craniological collection of the Liskun Museum of Animal Husbandry (RSAU — Timiryazev Moscow Agricultural Academy). For genotyping, a multiplex panel was used which included 11 microsatellite loci recommended by the International Society of Animal Genetics (ISAG), according to protocols adopted at the Ernst Federal Research Center for Animal Husbandry. The success of amplification for each locus in the sample was assessed by calculating genotyping quality indices (QI). The most frequently occurring genotypes were coded as 1, and the genotypes that differed from those coded as 1 due to allelic drop-out (ADO) or false alleles (FA) were defined as 0. Next, the proportion was calculated of genotypes with the value 1 to the total number of repetitions. The threshold value for QI was set at 0.75. The genotypes that showed a frequency of occurrence above the threshold value for each locus were included in the consensus genotype. The algorithm was tested on 144 museum samples of black-and-white, Turano-Mongolian, pale-and-white and brown cattle. A complete profile (11 microsatellite loci) was obtained for 60.42 % of accessions. The quality of genotyping at most loci (9 out of 11 loci examined) was above 0.950, ranging from 0.951±0.011 at the TGLA122 locus to 0.995±0.003 at the BM2113 locus. An assessment of genotyping efficiency showed that the TGLA53 and BM1818

loci had the lowest genotyping success (74.86 % and 61.45 %, respectively). A positive correlation at the trend level ($r^2 = 0.53$, $p = 0.09$) between the size of alleles at the locus and the proportion of genotyping errors was revealed. Since studying the allele pool of populations is impossible without obtaining correct genotypes, our proposed algorithm, which ensures the probability of correct genotyping $p < 0.001$, can be used when working with museum and other samples containing highly degraded DNA.

Keywords: microsatellites, genotyping errors, consensus genotype, cattle, museum samples

DNA analysis of ancient and historical samples is an invaluable source of genetic information for reconstructing the origins of local breeds [1-3]. Particularly valuable specimens are accessions from museum [4, 5] and craniological collections [6]. Given substantial DNA degradation in most of these samples, the mitochondrial genome is typically studied [7-9], since it is present in hundreds or even thousands of copies per cell. However, in some cases, mitochondrial DNA (mtDNA) analysis fails to fully trace the demographic history of animal species and breeds [10, 11]. For example, creation of some domestic cattle breeds practiced introductory crossings with bulls of foreign breeds [6]. Mitochondrial genome analysis is not helpful in estimating such events and tracing involvement of foreign breeds in the allele pool formation of modern breed populations, since mtDNA has a maternal type of inheritance. An informative tool for analyzing these types of demographic events is the study of microsatellites, or short tandem repeats (STR) [12]. Microsatellite analysis is recognized as the gold standard for pedigree testing in cattle breeding programs [13], and has provided a wealth of information on the genotypes of modern local and transboundary breeds. The availability of large genetic data sets from modern populations is important for the study of historical samples because it can help trace the persistence of historical alleles in modern populations.

The main problem with microsatellite genotyping for DNA from archaeological and museum samples is amplification errors, in particular, false homozygotes, or allele drop-out (ADO), and false alleles (FA) [14, 15] which are polymerase chain reaction (PCR) artifacts {16]. To improve the genotyping reliability for highly degraded or low-concentration DNA, a multiplex approach based on repeated independent amplifications of each DNA sample has been proposed [15, 17]. Compared to the standard procedure, this technique allows the error to be quantified for each possible genotype [17].

Despite numerous investigations of the cattle allele pool in Russia, until recently there were no protocols for constructing consensus genotypes by microsatellite markers for samples from craniological collections. Therefore, a retrospective investigation of the genetic pool in ancestral populations of local breed was unavailable. Studies of phylogenetic relationships between archaeological samples and modern breeds using STR markers have already been carried out around the world. However, very labor-intensive and expensive methods were used with a large number of repetitions of DNA extraction and amplification of single loci for correct genotyping [2].

This paper is the first to describe and validate a genotyping protocol we have developed for museum specimens that most efficiently provides correct data for microsatellites.

Our goal was to improve the algorithm for determining consensus STR marker genotypes for highly degraded DNA and to evaluate its effectiveness on museum samples of cattle.

Materials and methods. DNA was isolated from museum exhibits of cattle skulls, dated from the end of the 19th to the first half of the 20th century (the craniological collection of the Liskun Museum of Animal Husbandry RSAU—

Timiryazev Moscow Agricultural Academy, Moscow, 1950). The study involved 144 museum specimens of biomaterial from black-and-white, Turano-Mongolian, pale-and-white and brown cattle genotyped in 2019-2021.

Sample preparation and DNA extraction were performed as described [18]. Teeth separated from skulls were washed with detergent and distilled water to remove present-day DNA contamination. Using a Dremel 3000-15 mini-drill (Dremel, USA) with a diamond bur, the tooth was sawed lengthwise at minimum speed, drilling out the powder from the inner part. The powder was dissolved in the lysis solution, impurities that inhibit PCR were washed away, and a purified DNA extract was prepared using commercial kits Prep Filer™ BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific, Inc., USA), COrDIS Decalcine extract (GORDIZ LLC, Russia), M-sorb-bone (Syntol LLC, Russia), QIAamp DNA Investigator Kit (Qiagen, USA) as recommended by the manufacturers. The quality of the resulting DNA was assessed by measuring double-stranded DNA (dsDNA) concentrations (Qubit™ fluorimeter, Invitrogen, Life Technologies, USA) and the $OD_{260/280}$ ratio (NanoDrop™ 8000 spectrophotometer (Thermo Fisher Scientific, Inc., USA). Given that increasing the quantity of good quality DNA reduces the risk of erroneous genotyping [19], a dsDNA concentration of at least 1 ng/μl and an $OD_{260/280}$ of 1.6-2.0 were the thresholds chosen for microsatellite analysis. DNA preparations that did not meet these requirements were not involved in the study.

Samples were genotyped using a multiplex panel with 11 microsatellite loci TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA122, INRA23, TGLA126, BM1818, ETH225, BM1824 recommended by the International Society of Animal Genetics ISAG [20] according to the protocols adopted at the Ernst Federal Research Center for Animal Husbandry — VIZH [21].

Multiplex PCRs were run in a final 10 μl reaction mix in PCR buffer with 200 mM dNTP, 1.0 mM $MgCl_2$, 0.5 mM primer mixture (the sequences are given at https://strbase-archive.nist.gov/cattleSTRs.htm), 1 unit Taq polymerase (Dialat LLC, Russia) and 1 μl of genomic DNA (> 1 ng/μl). PCR was performed as follows: initial denaturation at 95 °C for 4 min; 95 °C for 20 s, 63 °C for 30 s (35 cycles); 72 °C for 1 min; the final elongation at 72 °C for 10 min. The size of the resulting fragments was determined (a genetic analyzer ABI3130xl with a GeneScan™ 350 ET ROX™ fragment length standard, GeneMapper™ v. 4 software; Applied Biosystems, USA). Allele sizes were standardized in accordance with ISAG STR typing comparative testing for the species *Bos taurus* 2018-2019.

A modified multiplex approach proposed previously was used as a prototype to determine consensus genotypes [22, 23]. Genotyping quality indices (QI) for each sample/locus were calculated according to S. Miquel et al. [24].

The most frequently occurring genotype at each locus was assigned a code of 1. Genotypes that differed from the most frequent genotype due to allele drop-out (ADO) or false alleles (FA) were considered genotyping errors and designated as 0. The proportion of genotypes with 1 vs. the total number of replicates was calculated. The threshold value for QI was set at 0.75.

Amplification efficiency (+PCR fraction) was calculated as the number of successful PCRs (+PCR) divided by the number of PCR replicates for each locus, expressed as a percentage. To calculate the percentage of unsuccessful amplifications, the percentage of +PCR was subtracted from 100%. The proportion of lost alleles (ADO) and the proportion of false alleles (FA) were calculated using the protocol proposed by T. Broquet and E. Petit [25]. The proportion of ADO for each locus was calculated for heterozygous genotypes (according to the corresponding consensus genotypes) as the number of replicates in which one allele was

lost divided by the total number of +PCRs. FA was calculated for homozygous or heterozygous genotypes as the number of PCRs with spurious alleles divided by the total number of +PCRs.

To analyze genotyping data for false results that affect the estimation of population parameters [26, 27], error rates (ER) were calculated as the number of genotypes differing from the consensus, divided by the total number of +PCRs. The probability of correct genotyping (p) for each locus was calculated as described by G. He et al. [28]. In addition, the means and standard errors of the QI genotyping quality index (M±SEM) values were calculated for each locus. Based on the assessed genotyping quality, Pearson correlation coefficients (r^2) were calculated to assess the relationship between the allele length and the proportion of genotyping errors in loci.

Results. Data on the studied microsatellite loci are summarized in Table 1.

1. Characterization of microsatellite loci for multiplex genotyping of cattle craniological museum samples (the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950)

Locus	BTA	The number of replicates		Allele length, bp	
		min	max	min	max
BM1818	23	13	25	256	280
BM1824	1	10	18	174	190
BM2113	2	12	23	121	143
ETH10	5	14	22	209	225
ETH225	9	19	29	140	160
INRA023	3	10	25	192	222
SPS115	15	17	28	240	262
TGLA122	21	14	38	137	185
TGLA126	20	12	22	105	125
TGLA227	18	9	25	71	103
TGLA53	16	17	40	150	196

N o t e. BTA — *Bos taurus* autosome.

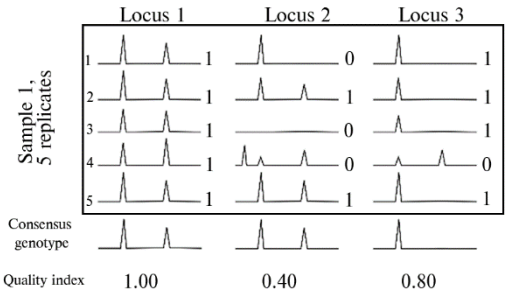

Fig. 1. Scheme for asessing the genotyping quality index (QI) for multiplex genotyping of cattle craniological specimens (the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950).

The scheme for assessment of quality indices for loci is shown in Fig. 1. In locus 1, the profiles of all c clearly show two alleles with different intensities. At locus 2, the most frequent genotype is visualized in replicates 2 and 5 (scored as 1). In replicate 1 for this locus there is an allele dropout (ADO), in replicate 4 a false allele (FA), replicate 3 shows no amplification, so all these replicates are scored as 0. In locus 3 in replicate 4 a false allele is observed (repeat score 0), in all other replicates a clear homozygous profile is visible (score 1). Thus, the QI value for these three loci were 1.00, 0.40 and 0.8, respectively.

Using museum accessions of cattle skulls as an example, we propose a modified algorithm to determine consensus genotypes for complex specimens (Fig. 2). After the initial multiplex amplification of microsatellite loci in duplicate, only those samples in which at least six loci were successfully amplified (+PCR) were selected for further analysis. For such samples, three additional independent PCR replicates were run using the same DNA preparations. Thus, each DNA sample was analyzed in at least five replicates. For samples in which less than six loci were amplified or less than four positive PCR results were obtained at each locus, DNA was re-extracted and PCR were performed as described above.

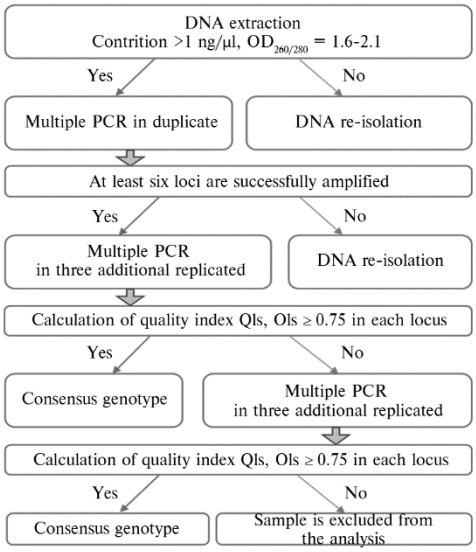

Fig. 2. Scheme for determination of consensus genotypes by microsatellites for cattle craniological specimens using multiplex genotyping (the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950).

As Figure 2 shows, samples having a QI = 0.75 or higher at each locus were considered correctly genotyped and were used in further analysis. For samples with a QI value less than the established threshold (QI < 0.75) at any of the loci, three additional multiplex PCRs were performed using the same DNA preparations, after which the quality indices were recalculated. In case the quality indices were low again (QI < 0.75), DNA was re-isolated and the multiplex PCR was performed as described above if a particular sample is extremely valuable, for example when the breed under study is represented by only 1-2 individuals, or were excluded from further analysis.

2. Efficiency of genotyping by microsatellites of cattle craniological specimens (n = 144, the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950)

Number of successfully genotyped loci	Number of specimens, n	Specimen proportion, %
11	87	60.42
10	21	14.58
9	9	6.25
8	3	2.08
7	3	2.08
6	3	2.08
< 6	27	18.75

The dsDNA concentration in the total sample varied from 1.01 to 63.40 ng/μl, the $OD_{260/280}$ from 1.64 to 2.00. As shown in Table 2, for 60.42% of the 144 genotyped specimens, a complete profile for 11 microsatellite loci was obtained, 14.58 and 6.25% of specimens were successfully genotyped for 10 and 9 microsatellite loci, respectively. For 18.75% of specimens, 5 or fewer loci were successfully genotyped, and as a result, these samples were not further investigated.

3. Quality of genotyping by microsatellite loci (QI index) of cattle craniological specimens (n = 144, the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950)

Locus	QI (M±SEM)	QI = 1		0,75 ≤ QI < 1,00		QI < 0,75		p
		1	2	1	2	1	2	
TGLA227	0.966±0.009	110	87.30	11	8.73	5	3.97	p < 0.001
BM2113	0.995±0.003	122	96.83	3	2.38	0	0.00	p < 0.001
TGLA53	0.759±0.030	68	53.97	20	15.87	38	30.16	p < 0.001
ETH10	0.972±0.008	112	88.89	10	7.94	4	3.17	p < 0.001
SPS115	0.953±0.011	104	82.54	18	14.29	4	3.17	p < 0.001
TGLA122	0.951±0.011	107	84.92	10	7.94	9	7.14	p < 0.001
INRA23	0.952±0.013	107	84.92	14	11.11	5	3.97	p < 0.001
TGLA126	0.960±0.009	106	84.13	15	11.90	5	3.97	p < 0.001
BM1818	0.700±0.040	82	65.08	5	3.97	39	30.95	p < 0.001
ETH225	0.953±0.017	115	91.27	5	3.97	6	4.76	p < 0.001
BM1824	0.961±0.013	113	89.68	7	5.56	6	4.76	p < 0.001

N o t e. 1 — number of samples, n, 2 — proportion of samples, %; p — probability of correct genotyping according to G. He et al. [27].

As Table 3 shows, the quality index of genotyping, assessed by the average values of the quality index QI, in most loci, 9 out of 11 studied, was above 0.950, ranging from 0.951±0.011 for the TGLA122 locus to 0.995±0.003 for the BM2113 locus. In two loci, TGLA53 and BM1818, the genotyping quality index was significantly lower, QI = 0.759±0.030 and QI = 0.700±0.040, respectively. The proportion of specimens with genotyping quality below the threshold value of 0.75 for these loci was maximum and amounted to 30.16% for TGLA53 and 30.95% for BM1818. The probability of correct genotyping for all studied loci was $p < 0.001$.

4. Efficiency and distribution of errors in genotyping of cattle craniological specimens by microsatellite loci ($n = 144$, the craniological collection of the Liskun Museum of Animal Husbandry RSAU—Timiryazev Moscow Agricultural Academy, Moscow, 1950)

Lous	Number of specimens, *n*	No amplification, %	ADO, %	FA, %	ER, %
TGLA227	125	4.33	4.20	0.73	4,23
BM2113	125	3.49	0.53	0.14	0,58
TGLA53	110	25.14	15.53	2.43	14,37
ETH10	126	3.63	3.93	0.43	2,17
SPS115	122	7.96	5.48	1.21	4,40
TGLA122	123	4.05	5.78	1.75	6,26
INRA23	121	8.38	3.89	0.76	3,66
TGLA126	122	7.40	4.60	0.90	4,22
BM1818	94	38.55	4.78	0.68	2,05
ETH225	120	9.36	2.51	0.31	2,00
BM1824	123	6.15	2.32	1.19	2,68
For 11 loci (*M*±SEM)		10.77±0.35	4.68±0.30	0.94±0.12	4.11±0.24

N o t e. Number of samples, *n* — number of animal skulls for which a genotype for the corresponding locus was idetified; ADO is the proportion of dropped alleles, FA frequency is the proportion of false alleles; ER is — total error rate.

An assessment of the genotyping performance for 11 microsatellite loci (Table 4) showed that the largest proportion of specimens with no amplification were detected for the BM1818 and TGLA53 loci, 38.55% and 25.14%, respectively. The BM1818 locus was successfully genotyped in a total of 94 specimens. At the other least successfully genotyped locus, TGLA53, consensus genotypes were obtained for 110 specimens, but the overall genotyping error rate (ER) was 14.37%, which was more than 3 times the average for the 11 loci examined. The number of repeated PCRs to successfully obtain a consensus genotype or to make a decision to exclude a DNA preparation varied from 5 to 8, which is comparable to the results reported for samples with DNA low concentrations or severe degradation [29-31].

We hypothesized that the high proportion of genotyping errors detected in the TGLA53 and BM1818 loci may be associated with the length of the amplified fragments, which serves as a limiting factor when working with degraded DNA. Calculations of r^2 showed a positive correlation between the length of alleles and the proportion of amplification errors, significant at the trend level ($r^2 = 0.53$, $p = 0.09$). Other researchers, when analyzing DNA from museum feather samples [32] or skin fragments from stuffed animals [33], also revealed a relationship between the size of the amplified fragment and the success of amplification.

Worldwide, the ancient DNA polymorphisms are currently studied primarily by sequencing mtDNA fragments [34, 35] or the entire genome [36], however, STR markers continue to be used to analyse complex samples [37]. This report does not present our data on mtDNA and whole-genome sequencing of museum specimens, since we aimed to describe the protocol for obtaining consensus genotypes using microsatellites as a type of DNA marker that remains the most used and accessible to a large number of laboratories.

Investigation of the population allele pool is impossible without obtaining

correct genotypes. The algorithm we described provides a probability of correct genotyping $p < 0.001$, and, therefore, can be used when working with accessions and other samples of highly degraded DNA. We also note that when studying complex samples, e.g., non-invasive samples from wild animals, museum samples, pilot studies are important to preliminaryly calculate the likelihood of genotyping errors and their probable impact on the population parameters [26, 27].

Thus, we propose a modified protocol for genotyping museum craniological specimens in which the DNA is highly degraded due to long-term storage. A multiplex panel of 11 microsatellite markers is used for analysis in one tube with calculation of the quality of genotyping and obtaining the consensus genotype for each locus separately, regardless of the other loci genotyping success in the same amplification repeat. Additionally, the success of genotyping each sample was assessed based on the average quality index in the first two amplification repeats, and a decision was made on the DNA sample prospects. The described modifications reduce the cost and labor intensity of the analysis, since, by quantifying the success of genotyping each locus and calculating the average quality index for each individuum, low-quality DNA samples were removed from the analysis at the initial stages, minimizing the number of PCR repetitions to obtain accurate genotyping data.

So, a modified multiplex PCR analysis method we suggest allows us to obtain, based on STR markers, correct consensus genotypes for cattle craniological speimens dating from the late 19th to the first half of the 20th century. We expect that the approach outlined in the article will help reduce the labor intensity and cost of historical DNA analysis and promote the involvement of museum specimens in studies of the genetic relationships between breeds of farm animals.

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ANALYSIS OF POLYMORPHISM IN THE MAJOR GENES FOR REPRODUCTIVE TRAITS IN SHEEP (*Ovis* spp.)

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Abstract

The reproductive traits significantly affect the cost of production of sheep products. The *BMP15, GDF9* and *BMPR1B* are the major genes for reproduction in sheep, mutations in which increase number of eggs per ovulation and litter size. The segregation of new mutations in an expanding breed diversity has been periodically reported. In this regard, the search for new SNPs in unexplored breeds is relevant to deepen knowledge about the genetic mechanisms underlying sheep prolificacy. In our work, for the first time, a comparative analysis of the complete nucleotide sequences of the *GDF9, BMP15* and *BMP15B* genes in the Romanov sheep was carried out in comparison with other breeds of domestic sheep (*Ovis aries* L.) and wild relatives. The most significantly varied SNPs were identified based on comparing the Romanov breed with autochthonous breeds of domestic sheep from Russia and the Persian Highlands, as well as wild *Ovis* species. Polymorphism in the major genes for reproduction in sheep was studied for argali (*O. ammon* L.) and mouflon (*O. orientalis* L.) for the first time. SNPs fixed in argali and domestic sheep were identified. The studies were conducted at Ernst Federal Research Center for Animal Husbandry in 2022-2023. We analyzed whole genome sequences of domestic sheep, Romanov (n = 9), other Russian breeds (n = 7), Iranian breeds (n = 6) and wild *Ovis* species, the Asian mouflon (*O. orientalis*, n = 16) and argali (*O. ammon*, n = 4). Alignment to the reference genome was performed using bwa-mem2 and SAMtools. The sequences of the *GDF9, BMP15,* and *BMP15B* genes were extracted from the whole genomes, in which the most different SNPs were searched based on the calculation of F_{ST} values for each SNP for each pair of groups. Gene sequences comparison of the Romanov breed with other breeds showed that the greatest differences were identified in the *BMPR1B* gene (F_{ST} = 0.562-0.749) when compared to the *BMP15* (F_{ST} = 0.051-0.374) and *GDF9* genes (F_{ST} = 0.037-0.660). Comparative analysis of gene sequences in the Romanov sheep and argali showed the presence of fixed SNPs (F_{ST} = 1), while one such SNP was identified in the *GDF9* gene. The highest F_{ST} values identified based on comparing Romanov breed sheep with mouflon were 0.702-0.780 (*BMPR1B* gene), 0.113-0.645 (*BMP15* gene) and 0.338-0.512 (*GDF9* gene). Thus, target SNPs were identified the effect of which on reproductive traits in the Romanov sheep should be studied in future work.

Keywords: SNP, candidate genes, *Ovis aries*, *Ovis ammon*, *Ovis orientalis*, domestic sheep, wild species, prolificacy

Prolificacy is an important economic trait that affects the cost of sheep

products. Mid- and high-latitude breeds still exhibit reproductive seasonality, with the reproductive season of ewes being shorter than that of rams, typically lasting from late summer to January [1]. To lengthen the reproductive season, the estrus cycle of sheep is synchronized, which is currently becoming an important element of reproduction programs [2]. However, breeders focus special attention on marker-assisted selection to fix desired alleles in target candidate genes associated with higher prolificacy in sheep. Many studies have reported a significant effect of the *BMP15*, *GDF9* and *BMPR1B* genes on reproductive traits in sheep. These genes are called the major candidate genes for sheep reproductive performance. The gene for bone morphogenetic protein 15 (BMP 15) *BMP15* and growth differentiation factor 9 (GDF9) *GDF9* are expressed in the ovaries and stimulate follicle growth [3, 4], promote the proliferation of granulosa cells [5, 6], influence cell viability signaling pathways [7, 8], and modulate other growth factors and hormones [9-11].

BMP15 is a key candidate gene controlling ovarian function. Several significant mutations have been identified in the gene, namely, $FecX^{I}$ (Fecundity Inverdale), $FecX^{H}$ (Fecundity Hanna) (12), $FecX^{B}$ (Fecundity Belclare), $FecX^{G}$ (Fecundity Galway) [13], $FecX^{L}$ (Fecundity Lacaune) [14], $FecX^{R}$ (Fecundity Rasa Aragonesa) [15, 16], and $FecX^{Bar}$ (Barbarine) [17]. The phenotypic expression of all of these mutations is generally uniform. A heterozygous genotype ensures higher prolificacy, and a homozygous genotype for the mutant allele leads to sterility. However, the French Grivette ($FecX^{Gr}$) and Polish Olkuska ($FecX^{O}$) sheep homozygous for the mutant allele have hyperprolificacy [18].

The first mutation ($FecB^{B}$ or Boorola allele) associated with fertility has been identified in the bone morphogenetic protein receptor 1B (*BMPR1B*) gene. This genetic variant, first identified in Booroola Merino, has an additive effect on the number of eggs at ovulation and a partially dominant effect on the number of lambs per lambing [19-21]. $FecB^{B}$ was then found in the Garole (or Bengal) sheep from India [22, 23], in the Javanese thin-tailed sheep in Indonesia [24], in the Hu, Small Tail Han, and Huyang, Cele, Duolang and Bayanbulak breeds from China [25-27], Bonpala from India [28] and Kalehkoohi from Iran [29].

Three mutations have been identified in the *GDF9* (*FecG*) gene, the $FecG^{H}$, $FecG^{T}$ and $FecG^{E}$. The $FecG^{H}$ (Fecundity High Fertility) [13] and $FecG^{T}$ (Fecundity Thoka) [30] mutations had a phenotypic inheritance pattern associated with the sterility of female carriers of homozygous mutations. The third mutation in the *GDF9* gene, $FecG^{E}$ (Fecundity Embrapa) associated with fecundity was identified in the Brazilian line of the Santa Inкs breed [31]. In this breed, a novel phenotype for the *GDF9* gene revealed for the first time was characterized not by sterility, but, on the contrary, by an increase in the number of eggs during ovulation in sheep homozygous for $FecG^{E}$ compared to non-mutant individuals, 2.22±0.12 vs. 1.22±0.11 with 1.78 lambs per lambing vs. 1.13 [31]. In 2014, another mutation in the protease cleavage site of *GDF9* ($FecG^{v}$, or Vacaria) was reported in the Île-de-France sheep [32]. In addition, several single nucleotide polymorphisms (SNPs) in the *GDF9* gene affect the number of lambs per lambing in the Chinese Hu local breed [33].

An analysis of scientific publications shows that in the global gene pool of sheep breeds, alleles have been identified that, both in hetero- and homozygous states are associated with increased fecundity, but the opposite effect has also been described when alleles are associated with sterility in homozygous females. Therefore, the identification of polymorphisms of the major candidate genes for reproductive qualities in sheep, especially in breeds that have not previously been studied in these aspects, is extremely important for practical breeding and a better understanding of the genetic mechanisms underlying the control of the reproductive

function of sheep.

In this work, we for the first time compared the complete nucleotide sequences of the *GDF9*, *BMP15* and *BMP15B* genes in highly prolific Romanov sheep, other domestic sheep breeds and their wild relatives. We have identified SNPs that differ most significantly between the Romanov sheep, autochthonous domestic sheep from Russia and the Persian Highlands, and wild species of the genus *Ovis*. Polymorphisms in the major candidate genes for reproductive qualities in argali (*O. ammon*) and mouflon (*O. orientalis*) are reported for the first time, and SNPs fixed in argali and domestic sheep are revealed.

The purpose of the work is to study polymorphism in the major genes of reproductive traits (*GDF9*, *BMP15* and *BMP15B*) in highly prolific Romanov breed sheep compared to low- prolific breeds and their wild relatives.

Materials and methods. The studied sample included domestic sheep (*Ovis aries* L.) of the Romanov breed (n = 9), Russian breeds Tushin (n = 3) and Karachaev (n = 4), and Iranian breeds Afshari, Ghezel, Gray Shiraz, Shal, Moghani, Karakul breed from a population bred in Iran (n = 6). Wild species were Asian mouflon (*Ovis orientalis* L.) (n = 16) and argali (*O. ammon* L.) (n = 4).

Ear tissue specimens of Tushin (n = 4), Karachaev (n = 4), Romanov (n = 9) sheep and argali (n = 4) were provided by the UNI Bank of Genetic Material of Domestic and Wild Species of Animals and Birds as part of the network bioresource collection of the sBRK SKhZh (Ernst Federal Research Center; agreement with the Ministry of Education and Science of Russia No. 075-15-2021-1037 of September 28, 2021).

DNA was isolated using the DNA-Extran-2 kit (Syntol LLC, Russia). The DNA concentration (a Qubit® 4.0 fluorometer, Invitrogen/Life Technologies, USA) and quality ($OD_{260/280}$, a NanoDropTM 8000 spectrophotometer, ThermoFisher Scientific, Inc., USA) were assessed. The minimum amount of DNA to create sequencing libraries is 3 μg, so the threshold DNA concentration was 30 ng/μl with at least 100 μl volume. The optimal $OD_{260/280}$ was 1.8 and higher. At lower concentrations, DNA was re-isolated to increase the amount of starting material.

For whole-genome sequencing, NGS (next generation sequencing) method was used (NovaSeq 6000 sequencer, Illumina, Inc., USA). Sequencing libraries were prepared with TruSeq DNA Nano Library Prep kits (Illumina, Inc., USA) and Accel-NGS® 2S Plus DNA Library Kit (IDT) for Illumina® Platforms (Swift Biosciences, Inc., USA).

Whole genome sequences of Iranian sheep breeds were provided by Professor A. Esmailizadeh.

Whole genome sequences of mouflons (*O. orientalis*) were downloaded from the publicly available online NCBI database (project PRJNA624020, ID 624020) [34, 35]. Alignment to the reference genome Ovis_aries_rambouillet.Oar_rambouillet_v1.0.dna.toplevel.fa.gz (https://www.ens-embl.org/Ovis_aries_rambouillet/Info/Index/,?db=core) was performed using bwa-mem2 tools [36] and SAMtools [37]. From complete genomes, complete nucleotide sequences of candidate genes for sheep reproductive traits (*GDF9*, *BMP15* and *BMP15B*) were extracted and searched for genetic variants. The genes were found according to their coordinates in the reference genome Ovis_aries_rambouillet.Oar_ram-bouillet_v1.0.dna.toplevel.fa.gz. The coordinates included the chromosome number, the nucleotide position of the beginning of the gene, the nucleotide position of the end of the gene, and the indication of the DNA chain (1 means forward, –1 means reverse). The coordinates were X:56594565-56601245: 1 for the *BMP15* gene (Ensembl entry ENSOARG00020012408); 5:46544645-46547585: 1 for the *GDF9* gene (Ensembl entry ENSOARG00020021050); 6:33990928-34214488: 1

for the *BMP15B* gene (Ensembl entry ENSOARG00020020206.1). Direct extraction of complete sequences of the studied genes was carried out using the bwa-mem2 and SAMtools tools according to the author's scripts.

The comparison groups were Romanov breed vs. other Russian breeds, Romanov breed vs. Iranian breeds, Romanov breed vs. mouflon, and Romanov breed vs. argali.

The most divergent SNPs in the analyzed genes were detected by calculation of F_{ST} values for each SNP within each gene, using the R package StAMPP [38].

Results. Based on F_{ST} values, SNPs were identified within the *GDF9*, *BMP15* and *BMP15B* genes, which were the most different when comparing the nucleotide sequence of these genes in Romanov sheep and a group including sheep bred in Russia and Iran (Table 1).

1. SNPs in candidate genes for reproductive traits of *Ovis aries* the most different between Romanov sheep and breeds with low prolificacy (Ernst Federal Research Center for Animal Husbandry — VIZh, 2020-2023)

Comparison group	SNP	SNP position	F_{ST}
Gene *BMP15* located on X chromosome			
Romanov breed (n = 9) and Iranian breeds (n = 6)	rs400940002	56599692	0.374
	rs426251007	56600582	0.185
	rs1090246541	56597586	0.149
	X:56597710	56597710	0.146
	rs1086873546	56596059	0.051
Romanov breed (n = 9) and Russian breeds (n = 7)	rs55628000	56595188	0.126
	X:56597676	56597676	0.111
	X:56597286	56597286	0.109
	X:56599717	56599717	0.109
	X:56600871	56600871	0.065
Gene *GDF9* located on chromosome 5			
Romanov breed (n = 9) and Iranian breeds (n = 6)	rs160076413	46545932	0.580
	rs418388291	46546176	0.490
	rs421019907	46545415	0.416
	rs399579080	46545431	0.369
	rs594156088	46544743	0.037
Romanov breed (n = 9) and Russian breeds (n = 7)	rs160076413	46545932	0.660
	rs418388291	46546176	0.432
	rs421019907	46545415	0.309
	rs399579080	46545431	0.227
	rs160076408	46545688	0.114
Gene *BMPR1B* located on chromosome 6			
Romanov breed (n = 9) and Iranian breeds (n = 6)	rs409507123	34036290	0.749
	rs421851559	34036546	0.749
	rs425275620	34009200	0.703
	rs410857597	34053188	0.630
	rs426525353	34035758	0.609
Romanov breed (n = 9) and Russian breeds (n = 7)	rs400119613	34016579	0.701
	6:34091908	34091908	0.623
	6:34092013	34092013	0.623
	6:34113605	34113605	0.562
	rs417658413	34113624	0.562

In general, the greatest differences in nucleotide sequences are identified in the *BMPR1B* gene, F_{ST} from 0.609 to 0.749 for the Romanov breed vs. Iranian breeds and F_{ST} from 0.562 to 0.701 for the Romanov breed vs. other Russian breeds. In the *BMP15* genes with F_{ST} from 0.051 to 0.374 and from 0.065 to 0.126, respectively, and the *GDF9* genes with F_{ST} from 0.037 to 0.580 and from 0.114 to 0.660 the differences are significantly smaller.

Analysis of the nucleotide sequences of the studied genes in Romanov sheep and argali showed fixed SNPs (F_{ST} = 1), while only one such SNP was identified in the *GDF9* gene (5:46545406) (Table 2). The highest F_{ST} values for SNPs identified when comparing Romanov sheep with mouflon were 0.702-0.780 for the *BMPR1B* gene, 0.113-0.645 for the *BMP15* gene and 0.338-0.512 for the

GDF9 gene).

2. SNPs in candidate genes for reproductive traits the most different between Romanov sheep (*Ovis aries*) and wild relatives mouflon and argali (Ernst Federal Research Center for Animal Husbandry — VIZh, 2020-2023)

Comparison group	SNP	SNP position	FST
Gene *BMP15* located on X chromosome			
Romanov breed (n =9) and	rs400940002	56599692	0.645
mouflon (*O. orientalis*) (n = 16)	rs403715147	56597068	0.511
	X:56597710	56597710	0.260
	rs420350765	56599601	0.159
	X:56596503	56596503	0.113
Romanov breed (n =9)	rs422668280	56595720	1
and argali (*O. ammon*) (n = 4)	X:56595843	56595843	1
	X:56595928	56595928	1
	rs412479434	56597103	1
	X:56597429	56597429	1
	X:56598037	56598037	1
	X:56598317	56598317	1
	rs417053670	56599070	1
Gene *GDF9* located on chromosome 5			
Romanov breed (n =9) and	rs399579080	46545431	0.512
mouflon (*O. orientalis*) (n = 16)	rs421019907	46545415	0.481
	5:46546592	46546592	0.373
	5:46546650	46546650	0.338
Romanov breed (n =9)	5:46545406	46545406	1
and argali (*O. ammon*) (n = 4)	rs425601341	46546485	0.767
	5:46546592	46546592	0.767
	rs160076408	46545688	0.746
	rs427433335	46546966	0.680
Gene *BMPR1B* located on chromosome 6			
Romanov breed (n =9) and	rs424055720	34152408	0.780
mouflon (*O. orientalis*) (n = 16)	rs400936557	34121377	0.772
	rs400817842	34051068	0.744
	rs414227223	34059131	0.734
	rs400453556	34053288	0.705
	rs408680692	34058728	0.702
	rs403920069	34059147	0.702
Romanov breed (n = 9)	rs420236481	33995134	1
and argali (*O. ammon*) (n = 4)	6:34002894	34002894	1
	rs402116959	34003208	1
	rs401004280	34003416	1
	rs428477215	34005483	1

Some SNPs were identified in more than one comparative analysis. The SNPs rs400940002 and X:56597710 in the *BMP15* gene differed between the Romanov sheep and both Iranian breeds and mouflon.

We identified the largest number of SNP matches in the *GDF9* gene. SNPs rs160076413 and rs418388291 were among the most divergent when comparing the Romanov sheep with both Iranian and Russian breeds. SNPs rs421019907 and rs399579080 differed when comparing the Romanov breed with both domestic sheep and mouflon breeds. SNP 5:46546592 was identified when comparing the sequences of this gene in the Romanov breed with mouflon and argali, but the difference with argali was higher, F_{ST} = 0.767 vs. F_{ST} = 0.373. Interestingly, SNP rs160076408 coincided when compared with both Russian breeds and argali.

In the *BMPR1B* gene, all identified SNPs were unique for each of the compared groups.

Next, genotypes were identified in positions with F_{ST} = 1 (Table 3). In the studied domestic sheep and argali, exclusively opposite homozygous genotypes were found. Polymorphism in a number of analyzed SNPs was detected only in Asian mouflons. In other SNPs in which there was no polymorphism, the mouflon genotype corresponded to the genotype of domestic sheep.

3. Genotypes for SNPs in the studied samples of domestic sheep (*Ovis aries*), mouflon (*O. orientalis*) and argali (*O. ammon*) (Ernst Federal Research Center for Animal Husbandry — VIZh, 2020-2023)

SNP	Identified genotypes		
	O. aries	*O. ammon*	*O. orientalis*
Gene *BMP15* located on X chromosome			
rs422668280	GG	AA	GG
X:56595843	CC	TT	TT, CC
X:56595928	TT	CC	TT
rs412479434	AA	TT	AA
X:56597429	GG	AA	GG
X:56598037	CC	TT	CC
X:56598317	AA	GG	AA
rs417053670	GG	AA	GG
Gene *GDF9* located on chromosome 5			
5:46545406	AA	CC	AA
Gene *BMPR1B* located on chromosome 6			
rs420236481	AA	CC	AA, CA
6:34002894	GG	AA	GG, AG
rs402116959	CC	GG	GG, CC, GC
rs401004280	AA	TT	AT, TT, AA
rs428477215	GG	AA	AG, GG

Reproductive traits, ultimately expressed in the number of lambs per lambing, significantly influence the profitability of sheep farming. Because most ewes produce one lamb, identifying the genes responsible for specific fertility traits is of great scientific and economic interest. The genes *SPOCK1* for age of first estrus, *GPR173* for mediator of ovarian cyclicity, *HB-EGF* for signals about successful onset of pregnancy, *SMARCAL1* and *HMGN3a* which regulate gene expression during embryogenesis [39], *B4GALNT2* for follicle development, the $FecL^L$ mutation has been described in the Lacaune breed [40], are considered as potential candidates influencing reproductive traits. In addition, some genes are not discussed as likely candidates for marker selection, but they may influence reproductive traits to varying degrees, in particular the genes *ESR1* [41], *FSHR* [42], *FTF* or *NR5A2* [43].

Nevertheless, it is the *BMP15*, *GDF9* and *BMPR1B* genes that continue to attract the greatest interest. For example, attempts have been made to link known mutations in these genes, especially the *GDF9* gene, in sheep bred in Russia. The frequencies of alleles of the *GDF9* gene (polymorphism c.260G>A) were studied in the Altai Mountain [44], Dagestan Mountain [45] breeds, and in the Manych Merino [46].

In the Romanov breed, genetic screening for the main mutations in the *BMP15* gene, the $FecX^G$, $FecX^H$, $FecX^I$, $FecX^L$, $FecX^B$, and in the *GDF9* gene revealed the absence of these mutant alleles in all animals in the sample [47]. Continuing screening in an expanded sample also did not bring a success [48]. However, it should be noted that in general these results are consistent with our data, since none of the identified SNPs coincided with previously known substitutions. It is likely that unique reproductive traits may be associated with genetic variants in other genomic regions.

Other researchers [44-48] have studied polymorphisms at established positions that have a known effect in certain breeds, which does not always guarantee positive results in other breeds. Therefore, we chose a different methodology based on the complete sequencing the *BMP15*, *GDF9* and *BMPR1B* genes to study polymorphisms in each SNP in these genes and identify SNPs. most significantly different between highly- and low prolific sheep breeds. We plan further research to determine whether these substitutions affect reproductive traits in Romanov sheep.

In addition, our work was the first to analyze the complete sequences of

the *BMP15*, *GDF9* and *BMPR1B* genes in argali (*O. ammon*) and mouflon (*O. orientalis*). It should be noted that the specific distribution of genotypes by fixed SNPs in mouflon, that is, common allelic variants with argali, could be due to introgression events that occurred before or after domestication. This hypothesis is consistent with complete genome sequencing of wild species, which revealed adaptive introgression from argali into the genomes of European and Asian mouflon [49].

Thus, here, we compared polymorphisms in the candidate genes *GDF9*, *BMP15* and *BMP15B* for reproductive traits of highly prolific Romanov sheep, low prolific breeds and wild species of the genus *Ovis*, the argali and mouflon. SNPs have been identified that differ most significantly between the Romanov breed, Russian and Iranian breeds, and wild species. Polymorphisms in the main candidate genes for reproductive traits in argali (*O. ammon*) and mouflon (*O. orientalis*) were examined for the first time, and SNPs fixed in argali and domestic sheep were identified.

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DEVELOPMENT OF MULTIPLEX PANEL OF MICROSATELLITES FOR GENETIC STUDIES OF SIBERIAN STURGEON (*Acipenser baerii*) BRED IN COMMERCIAL AQUACULTURE

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Abstract

The Siberian sturgeon (*Acipenser baerii* Brandt, 1869) is one of the most important aquaculture fish species in Russia. Due to the high demand for valuable commercial products, breeding of Siberian sturgeon is promising in the industry. However, breeding this species is significantly complicated by because of its tetraploid genome, which, together with the need to mix fidh roe and sperm from several producers when obtaining offspring, prevents the introduction into practice of well-proven molecular genetic methods, e.g. microsatellite analysis. In this work, for the first time, the known microsatellite loci in the Siberian sturgeon are characterized from the point of view of the possibility of effectively accounting for the doses of their alleles in the tetraploid genome. Seven loci were found that met this criterion. The goal of our work was to create a panel of microsatellite markers adapted for use in the selection of Siberian sturgeon from the Lena population. The research was carried out in 2023. As biological material, we used sections of fin tissue of the Siberian sturgeon of the Lena population, taken from the fish of an experimental herd kept in a closed water supply installation of the Ernst Federal Research Center for Animal Husbandry. The experimental herd contained fish from the Mozhaisk production and experimental fish hatchery (Goretovo village, Mozhaisk urban District, Moscow Province; group I, $n = 42$) and fish obtained from RTF Diana LLC (village Kaduy, Kaduysky District, Vologda Province; group II, $n = 47$). DNA was isolated using the DNA-Extran-2 kit (NPK Synthol, Russia) according to the manufacturer's protocol. Qualitative assessment of DNA was carried out by electrophoresis in 1.2 % agarose gel. PCR was performed in a Thermal Cycler SimpliAmp amplifier (Thermo Fisher Scientific, Inc, USA). Electrophoretic separation of amplification products was carried out in a Nanofor 05 capillary electrophoresis system (NPK Synthol, Russia). Allele sizes were determined using GeneMarker software (Version 3.0.1). For each locus, the dose of each allele was determined. Twenty seven microsatellite markers known for sturgeon fish species were used (Ls 19, Aox 45, Aox 9, Ls 68, Agu 38, Ag 49a, Agu 37, Agu 41, Agu 15, Agu 51, Agu 59, Agu 34, Agu 36, Agu 46, Agu 56, Agu 54, AoxD 161, AfuG 63, AfuG 51, AfuG 112, An 20, Aru 13, Aru 18, Afu 68 b, Spl 163, AfuG41, and Ls 39). Of these, seven were selected for multiplex panels (Agu 38, An 20, Aru 18, Ls 19, Ag 49a, Agu 37, Agu 41). Based on the polymorphism of seven microsatellite loci for the two studied groups of Siberian sturgeon individuals, classical population genetic indicators were calculated, the average number of alleles per locus (Na = 6.86), the number of effective alleles (Ne = 3.61), observed ($H_o = 0.839$) and expected ($H_e = 0.6535$) heterozygosity. In the studied groups, inbreeding was not revealed ($F_{IS} = -0.340$ and -0.173) while a significant genetic differentiation occurred (Nei's GD = 0.1340, Fst = 0.0796). The groups formed two clear, practically non-overlapping PCA clusters despite the fact that the ancestors of Siberian sturgeon individuals in both groups were of related origin. The contribution of the allele dose of the tetraploid locus to the efficiency of microsatellite analysis was assessed. On average, the information content of each locus increased by 32 %. A comparison of the results of genetic analysis with the available research publications allows us to assume that in aquaculture

herds of Siberian sturgeon from the Lena population, processes associated with changes in allele frequencies of microsatellite loci occur, which gradually enhance their genetic differentiation. As a result of the work, the high efficiency of the created panels of microsatellite markers and their potential suitability for use in genetic certification were confirmed, each individual Siberian sturgeon had its own genetic profile. The distribution of alleles at seven microsatellite loci indicated a unique genetic structure in Siberian sturgeon stocks in each of the two fish hatcheries that were sources of fish seeding material.

Keywords: Siberian sturgeon, microsatellites, tetraploids, sturgeon breeding, null alleles

Siberian sturgeon (*Acipenser baerii* Brandt, 1869) is one of the most important fish species grown in commercial aquaculture. The main advantages of the Siberian sturgeon are rapid weigh gain and high survival rate in farm conditions [1, 2]. The high-quality caviar produced by Siberian sturgeon is of special interest. Due to the economic importance and high demand for Siberian sturgeon products, its breeding has significant potential for the development of aquaculture [3].

In the wild, the Siberian sturgeon is in the status of an endangered species because of environment pollution, uncontrolled fishing, and degradation of the natural habitat. The species is listed in the IUCN (International Union for Conservation of Nature and Natural Resources,) Red List [4]. In addition, the Siberian sturgeon is included in Appendix 1 and Appendix 2 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [5]. The unsatisfactory state of wild populations dictates the need to preserve the biodiversity of the Siberian sturgeon population at fish hatcheries and wisely use the genetic potential in aquaculture [6].

Modern breeding methods rely on molecular genetics data to significantly speed up and simplify improvemrnt of agricultural animals and plants in turmes of productivity [7-9]. However, in sturgeon farming, the introduction of these technologies into everyday breeding practice has slowed down. Today, molecular genetic markers, primarily microsatellites and mitochondrial DNA, are used to control species identity for further using juveniles to replenish natural populations [10]. Developing molecular genetic panels for individual identification of Siberian sturgeon faces difficulties since the species has a tetraploid genome [11], while certain loci can exhibit a diploid or even hexaploid character [12, 13].

The direct use of molecular genetic methods in breeding Siberian sturgeon is also hampered by the long maturation period characteristic of this species, and by the mixing of reproductive material from several individuals practiced at fish hatcheries. This significantly reduces waste, increases the percentage of fertilized eggs, but also prevents the exact correlation of parents and their offspring, which is necessary in breeding. The mass individual tagging of breeder fish with electronic chips at sturgeon hatcheries promotes using molecular genetic methods in breeding.

In this work, for the first time, the known microsatellite loci in the Siberian sturgeon are characterized in turms of effective accounting for their allele doses in the tetraploid genome. Seven loci were found that met this criterion.

The goal of our work was to create a panel of microsatellite markers adapted for use in the selection of Siberian sturgeon from the Lena population.

Materials and methods. Sections of fin tissue of the Siberian sturgeon from the Lena population were collcected from the fish of an experimental herd kept in a closed water supply installation (the Ernst Federal Research Center for Animal Husbandry — VIZh, 2023). The herd consisted of fish from the Mozhaisk production and experimental fish hatchery (Goretovo village, Mozhaisk urban district, Moscow region; group I, $n = 42$) and fish from RTF Diana LLC (working Kaduy village, Kaduysky District, Vologda Province; group II, $n = 47$).

DNA was isolated using the DNA-Extran-2 kit (NPK Synthol, Russia) as described in the manufacturer's protocol. DNA quality was assessed by 1.2% agarose gel electrophoresis. PCR reaction mixture was 1.5 μl of 10× Turbo buffer

(Evrogen, Russia), 1.5 μl of a 2 mM dNTPs solution, 0.3 μl of a 10 mM primer mixture, 1 unit Smart Taq polymerase (JSC Dialat Ltd., Russia), ~ 50-100 ng of the studied genomic DNA. The reaction mixture was added with deionized water to the final volume of 15 μl. PCR was performed on a Thermal Cycler SimpliAmp amplifier (Thermo Fisher Scientific, Inc, USA) in the following mode: 10 min at 94 °C (primary denaturation); 30 s at 95 °C (denaturation), 40 s at 58 °C (annealing of primers on the DNA template), 35 s at 72 °C (chain elongation) (38 cycles); 5 min at 72 °C (final elongation). Amplification products were electrophoretically separated (a Nanofor 05 capillary electrophoresis system, NPK Synthol, Russia). Allele sizes were determined with GeneMarker software (Version 3.0.1). For each locus, the dose of each allele was determined [14].

The assembled panels were tested on groups I and II of the experimental herd of Siberian sturgeon. For each group, classical population genetic indicators were calculated, namely, expected (H_e) and observed (H_o) heterozygosity, average number of alleles per locus (Na), average number of effective alleles (Ne), coefficient of inbreeding (F_{IS}), genetic distances by M. Nei method [15], index F_{st} [16, 17].

Microsatellite analysis data were processed using R package Polysat (Version 1.7) [18], STRUCTURE (Version 2.3.4) [19], and SPA Ge Di1-5d [20] software. PCA (Principal Coordinate Analysis) plot was constructed according to R. Bruvo et al. [21] based on the genetic distances.

Results. Since we needed to create multiplex microsatellite panels with a uniform ploidy pattern of loci and the absence of null alleles, the microsatellite markers had to strictly comply with certain parameters. The locus should not have more than four alleles in one sample, should not have zero alleles, should have polymorphism and stable PCR amplification subject to multiplexing, and the dose of each allele should be visually well determined.

To create a panel of microsatellite markers for individual identification and control of the Siberian sturgeon origin, we tested 27 microsatellite loci, the Ls 19, Aox 45, Aox 9, Ls 68 [22, 23], Agu 38, Ag 49a, Agu 37, Agu 41, Agu 15, Agu 51, Agu 59, Agu 34, Agu 36, Agu 46, Agu 56, Agu 54 [24], AoxD 161, AfuG 63, AfuG 51, AfuG 112, An 20, Aru 13, Aru 18, Afu 68 b, Spl 163, AfuG41, and Ls 39 [25, 26]. A number of loci (e.g., AoxD 161, AfuG 41, Agu 59) where more than 4 alleles were identified (Fig. 1) we excluded from further analysis.

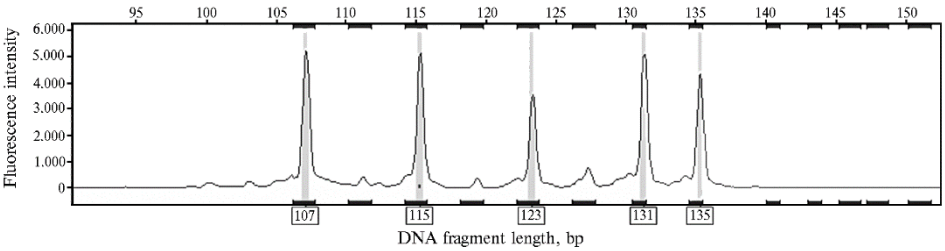

Fig. 1. Genetic profile of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) individuals of the Lena population at the AoxD 161 locus with five identified alleles (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

Null alleles are known to distort statistical calculations, overestimating homozygosity [27, 28]. Species with a polyploid genome acquires a higher risk of having null alleles [29]. Polyploidization can be caused by the fusion of genomes that are polymorphic at the primer annealing site. In addition, it is believed that the polyploidization itself stimulates transposon activity and loss of DNA sections due to genomic rearrangements [30], which can also destroy primer binding sites [31]. Therefore, determining allele dosage was a critical requirement in loci selection. Determining the dose of each allele (Fig. 2) identified loci AfuG 51, Aru 13,

Agu 15 that presumably had null alleles.

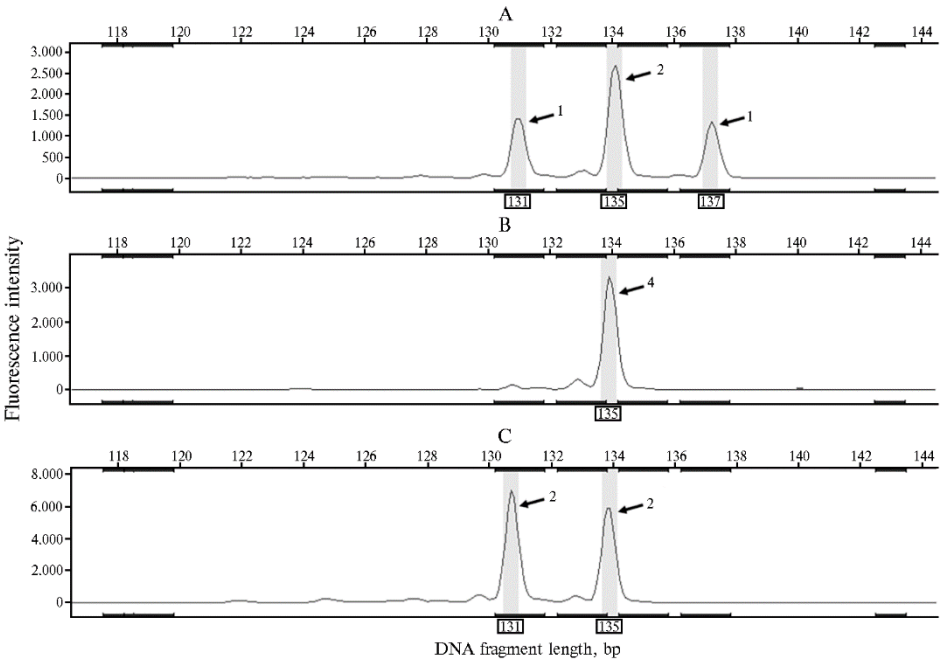

Fig. 2. Assessment of the each allele dose at the microsatellite locus Ls 19 in individuals of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) of the Lena population: A — with allele ratio of 1/2/1, B — with allele ratio of 4/0, C — with allele ratio of 2/2. Arrows indicate peaks corresponding to alleles. 1, 2, 3, 4 indicate allele doses (the number of chromosomes in a tetraploid genome carrying a given allele) (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

Thus, for the AfuG 51 microsatellite locus, three alleles with approximately the same amount of the resulting PCR product were identified (Fig. 3). Since the Siberian sturgeon genome is tetraploid, the fourth allele should most likely be null, but we observed the presence of a fourth peak with a weak signal which was probably the fourth allele with a modified primer annealing site.

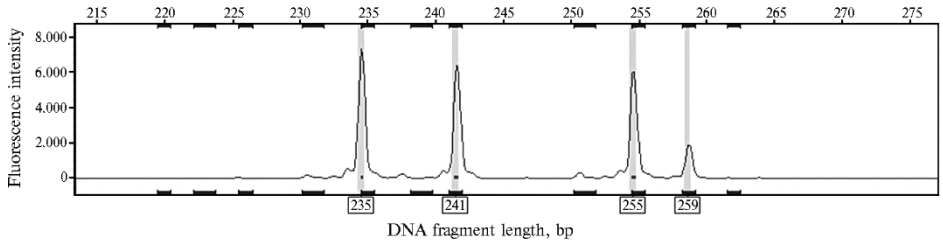

Fig. 3. Identification of the null allele on the example of the AfuG 51 locus in individuals of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) of the Lena population: 235, 241, 255 are three alleles with approximately the same efficiency in PCR, 259 is the fourth peak with a low fluorescence that probably corresponds to an allele with a modified primer annealing site (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

On a small sample of Siberian sturgeon DNA (n = 16), we preliminarily tested if microsatellite loci might be multiplexed, and whether there was a clear deviation from tetraploid inheritance. One more requirement was to retain the opportunity of accounting the dose of alleles in PCR multiplexing. Of 27 microsatellite markers, 7 loci, the Agu 38, An 20, Aru 18, Ls 19, Ag 49a, Agu 37, and Agu 41 met the requirements and was chosen for multiplex PCR (Table 1, Fig. 4). The loci were arranged into two multiplex panels, Agu 38, An 20, and Aru 18 (panel 1), Ls 19, Ag 49a, Agu 37, and Agu 41 (panel 2).

1. Microsatellite loci for testing individuals of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) of the Lena population (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023)

Locus	Dye	Allele length expected, bp [22-26]	Allele length observed, bp	Primer sequence
		Panel 1		
Agu 38	6-FAM	108-114	90-112	F: ACTGGGGTTGAAGGACAGTG R: TCCGTCTCATGTCCAAGGGTA
An 20	6-FAM	151-207	143-185	F: AATAACAATCATTACATGAGGCT R: TGGTCAGTTGTTTTTTTATTGAT
Aru 18	R6G	138-154	137-145	F: CCTGGAACACGTCCAGTTTT R: TGGGTGAATGTTTTGGTGTG
		Panel 2		
Ls 19	6-FAM	118-145	119-137	F: CATCTTAGCCGTCTGGGTAC R: CAGGTCCCTAATACAATGGC
Agu 37	R6G	128-136	124-128	F: ACATGGTAGCAAAATCCCAA R: CAGCAAGCTTAGATGCATGG
Agu 41	ROX	178-218	177-229	F: AAGACAAACAGTGGCCCAAC R: CAATGGCAGGTGCTACTGAA
Ag49a	6-FAM	198-219	192-218	F: TGTTATCTGCTCTGATATTGATTCG R: CGTTTTAAAGTTTGAACGGCA

Table 2 submits results of testing these panels in two groups of individuals from the experimental Siberian sturgeon.

2. Allele frequency for 7 microsatellite loci in individuals of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) of the Lena population (calculated in the SPAGeDi1-5d program; Ernst Federal Research Center for Animal Husbandry — VIZh, 2023)

Locus	Group	Na	Ne	H_e	H_o	F_{IS}	PIC
All (*M*±SEM)	I + II	6.86±1.506	3.61±0.731	0.6535±0.07441	0.839±0.0976	–0.191±0.0778	
	I	4.43±0.519	2.90±0.476	0.5770±0.10235	0.816±0.1413	–0.340±0.1106	
	II	6.14±1.300	3.63±0.657	0.6744±0.05663	0.860±0.0653	–0.173±0.0590	
Agu 38	I + II	6	3.45	0.7102	0.989	–0.366	0.639
	I	5	3.13	0.6809	1.000	–0.460	0.609
	II	5	3.32	0.6993	0.979	–0.359	0.608
An 20	I + II	13	6.17	0.8379	0.989	–0.167	0.813
	I	6	3.98	0.7486	1.000	–0.335	0.688
	II	12	6.11	0.8365	0.979	–0.156	0.803
Aru 18	I + II	4	1.45	0.3122	0.337	0.231	0.396
	I	3	1.05	0.0471	0.048	0.330	0.301
	II	4	1.98	0.4943	0.596	0.091	0.447
Ls 19	I + II	5	2.47	0.5953	0.854	–0.158	0.526
	I	3	2.01	0.5028	0.857	–0.397	0.429
	II	5	2.67	0.6258	0.851	–0.172	0.526
Ag 49a	I + II	6	3.56	0.7193	0.966	–0.291	0.653
	I	4	3.05	0.6723	0.976	–0.411	0.591
	II	5	3.98	0.7491	0.957	–0.233	0.687
Agu 37	I + II	4	2.33	0.5703	0.753	–0.078	0.502
	I	4	2.56	0.6090	0.833	–0.184	0.519
	II	3	2.01	0.5036	0.681	–0.028	0.449
Agu 41	I + II	10	5.86	0.8295	0.989	–0.180	0.793
	I	6	4.51	0.7781	1.000	–0.282	0.724
	II	9	5.33	0.8123	0.979	–0.185	0.767

N o t e. Group I is fish from the Mozhaisk production and experimental fish hatchery (Goretovo village, Mozhaisk urban District, Moscow Province; $n = 42$), Group II is fish from RTF Diana LLC (working village Kaduy, Kaduysky District, Vologda Province; $n = 47$). H_o — observed heterozygosity, H_e — expected heterozygosity, Na — average number of alleles per locus, Ne — average number of effective alleles per locus, F_{IS} — inbreeding coefficient, PIC— locus polymorphic information content index.

Of the seven microsatellite loci, An 20 and Agu 41 were the most effective as followes from the locus polymorphic information content index (PIC) values. Despite the fact that the domesticated fish originated from the wild Siberian sturgeon of the Lena population, we revealed a clear genetic differentiation between the studied groups. Given the fixation index $F_{st} = 0.0796$, 7.96% of the variability was due to intrapopulation differences, and 92.04% to interpopulation differences. The genetic distance calculated by M. Nei based on the allele frequencies of the

seven microsatellite loci was 0.1340. The genetic differentiation of Siberian sturgeon groups was clearly identified when clustering using the STRUCTURE program (Fig. 5) and by PCA analysis (Fig. 6).

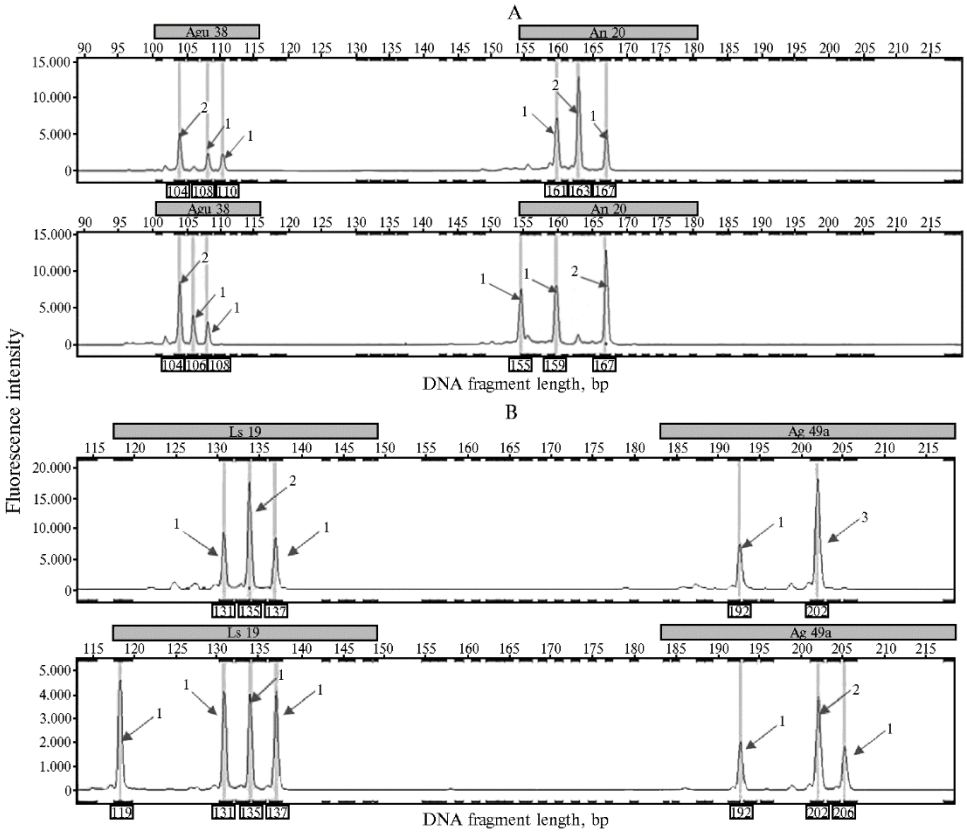

Fig. 4. An example of multiplexing loci with preservation of the allele dose effect for individuals of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) of the Lena population: A — panel 1 (loci Agu 38, An 20; FAM channel), B — panel 2 (loci Ls 19, Ag 49a; FAM channel). The arrows indicate the peaks corresponding to the alleles, 1, 2 and 3 indicate the allele doses of the (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

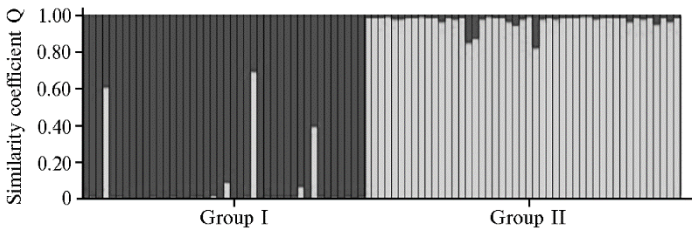

Рис. 5. Cluster analysis of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) individuals of the Lena population based of allele frequencies of seven microsatellite loci: group I — fish from the Mozhaisk production and experimental fish hatchery, Goretovo village, Mozhaisky urban District, Moscow Procine, group II — fish obtained from RTF Diana LLC (working village of Kaduy, Kaduysky District, Vologda Province). The STRUCTURE program for the number of clusters K = 2 (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

Considering the obtained values of population genetic indicators, it can be argued that the analyzed groups of Siberian sturgeon from the Lena population are not inbred. Taking into account the relatively lower values of Na, Ne, H_o and H_e, we can conclude that there is less genetic diversity of group I from the Mozhaisk production and experimental fish hatchery. This effect could result from

the fish hatchery ecomovic model targereted to the release of juvenile sturgeon to replenish natural populations and more stringent control for the producers' origin.

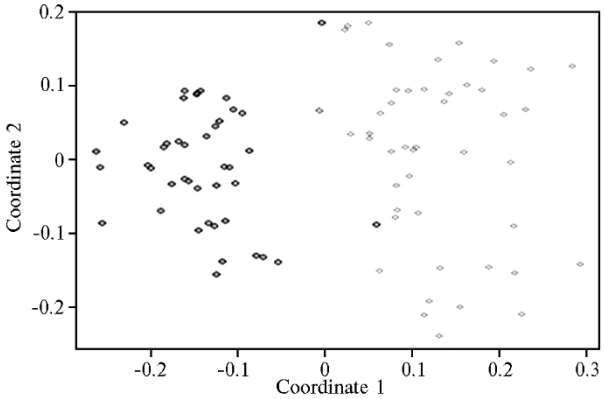

Fig. 6. Principal component analysis of the domesticated Siberian sturgeon (*Acipenser baerii* Brandt, 1869) individuals of the Lena population based on genotyping for 7 microsatellite markers. Group I **(black color)** — fish from the Mozhaisk production and experimental fish hatchery, Goretovo village, Mozhaisky urban District, Moscow Procine, group II **(gray color)** — fish obtained from RTF Diana LLC (working village of Kaduy, Kaduysky District, Vologda Province). PCA plot is built based on the calculation of genetic distances accridng to R. Bruvo et al. [21] (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023).

Since the allele dose effect for each microsatellite locus was accounted, we compared the resolution of microsatellite analysis when ignoring data on this effect and when taking it into account. When accounting for the allele dose effect, the number of genotypes increased on average by 31.75% for each locus (Table 3).

3. Number of the Lena population Siberian sturgeon (*Acipenser baerii* Brandt, 1869) genotypes as influenced by allele dose effects for 7 microsatellite loci (Ernst Federal Research Center for Animal Husbandry — VIZh, 2023)

Accounting for unique genotypes	Locus							
	Agu 38	An 20	Aru 18	Ls 19	Ag 49a	Agu 37	Agu 41	∑
Without allile dose	11	34	5	11	21	5	39	126
With allile dose	19	48	6	14	27	6	46	166

Unfortunately, it should be recognized that modern software for calculating population genetic indicators is poorly adapted to process microsatellite locus allele dose data. Thus, to convert the data file with genotyping results into the STRUCTURE and SPAGeDi program formats, we used the Polysat R package, specially designed for the analysis of polyploid genotypes. In this case, data on allele dose were removed. The decrease in the quality of the analysis, however, did not lead to critical errors in the calculation of population statistics. However, when comparing the genetic profiles of parents and offspring, this can have a significant impact and lead to the need of a larger number of microsatellite markers involved in the analysis. The situation will be especially noticeable for herds where there is a large proportion of siblings and half-siblings among the sires.

Microsatellite polymorphism of the wild Lena population of Siberian sturgeon has been studied in sufficient detail [10, 14]. Many researchers have investigated the genetic structure of aquaculture stocks of this species, but among similar works we can highlight the report of A.E. Barmintseva et al. [32] who compared the polymorphisms for five microsatellite loci in Siberian sturgeon stocks from the Lena population at nine farms. As in our study, the authors identified two clear genetic clusters, but their formation significantly differed. The authors note an unusual effect that the year of birth had a decisive influence on whether a particular

individual belonged to a cluster. Regardless of their belonging to the farm, individuals of the 1990-1996 generations grouped in a single cluster with wild representatives of the Lena population, and the aquaculture cluster was formed from individuals starting from 2001. In our study, this effect was not repeated. We obtained a clear genetic differentiation of individuals born in 2022 solely depending on their origin. The Siberian sturgeon of the Lena population was domesticated in the recent past (1993) [33]. In the work of A.E. Barmintseva et al. [32] testing was carried out on samples of the generations 1990-2008. If we take into account the rather significant time period as compared to the time of domestication (2008-2022), then a quite likely explanation may be the gradual accumulation of differences in allele frequencies in current broodstocks of the Siberian sturgeon of the Lena population at different farms due to ongoing selection. In addition, the detection of genetic differentiation could be influenced by an increase in the number of microsatellite markers used. However, these assumptions need to be tested on other samples.

Thus, multiplex panels of seven microsatellite loci that we have developed allows us to obtain for each individual Siberian sturgeon its unique genotype even without accounting the dose of each allele. This gives the opportunity to create individual genetic passports. A clear genetic differentiation between the groups of Siberian sturgeon from two different enterprises reflects the influence of the selection carried out. Such genetically differentiated herds may be useful in creation of new breeds and lines. Before releasing juvenile Siberian sturgeon into natural populations, it is advisable to compare the genetic profiles of wild and released fish to control and preserve the genetic structure of native populations. The development of software for processing data that include doses of alleles in polyploid loci is very urgent. Such a tool will significantly improve genetic analysis of polyploid species.

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GENOME-WIDE ASSOCIATION STUDIES OF CHICKEN (*Gallus gallus* L.) BREAST MEAT COLOR CHARACTERISTICS

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Abstract

One of the most important parameters of meat quality is its color characteristics, which largely determines consumer demand for these products. Special color scales are used to assess the quality of meat based on its color spectrum. The L*a*b* scale is common the effectiveness of which has been shown in meat livestock farming. A number of studies have established the genetic determination of meat color characteristics for farm animals and poultry. SNPs and candidate genes that determine the expression of this trait have been identified (J. Sun et al., 2022; X. Guo et al., 2023). Here, we submit data on genome-wide association studies of the spectrum of color parameters of breast meat of F_2 chickens of the resource population based on genome-wide genotyping data. The aim of research was to search for SNPs and identify genes associated with meat color in chickens. For the research, an F_2 model resource chickens population (n = 260, vivarium of the Ernst Federal Research Center for Animal Husbandry, 2021-2023) was obtained by crossing two chicken breeds contrasting in meat quality, the Russian White (slow growth) and Cornish (fast growth). The poultry of F_2 resource population was genotyped using high-density Illumina Chicken iSelect BeadChip 60k (Illumina, Inc., USA). At the age of 9 weeks, birds were slaughtered. The spectra of breast meat were measured according to the L*a*b* color scale using a portable spectrophotometer CM-700d (Konica Minolta, Japan). Based on the genotype and phenotype data, genome-wide association studies were carried out using PLINK 1.9 software with accepted restrictions (geno 0.1, mind 0.1, maf 0.03). The threshold significance criterion was set to $p < 0.000001$. The chickens of F_2 resource population was characterized by a high coefficient of variability in the green (a*) and blue (b*) spectrum of meat color, from 19.99 % to 97.23 %. According to the L parameter, chickens showed relatively low variability not exceeding 9.75 %. Based on the GWAS analysis, 60 significant SNPs were identified, including those associated with the color spectrum L* (28 SNPs), a* (48 SNPs), and b* (4 SNPs). These SNPs were located on chromosomes GGA1 (10 SNPs), GGA2 (3 SNPs), GGA3 (18 SNPs), GGA7 (2 SNPs), GGA8 (4 SNPs), GGA10 (2 SNPs), GGA12 (7 SNPs), GGA13 (9 SNPs), GGA17 (4 SNPs), and GGA18 (1 SNP). We identified 270 candidate genes associated with the studied traits, including 30 genes that contain the identified SNPs. The results of the study can be helpful in further genomic selection of chickens for improving meat quality.

Keywords: *Gallus gallus*, chicken, SNP, GWAS, candidate genes, meat quality, meat color, L*a*b* color scale

Progress in poultry farming is associated with high demand for the poultry products. Every year the requirements for the quality of poultry meat and its marketable yield are increasing [1, 2]. According to FAO, total production and consumption of this product is expected to increase annually by 1.8% until 2050, which is significantly higher than the expected growth in pork production and

consumption of 0.8% annually [3]. Chicken meat is a source of protein with high biological value, especially compared to plant proteins, in particular, in terms of the content of iron, phosphorus, vitamin A, thiamine, nicotinic acid [4]. In addition, the low energy value makes chicken meat a healthy food with a reduced fat content and a higher content of polyunsaturated fatty acids (PUFAs) compared to other types of meat [5].

Currently, poultry production emphasizes on improving quality for various characteristics of the final product, including appearance, texture and firmness, water holding capacity, color, pH, shelf life, collagen content, protein solubility, fat binding capacity [6]. Many of these parameters are significantly by poutry feeding, housing conditions [7], sex, age and breed [7, 8].

Color is an important indicator of meat quality and largely determines consumer demand [9]. Products with the desired color and without defects in appearance ensure better sales and final price [9, 10]. Pale, soft and exudative (PSE) meat is a color defect. PSE is becoming a growing problem in the meat industry. In the PSE condition, the water holding capacity (WHC) of the meat decreases and its texture becomes softer [11]. In broilers, meat PSE is influenced by various pre-slaughter factors, stunning methods, and cooling regimes [3, 12].

Poultry is the only animal species that has dark and light meat depending on the type of muscle. The breast meat is pale pink, the thighs and legs are dark red [13]. Direct correlations have been established between the meat color and pH. Dark meat, as a rule, has a higher pH, and very light meat has a lower pH value [14]. In the meat industry, pH also influences the PSE (pale, soft, exudative) and DFD (dark, firm, dry) appearance [15]. Fresh poultry meat is often classified as PSE based solely on high L* (lightness) color parameter and low pH, which also reduces WHC [16]. A number of studies have reported the genetic basis of meat color of farm animals and poultry, including loci of quantitative traits [17, 18], SNPs [19, 20] and candidate genes [19-22] for color parameters.

This paper results from a genome-wide association study of the color spectrum indicators of breast meat in F_2 chicken resource population. We identified novel SNPs and candidate genes that are highly significantly ($p < 0.00001$) associated with meat color parameters in chickens. In the future we plan to assess the discovered SNPs as genetic markers in breeding for chicken meat quality.

The goal of the work is to search for SNPs and identify genes associated with meat color in chickens.

Materials and methods. Experiments were carried out in 2021-2023 at vivarium of the Ernst Federal Center for Livestock Husbandry — VIZh (Moscow Province) on an F_2 chicken (*Gallus gallus* L.) model resource population (n = 260) derived from crossing Cornish meat breed and Russian White egg breed. Chicks up to 3 weeks of age were raised in brooders with a gradual decrease in temperature from 34 °C (in the first hours after hatching) to 23 °C, and then was floor-housed. The keeping conditions met the birds' age requirements and provided free access to complete feed, fresh water and normal lighting, good ventilation ensured the absence of dampness, drafts and gas pollution. The birds aged 9 weeks were slaughtered after starvation period of 8-10 h in accordance with the Russian Federation national standard the GOST R 52837-2007 "Agricultural poultry for slaughter. Technical conditions". After slaughter, the carcasses were scalded, the plumage was removed and the carcasses were deboned.

The color parameters of breast meat were measured using a portable spectrophotometer CM-700d (Konica Minolta, Japan) based on the L*a*b* system which is a three-dimensional space where negative values of a and b correspond to cold colors, positive values correspond to warm colors. The color index L characterizes the light reflection from meat surface [23]. Color spectra were recorded

24 h after slaughter at five points of the breast fillet sample and the average value was determined for each scale of the spectrum.

DNA was isolated from feather pulp using the commercial kit DNA Ekstran-2 (NPF Syntol LLC, Russia) according to the manufacturer's recommendations. The quality and integrity of the isolated DNA was assessed by a 1% agarose gel horizontal electrophoresis, the DNA purity spectrophotometrially (a NanoPhotometer® N60 spectrophotometer, Thermo Fisher Scientific, USA), samples with an $OD_{260/280} > 1.8$ were used. DNA concentration was assessed fluorometrically (a Qubit® 2.0 fluorometer, Invitrogen/Life technologies, USA) with the Qubit™ dsDNA BR Assay kit for 2-1000 ng DNA quantification (Invitrogen/Life technologies, USA).

Whole-genome genotyping was performed with the Illumina Chicken iSelect BeadChip DNA chip (Illumina, Inc., USA) for 60 thousand SNPs. Quality control and data filtering for each sample and each SNP were performed using PLINK 1.9 software in R (http://zzz.bwh.harvard.edu/plink/). The filters were --mind 0.10, --geno 0.10, --maf 0.05, --hwe 1e-3. Regression analysis commands –assoc, --adjust, --qt-means were used to perform genome-wide association studies (GWAS) and identify SNPs associated with muscle color indices. To confirm the influence of SNPs and identify significant regions in the chicken genome, a Bonferroni test for null hypotheses was used. Data were visualized in the qqman package (https://github.com/qqman). The candidate genes in the regions of identified SNPs were searched with the Genome Data Viewer in the NCBI Gallus gallus (chicken) database (https://www.ncbi.nlm.nih.gov/datasets/genome/). For functional annotations, the GeneCards database (http://www.genecards.org/) and the DAVID program (https://david.ncifcrf.gov/) were used.

Mean values (*M*), standard errors (±SEM), minimum (min), maximum (max), coefficient of variation (*Cv*, %) were calculated using the Microsoft Office 365 package.

Results. Table 1 shows the color parameters of breast meat in F_2 chickens of the resource population studied.

1. Breast meat color parameters of chickens (*Gallus gallus* L.) from the F_2 resource population (n = 260, vivarium of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2021-2023)

Parameter	*M*	±SEM	min	max	*Cv*, %
L	38.88	1.98	42.08	62.28	7.62
a	2.12	0.19	-0.31	14.07	97.23
b	10.07	0.18	5.73	16.81	19.99

N o t e. a — spectrum from green (–128) to purple (127); b - spectrum from blue (–128) to yellow (127).

According to Table 1, the values of the a* and b* spectra of breast fillet highly fluctuated coefficient of variation, *Cv* of 97.23 and 19.99%, respectively. This indicates the influence of crossbreeding on the variability of these traits, when birds' feeding, housing and clinical health are controlled and stress during slaughter is minimized.

After data filtering, 16,630 SNPs were involved in genome-wide association studies. Figure 1 shows the distribution of identified SNPs across chromosomes.

Based on all the studied color indicators of meat in chickens from the F_2 resource population, we eventually identified 60 significant SNPs ($p < 0.00001$) on chromosomes GGA1, GGA2, GGA3, GGA7, GGA8, GGA10, GGA12, GGA13, GGA17, GGA18 (Fig. 2).

Table 2 shows significantly significant SNPs ($p < 0.00001$) associated with the color characteristics of breast meat in chickens from the F2 resource population. It was found that 28 SNPs are associated with the color parameter L*.

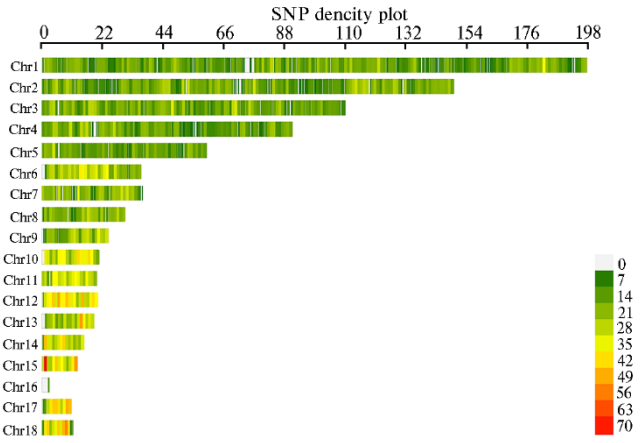

Fig. 1. Distribution density of SNPs for meat color parameters across chromosomes of chickens (*Gallus gallus* L.) from the F2 resource population (*n* = 260, vivarium of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2021-2023).

These SNPs are located at GGA1 (4 SNPs), GGA2 (5 SNPs), GGA3 (8 SNPs), GGA8 (1 SNP), GGA12 (4 SNPs), GGA13 (3 SNPs), GGA17 (2 SNPs) and GGA18 (1 SNP). In total, we identified 48 significant SNPs for the a* parameter on chromosomes GGA1, GGA2, GGA3, GGA7, GGA8, GGA10, GGA12, GGA13, GGA17 and GGA18. The largest number of SNPs was found on GGA3 (12 SNPs), the smallest on GGA18 (1 SNP). For breast meat color criterion b*, only 4 SNPs were identified on GGA3.

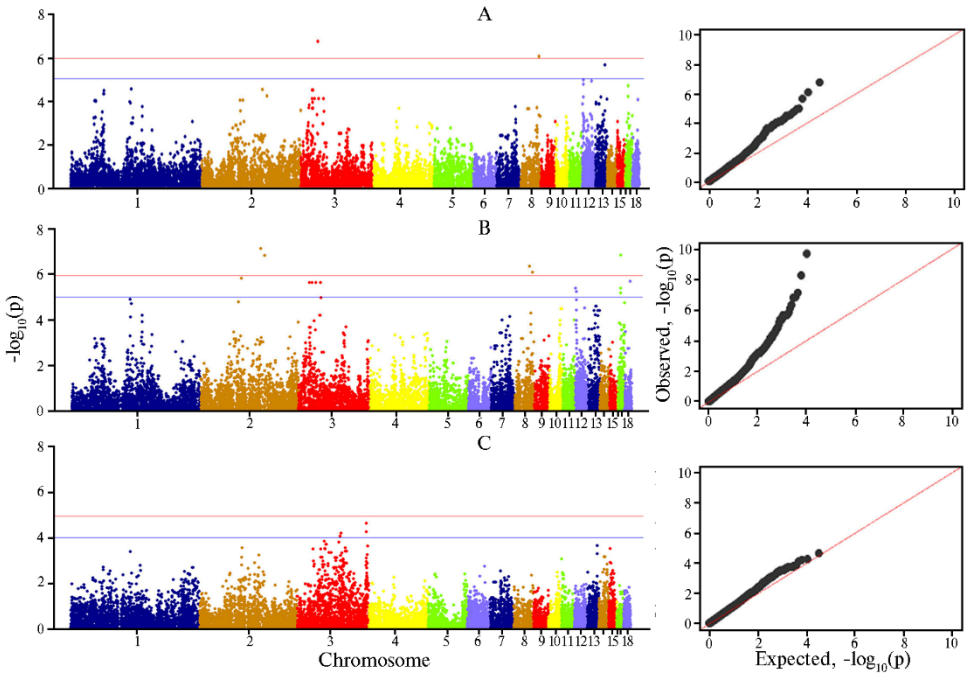

Fig. 2. Genetic structure of breast meat color parameters in chickens (*Gallus gallus* L.) from the F2 resource population: on the left — genome-wide plots, on the right — Q-Q (probability) graphs; A — spectrum of color L*, B — spectrum of color a*, — spectrum of color b* (*n* = 260, vivarium of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2021-2023).

2. Significant ($p < 0.00001$) **SNP associations with breast meat color parameters in chickens (*Gallus gallus* L.) from the F2 resource population** (*n* = 260, vivarium of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2021-2023)

Chromosome GGA	SNP	Position	p	Trait
1	GGaluGA017028	49518104...50473515	4.87E-05	L
	Gga_rs13865002		4.87E-05	
	GGaluGA017292		3.33E-05	
	Gga_rs13899455	88864063...90363842	1.27E-05	a
	Gga_rs14856616		7.36E-05	

Continued Table 2

	GGaluGA031490	91050938...91450938	2.87E-05	L, a
			1.95E-05	
	Gga_rs13917314	107440599...107940361	6.13E-05	a
	Gga_rs13917480		6.13E-05	
2	Gga_rs14187600	57548810...58000666	9.71E-05	L, a
			1.62E-05	
	Gga_rs14187774		9.71E-05	
			1.62E-05	
	GGaluGA150095	62037051...62437051	9.72E-05	L, a
			1.47E-06	
	Gga_rs14219701	91397215...91797215	2.99E-05	L, a
			7.47E-08	
	Gga_rs15133231	97797354...98197354	5.91E-05	L, a
			1.49E-07	
3	Gga_rs16228851	16704934...17317837	7.91E-05	L, a
			2.24E-06	
	GGaluGA210154		3.22E-05	
	Gga_rs14321392	18109888...18509888	3.22E-05	L
	Gga_rs14323710	20423383...20823383	7.91E-05	L, a
			2.24E-06	
	Gga_rs16239991	25213895...25613895	1.85E-07	L, a
			2.39E-11	
	Gga_rs15303835	26562059...26962059	7.91E-05	L, a
			2.24E-06	
	GGaluGA215531	32726056...34925170	6.15E-05	L, a
	Gga_rs16250047		6.15E-05	
	Gga_rs14337156		6.15E-05	
	Gga_rs16250652		6.15E-05	
	Gga_rs14337823		6.15E-05	
	GGaluGA215952		7.91E-05	
			2.24E-06	
	Gga_rs16251735		7.91E-05	
			2.24E-06	
	GGaluGA216144		1.04E-05	
	Gga_rs15375140	65279421...65679421	8.35E-05	b
	GGaluGA226948	66888191...67288191	6.24E-05	
	GGaluGA238450	105376493...105875531	5.50E-05	
	Gga_rs16338886		2.25E-05	
7	GGaluGA313956	16384962...16784962	9.96E-05	a
	Gga_rs13598324	28338012...28738012	6.98E-05	
8	GGaluGA330168	22596941...22996941	4.46E-07	a
	Gga_rs14653321	24397555...24797555	4.96E-05	a
	GGaluGA332278	27324733...27840907	8.92E-07	L, a
			2.06E-10	
	Gga_rs15937915		7.96E-07	
10	Gga_rs14953406	18446784...19677731	3.25E-05	a
	GGaluGA072861		3.25E-05	
12	GGaluGA080532	366564...1003693	4.08E-06	a
	GGaluGA080537			
	Gga_rs15628463			
	Gga_rs15630811	1703306...2418508	6.82E-05	L, a
			1.33E-05	
	GGaluGA081274		1.09E-05	
			5.67E-06	
	Gga_rs14031390		1.71E-05	
			3.72E-05	
	GGaluGA087110	14173406...14573406	1.21E-05	L, a
			3.01E-05	
13	GGaluGA093806	9428125...9828125	6.88E-05	L, a
			3.86E-05	
	Gga_rs15698305	11860386...13195035	5.92E-05	a
	Gga_rs14060024		2.46E-05	
	GGaluGA095191		2.46E-05	
	GGaluGA001139		2.46E-05	
	Gga_rs14063043	15435179...16143937	6.59E-05	L, a
	Gga_rs14063186		2.26E-06	
			5.55E-09	
	GGaluGA097116		3.85E-05	
	Gga_rs14065976	18469260...18869260	8.32E-05	L, a
			3.78E-05	
17	GGaluGA114289	4212179...5004176	6.34E-05	L, a
			4.00E-06	

Continued Table 2

	GGaluGA114391		2.04E-05	
			1.44E-07	
	GGaluGA114420		6.48E-06	
	Gga_rs15788572	10517209...10917209	1.78E-05	a
18	Gga_rs14114367	8362502...8762502	9.17E-05	L, a
			2.02E-06	

We identified candidate genes containing or linked to the identified SNPs (±0.2 Mb). Structural annotation revealed 270 genes that, according to a preliminary assessment, are responsible for the color spectra of breast sirloin, including 30 genes in the positions of identified SNPs (Table 3).

3. Structural annotation of candidate genes in the region of identified SNPs associated with breast meat color parameters in chickens (*Gallus gallus* L.) from the F_2 resource population (n = 260, vivarium of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2021-2023)

Chromoosme GGA	Candidate gene			Trait
	SNP location		linked with SNP (±0,2 Mb)	
	gene ID	positions		
1	*TEF*	49701507..49717640	*CD200, CD200L, CRYBG3, KCNJ15, MEI1,*	L, a
	MKL1	50003390..50101264	*EP300, BTLA, CGGBP1, ARL6, KCNJ6,*	
	TNRC6B	50146094..50287628	*ETS2*	
	EPHA6	90900356..91399201		
	ERG	107658703..107811187		
2	*VAPA*	97965836..97997739	*ACO2, RANGAP1, C1H3ORF52, EPHA3, CCDC134, L3MBTL2, ARL13B, PROS1, CSDC2, RBX1, NSUN3, STX19, XRCC6, XPNPEP3, POLR3H, ST13P5, TOB2, SGSM3, SREBF2, ADSL, SHISA8, GRAP2, PHF5A, FAM83F, DESI1, SNU13, DCDC2, NRSN1, MRS2, GPLD1, PHACTR1, EDN1, CNDP1, CNDP2, MOG, ALDH5A1, HIVEP1, CYB5A, FAM69C, APCDD1, PPP4R1, FBXO15, TIMM21, RAB31, RALBP1, TWSG1*	L, a
3	*TMEM63A*	16879683..16908662	*PARP1. CNIH3, HLX, SHKBP1, CAMKMT,*	L, a, b
	KCNK2	20526712..20653121	*SIX3, MCFD2, SCCPDH, PRKD3, SPAST,*	
	TTC7A	26628446..26790506	*SRD5A2, QPCT, CEBPZ, ELP3, LIN9,*	
	KIF28P	33508057..33530730	*MIXL1, DNAH14, MTARC1, CENPF,*	
	AHCTF1	33454877..33507931	*PTPN14, SIX2, CALM2, PPP1CB, NDUFAF7,*	
	KIF26B	34003714..34297816	*SULT6B1, DPY30, MEMO1, SMYD3, CNST,*	
	AKT3	34651696..34796287	*TFB2M, ADSS2, COX20, DESI2, TUBE1,*	
	LAMA4	65400812..65497195	*ARMC2, FBXO16, EXTL3*	
	AFG1L	67086571..67151321		
	FZD3	105634168..105686155		
7	–	–	*ACBD3, SDE2, WDR26, PFN3, EFCAB2, HNRNPU, SDCCAG8, WISP3, FOXO3, INTS9, LEFTY1, ENAH, DEGS1, MARK1, FYN, SNX3, NR2E1, OSTM1, SEC63, CHN1, INSIG2, WIPF1, CCDC93, CHRNA1, DDX18*	L, a
8	*BEND5*	22380811..23220947	*GPR155, CIR1, SCRN3, SP9, OLA1, SP3,*	L, a
	GLIS1	24591950..24764561	*FOXD3, ALG6, LRP8, ITGB3BP, PGM1,*	
	EFCAB7	27494881..27547348	*ROR1, DMRTB1, YIPF1, NDC1*	
10	–	–	*DIO1, MEGF11, ANP32A, DIS3L, NOX5, MAP2K1, GLCE, TIPIN, KIF23, ZWILCH, PAQR5, LCTL, TLE3, RPL4, UACA, SNAPC5, SMAD6, SMAD3*	L, a
12	*PBRM1*	771096..833446	*TNNC1, RPL29, MAPKAPK3, CISH, NISCH,*	L, a
	HEMK1	1847096..1930905	*STAB1, NT5DC2, SMIM4, GNL3, SPCS1,*	
	DOCK3	2034647..2317912	*GLT8D1, NEK4, ITIH3, MUSTN1, SFMBT1*	
	MAGI1	14181633..14474643		
13	*GLRA1*	12434258..12486014	*SFXN1, DRD1, MFAP3, GRIA1, UBE2B,*	L, a
	FLT4	12951742..12998047	*SKP1, BRD8, KIF20A, NMUR2, G3BP1,*	
	VDAC1	15589702..15650724	*SPARC, ATOX1, PPP2CA, TCF7, RAPGEF6,*	
	FSTL4	15845754..16032315	*GRK6, LMAN2, FAT2, CCDC69, GM2A, ANXA6, RGS14, ARL2, LACAAT2L, TNIP1, GPX3, DCTN4, PRR7, PDLIM7, NDST1, RPS14, CD74, TCOF1, B4GALT7, ADRA2BL2, SMIM3, RBM22, MYOZ3,*	

Continued Table 3

			SYNPO, DOK3, DBN1, ARSI, CAMK2A, PDGFRB, CDX1, DDX41, RAB24, HMGXB3, CSF1R, TRIM105, NPY7R, PRELID1, NSD1, TBC1D9B, MGAT4B, SQSTM1, MAML1, CANX, HNRNPH1, DCK2	
17	*BRINP1*	4664527..4744154	*TLR4*	L, a
18	*LMX1B*	10665426..10802338	*GNA13, MVB12B, RGS9, ARSG, ALC,*	L, a
	ABCA9	8559320..8581766	*WIPI1, PRKAR1A, ANGPTL2, ABCA8, MAP2K6, RALGPS1, ABCA5*	

N o t e. Dashes in the table mean that the found SNP position was not localized within the gene

Of the 270 identified genes associated with the color characteristics of breast meat, 39 significant candidate genes for biological functions were selected, including 3 genes in which the identified SNPs were located (Table 4).

4. Functional annotation of genes associated with breast meat color parameters in chickens (*Gallus gallus* L.) from the F_2 resource population (n = 260, vivarium of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2021-2023)

Gene	Position	Biological functions
In the SNP position:		
BRINP1	4664527..4744154	Cell cycle, cell death, behavior
FSTL4	15845754..16032315	Development of multicellular organisms, development of the nervous system
TTC7A	26628446..26790506	Cellular homeostasis of iron ions
Linked to the SNP position (±0,2 Mb):		
ARL13B	89891671..89940839	Looping the heart, forming a neural tube pattern
ABCA5	8582298..8608484	Lipid transport
ABCA8	8536924..8557588	Lipid transport
CD200L	88913076..88921182	Regulation of the immune response
DMRTB1	24557324..24563156	Development of germ cells, sex differentiation
FBXO15	91609665..91633896	Protein binding
FBXO16	105598226..105634014	Protein binding
FYN	65580445..65711082	Cardiac process, forebrain development, innate immune response
GNA13	8386712..8414335	Aging of a multicellular organism
G3BP1	12485905..12506960	Innate immune response, a protective response to the virus
GM2A	12614487..12617622	Lipid transport
LRP8	24349072..24499027	Regulation of the innate immune response
NDST1	12716758..12736200	Polysaccharide chain biosynthesis process
PDLIM7	9768860..9788764	Heart development
PRELID1	9812879..9814674	Lipid transport
SIX3	25520822..25523953	Eye development, maturation of epithelial cells
SMAD3	18814590..18878841	Response to hypoxia, development of the immune system
SP3	16704259..16735630	Liver development
B4GALT7	9710664..9712643	Carbohydrate metabolism, glycosaminoglycan biosynthesis
CCDC134	49550539..49554893	Angiogenesis, embryonic hematopoiesis, embryonic liver development
DIO1	24792883..24797740	Biosynthesis of hormones
DEGS1	17295979..17300997	Biosynthesis of fatty acids
EDN1	62406640..62411539	Cellular homeostasis of calcium ions, contraction of smooth muscle veins
GPX3	12661488..12663434	Response to oxidative stress
INSIG2	28449946..28462775	Cholesterol biosynthesis
LCTL	18547541..18551236	Carbohydrate metabolism
LEFTY1	16864275..16879586	Spleen development
LACAAT2L	12628444..12633004	Amino acid transport
MAP2K1	18496305..18527713	Heart development, thyroid development
NRSN1	57666592..57673732	Nervous system development
PGM1	27548636..27567569	Carbohydrate metabolism, glucose metabolism process
PPP1CB	26901055..26932174	Glycogen metabolism
SCCPDH	33530313..33540359	Biosynthesis of glycolipids
SFXN1	9634295..9669465	Ion transport, amino acid transport
STX19	89905548..89921452	Intracellular protein transport
TNNC1	643058..648527	Contraction of skeletal muscles, regulation of muscle contraction

Color is an important quality indicator closely related to glycolysis and intramuscular fat metabolism. The functions of a number of identified candidate genes (transport of lipids, amino acids, proteins, cholesterol, biosynthesis of fatty acids, development of the spleen, liver, heart, protein binding) directly or indirectly affect chicken meat color. The identified genes are responsible for the development of a multicellular organism and its organs, fatty acid homeostasis, and

biochemical processes. As is known, stress greatly affects the organoleptic characteristics of meat, therefore the function of nervous system is important meat color spectra. For the 7 candidate genes we identified, there are many reports about their connection with valuable traits in chickens. In particular, highly reliable associations of the *AKT3* gene with feather pigmentation in chickens [24] and the development of muscle fibers [25] have been established. The influence of the *FSTL4* and *VDAC1* genes on the moisture content in the egg yolk has been shown [26], and the *TTC7A* gene has been shown to influence the accumulation of glycogen in the muscles of chickens [27]. Association of the *FSTL4* gene with rapid muscle growth in broilers has also been reported [28]. The *MAGI1* and *VDAC1* genes influence immunity [29-31], and the *FZD3* and *EPHA6* genes influence the function and development of the nervous system [32]. For other candidate genes we identified, a number of studies have found a connection with indicators of meat productivity and quality in other species of farm animals. E.g., the *TTC7A* and *AFG1L* genes are associated with back fat thickness in pigs [33, 34], and the *BRINP1* gene is associated with growth indices in pigs [35] and linear measurements in goats [36].

Thus, we identified 60 significant SNPs associated with meat color in chickens, 28 SNPs for the L* color spectrum, 48 SNPs for the a* spectrum, and 4 SNPs for the b* spectrum. The SNPs we discovered are located in the chromosomes GGA1 (10 SNPs), GGA2 (3 SNPs), GGA3 (18 SNPs), GGA7 (2 SNPs), GGA8 (4 SNPs), GGA10 (2 SNPs), GGA12 (7 SNPs), GGA13 (9 SNPs), GGA17 (4 SNPs), and GGA18 (1 SNP). We also identified 270 candidate genes associated with the studied traits, of which 30 genes harbored SNPs. These results can promote genomic selection of chickens for meat quality.

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IDENTIFICATION OF SNPs AND CANDIDATE GENES ASSOCIATED WITH ABDOMINAL FAT DEPOSITION IN QUAILS (*Coturnix japonica*)

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Abstract

The rate of fat deposition, including abdominal fat, is one of the important indicators characterizing both meat performance and product quality, as well as the poultry welfare in general. This trait positively correlates with the bird's rapid growth and largely depends not only on feeding and housing conditions, but also on genetic factors. Mostly, data on the genetic mechanisms that determine fat metabolism and fat deposition rate have been obtained in chickens; SNPs and candidate genes that determine the deposition of both intramuscular and abdominal fat have been identified. The number of similar studies on quail is relatively small. To date, there is not enough information in the specialized literature about quantitative trait loci (QTLs) that are reliably associated with fat metabolism indices in quails. The present work reports for the first time the identified SNPs that are highly significantly ($p < 0.00001$) associated with the intensity of abdominal fat deposition in 8-week-old quails from the F_2 model resource population. In the region of identified SNPs, candidate genes reliably associated with this trait were established. The objective of the study was to search for SNPs and identify candidate genes associated with abdominal fat deposition in quails. The studies were carried out on F_2 males of the model resource population ($n = 146$) obtained by crossing two quail breeds contrasting in growth rate and meat quality, Japanese (slow growth) and Texas (fast growth). F_2 individuals were genotyped using the GBS (genotyping-by-sequencing) method. To identify associations between genome-wide genotyping data and the amount of abdominal fat, PLINK 1.9 software was used with accepted filter settings (geno 0.1, mind 0.1, maf 0.05). The threshold significance criterion was set to $p < 0.00001$. The resultant F_2 resource population of quail was characterized by high variability in the content of abdominal fat in the carcass. At the age of 56 days, this indicator varied from 0.01 to 10.46 g and averaged 2.41±0.16 g. Based on the GWAS (genome-wide association study) analysis, we identified 29 SNPs and 11 candidate genes located in the regions of these SNPs that were associated with abdominal fat deposition in quail. The determined SNPs are localized on chromosomes 1, 2, 7, 8, 17, 19, 21, 24 and 28. The candidate genes identified (*CNTN5*, *GNAL*, *PDE1A*, *RBMS1*, *PTPRF*, *SH3GLB2*, *SLC27A4*, *TRIM62*, *IGSF9B*, *USHBP1*, and *NR2F6*) were established on chromosomes CJA1 (1 gene), CJA2 (1 gene), CJA7 (2 genes), CJA8 (1 gene), CJA17 (2 genes), CJA21 (1 gene), CJA24 (1 gene) and CJA28 (2 genes). The detected SNPs and candidate genes can serve as genetic markers in breeding programs to improve the meat quality of quails and reduce the fat content in carcasses.

Keywords: *Coturnix japonica*, quail, QTL, SNP, genotyping-by-sequencing, GBS, genome-wide association study, GWAS, candidate genes, abdominal fat

Poultry products occupy a strong position in the overall structure of food products of animal origin [1, 2]. Both eggs and poultry meat are in great demand [3, 4]. Unlike other types of farm animals, poultry meat is a dietary product with high nutritional value and good taste [5, 6]. Poultry meat typically contains a small

amount of fat and a significant proportion of protein [3, 6].

The basis of a sustainable and competitive poultry meat industry is highly productive breeds and crosses. Therefore, constant breeding is required to search and identify valuable genotypes. For this purpose, modern methods are used to identify the molecular genetic mechanisms underlying economically valuable traits. One of the tasks of genomic selection to improve the quality of poultry products and increase the profitability of the industry is the search for SNPs and identification of candidate genes that determine selection-significant traits. In recent years, significant databases of SNPs and candidate genes associated with indicators of meat productivity of poultry have been formed [7, 8].

An important indicator of the meat quality of poultry is the intensity of fat deposition [9, 10]. There are intramuscular and internal (abdominal, abdominal) fat. Intramuscular fat determines the nutritional value of meat, its taste and texture [9]. Abdominal fat is deposited in poultry in the abdominal cavity and serves as a source of energy. Its content in the carcass can reach 3-4% of its weight [11]. Excessive abdominal fat deposition negatively affects poultry health [12, 13] and carcass quality [10, 11]. A number of studies have examined the use of abdominal fat to improve texture and palatability and to improve the nutritional value and nutritional value of poultry products [14, 15]. A number of factors influence fat metabolism and accumulation of fat deposits, e.g., living conditions [10], feeding [11, 16, 17], age [18, 19], sex [19), genetic predisposition [20-22]. Females have larger body fat than males [19]. The intensity of fat accumulation positively correlates with the rapid growth of birds, which makes it difficult to select birds simultaneously for growth rate and fat reduction in in carcasses [20].

Genome-wide association studies (GWAS) of single nucleotide polymorphisms (SNPs) with economically significant traits allow effective identification of candidate genes associated with their expression [23]. In chickens, compared to other types of poultry, the genetic mechanisms associated with fat metabolism and the intensity of fat deposition have been studied in more detail [24, 25], SNPs and candidate genes have been identified that determine the deposition of both intramuscular and abdominal fat [26-28]. For quail, such data are limited [29]. To date, there is insufficient information on quantitative trait loci (QTLs) reliably associated with fat metabolism in quail.

In this report, novel SNPs and candidate genes were identified in the F_2 model resource population of quails, with high confidence ($p < 0.00001$) associated with the intensity of fat deposition. The detected SNPs can be genetic markers in breeding to improve the meat qualities of quails and reduce the fat content in carcasses.

The purpose of this work is to search for SNPs and identify candidate genes associated with abdominal fat deposition in quails.

Materials and methods. The experiments were carried out on F_2 male quails of a model resource population ($n = 146$; vivarium of the Ernst Federal Research Center — VIZh, 2022-2023) derived from crossing two breeds contrasting in growth rate and meat qualities, the Japanese slow growing and Texas fast growing quails. At the first stage, four families were formed, each consisting of one male and five females of the original breeds. Among 20-30 F_1 individuals from each family, 12 families (F_1_1-F_1_12) were composed, each of one F_1 male and three F_1 females who were not close relatives, to create offspring. Of their F_2 progeny F_2 male groups F_2_1-F_2_12 were assembled for study.

After experimental slaughter at the age of 8 weeks, the carcass was cut up, and the carcass and abdominal fat were separately weighed. GWAS analysis was performed for both absolute and relative abdominal fat content. The relative abdominal fat content was calculated as the percentage of abdominal fat weight to

total carcass weight.

DNA was extracted from feather pulp using a Syntol kit for extracting DNA from animal tissue (NPF Syntol LLC, Moscow). The DNA concentration was measured (a Qubit® 3.0 fluorometer, Thermo Fisher Scientific, USA) and $OD_{260/280}$ was assessed (NanoDrop-2000, Thermo Fisher Scientific, USA) to control its purity. Quail genotyping was performed using the GBS (genotyping-by-sequencing) method according to the protocol described previously [30]. A reference genome was the Japanese quail (Coturnix_japonica_2.0) (https://www.ensembl.org/Coturnix_ja-ponica/Info/Annotation). Removal of adapters and the fastq file demultiplexing were carried out (https://cutadapt.readthedocs.io/en/stable/). Quality control of fastq files was carried out in the FastQC program [31]. For alignment to the reference genome, the bowtie2 package was used [32]. The R software system was used to convert to a file format suitable for further analysis [33]. Quality of SNPs detection was controlled in the PLINK 1.9 program (https://zzz.bwh.harvard.edu/plink/plink2.shtml). The genotyping efficiency filters (mind 0.1; maf 0.05) was applied, SNPs genotyped in less than 90% of samples (geno 0.1) were excluded.

To identify associations of SNPs with the abdominal fat content, regression analysis was performed PLINK 1.9 software. The significance of the SNP influence and the identification of significant regions in the quail genome were assessed by null hypothesis testing with a threshold value of $p < 0.00001$. Data were visualized in the qqman package in R [33]. The search for candidate genes located in the region of identified SNPs was carried out using the Ensembl Coturnix japonica 2.0 genomic resource (https://www.ens-embl.org/Coturnix_japonica/Info/Annotation).

Statistical indicators were calculated in Microsoft Excel 2013. Mean values (*M*), standard errors of means (±SEM), minimum (min) and maximum (max) values, and coefficient of variation (*Cv*, %) are submitted.

Results. The content of abdominal fat in the carcasses of 56-day-old F_2 male quails varied from 0.01 to 10.46 g and averaged 2.41±0.16 g. Note the high variability of the trait among the studied birds ($Cv = 78.7\%$), whereas the proportion of abdominal fat from the total carcass weight varied from 0.01% to 4.82% with $Cv = 73.2\%$ on average.

We also revealed a high variation in the absolute and relative content of abdominal fat in carcasses in the quail groups F_2_1-F_2_12 (Table 1).

1. Content of abdominal fat in carcasses of male quails (*Coturnix japonica*) of F_2 model resource population ($n = 146$; vivarium of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022-2023)

Group	*n*	Fat weight, г					Fat weigh to carcass weight, %				
		M	±SEM	max	min	*Cv*, %	*M*	±SEM	max	min	*Cv*, %
F_2_1	8	1.93	0.51	4.24	0.01	74.6	1.09	0.29	2.48	0.01	75.2
F_2_2	15	2.95	0.48	6.10	0.10	63.5	1.53	0.25	3.19	0.06	63.2
F_2_3	7	1.35	0.15	2.02	0.82	29.5	0.71	0.06	1.00	0.48	23.8
F_2_4	18	2.49	0.40	7.64	0.35	68.6	1.31	0.21	4.03	0.22	67.3
F_2_5	7	2.54	0.87	6.95	0.71	90.9	1.53	0.51	4.13	0.46	87.4
F_2_6	14	2.84	0.68	10.46	0.55	89.7	1.47	0.31	4.82	0.30	80.1
F_2_7	18	1.96	0.46	8.72	0.10	99.5	1.05	0.23	4.29	0.06	92.9
F_2_8	11	2.30	0.52	5.39	0.10	75.1	1.32	0.29	2.92	0.08	71.7
F_2_9	10	2.13	0.43	5.41	0.73	63.5	1.18	0.19	2.44	0.45	50.2
F_2_10	6	2.13	0.66	3.63	0.01	76.3	1.22	0.35	1.99	0.01	71.0
F_2_11	18	2.97	0.56	8.83	0.49	80.3	1.52	0.26	4.13	0.32	71.2
F_2_12	14	2.32	0.51	5.36	0.01	78.6	1.31	0.28	3.04	0.01	76.2
On average	146	2.41	0.16	10.46	0.01	78.7	1.30	0.08	4.82	0.01	73.2

A diagram (Fig. 1) shows the distribution of quails from the study sample according to the content of abdominal fat in the carcass depending on the genotype.

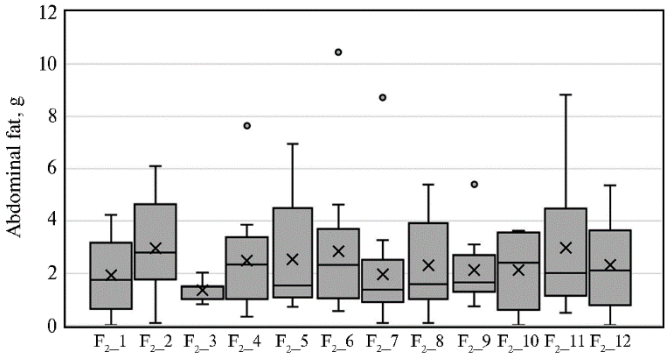

FIg. 1. Distribution of male quails (*Coturnix japonica*) of F2 model resource population according to the abdominal fat content in the carcass depending on the genotype (groups F2_1-F2_12, for the group size, see Table 1; vivarium of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022-2023). *Me*, *M*, min, max, and outliers of single data are given.

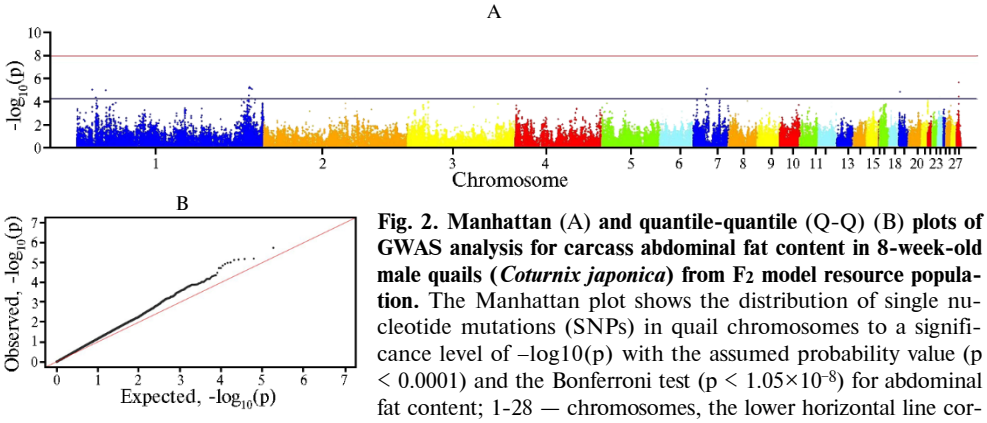

Fig. 2. Manhattan (A) **and quantile-quantile** (Q-Q) (B) **plots of GWAS analysis for carcass abdominal fat content in 8-week-old male quails (*Coturnix japonica*) from F2 model resource population.** The Manhattan plot shows the distribution of single nucleotide mutations (SNPs) in quail chromosomes to a significance level of –log10(p) with the assumed probability value ($p < 0.0001$) and the Bonferroni test ($p < 1.05\times10^{-8}$) for abdominal fat content; 1-28 — chromosomes, the lower horizontal line corresponds to $p < 0.0001$, the upper line is $p < 1.05\times10^{-8}$ (vivarium of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022-2023).

GWAS analysis for the abdominal fat rate in a carcass showed that in the F2 quails aged 8 weeks, this trait is associated with 29 SNPs ($p < 0.00001$) on chromosomes 1, 2, 7, 8, 17, 19, 21, 24 and 28 (Fig. 2). Chromosomes 1 and 7 contain the largest number of SNPs, 11 and 7 SNPs, respectively, and chromosomes 2, 17, 19, 21 and 24 the smallest number, 1-2 SNPs.

The identified SNPs ($p < 0.00001$) were used to annotate candidate genes associated with abdominal fat deposition in quail. Structural annotation identified 124 genes, including 11 genes, the *CNTN5*, *GNAL*, *PDE1A*, *RBMS1*, *PTPRF*, *SH3GLB2*, *SLC27A4*, *TRIM62*, *IGSF9B*, *USHBP1* and *NR2F6* located in the region of identified SNPs (Table 2). These genes were identified on 8 chromosomes, the CJA1 (1 gene), CJA2 (1 gene), CJA7 (2 genes), CJA8 (1 gene), CJA17 (2 genes), CJA21 (1 gene), CJA24 (1 gene) and CJA28 (2 genes).

The detected candidate genes and significant SNPs ($p < 0.00001$) associated with abdominal fat deposition in quail are submitted in Table 2.

Analysis of published research showed that for none of the 11 candidate genes (*CNTN5*, *GNAL*, *PDE1A*, *RBMS1*, *PTPRF*, *SH3GLB2*, *SLC27A4*, *TRIM62*, *IGSF9B*, *USHBP1*, and *NR2F6*) we identified in the region of the novel SNPs an association was previously reported with the deposition of abdominal fat in quails. However, seven candidate genes identified have been previously shown to influence lipid metabolism and body fat accumulation in other animal and poultry species. For example, it was reported that the *PTPRF* and *GNAL* genes are associated with the development, formation and accumulation of adipose

tissue [34] and abdominal fat deposition [35] in chickens, and the *TRIM62* and *SLC27A4* genes with the thickness [36] and lipid composition [37] of pig backfat. The influence of the *NR2F6*, *PDE1A* and *RBMS1* genes on adipogenesis and lipid metabolism, in particular on the accumulation of fat deposits in cold conditions [38] and obesity [39-41], has been shown in laboratory mice.

2. SNPs and potential candidate genes ($p < 0.00001$) **associated with abdominal fat deposition in the carcass of 8-week-old male quails (*Coturnix japonica*) of F_2 model resource population** (the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022-2023)

Chromosome	Number of SNPs	SNP	p	Gene	
				in the SNP region	±0,2 Mb
1	11	1:14239735	1.03E-05	–	*GTSE1*, *TRMU*, *RAMD4*, *CERK*
		1:17986717	4.74E-05	–	*IL17REL*, *MLC1*, *MOV10L1*, *PANX2*, *TRABD*, *SELENOO*, *TUBGCP6*, *HDAC10*
		1:161416406	8.12E-05	–	*ATM*, *NPAT*, *ACAT1*, *ELMOD1*
		1:161611015	7.37E-06	–	*ALKBH8*, *CWF19L2*
		1:161611163	3.26E-05	–	*ELMOD1*, *ALKBH8*, *CWF19L2*, *GUCY1A2*
		1:161950040	6.55E-06	–	*GUCY1A2*, *ASDHPPT*, *KBTBD3*, *MSANTD4*
		1:162669404	6.99E-06	–	*PDGFD*
		1:164041425	6.53E-05	–	*PGR*, *ARHGAP42*, *CNTN5*
		1:164300065	9.84E-06	*CNTN5*	-
		1:18579785	9.30E-05	–	*PLXNB2*, *ZNF800*, *GRM8*
2	2	2:85104703	5.62E-05	–	*TMX3*
		2:87734460	1.35E-05	*GNAL*	*SPIRE1*, *AFG3L2*, *PRELID3A*, *MPPE1*
7	7	7:11751354	6.63E-05	–	*PARD3B*, *NRP2*, *INO80D*
		7:11751372	6.63E-05	–	
		7:12205885	1.88E-05	–	*DNAJC10*, *PIKFYVE*, *CYP20A1*, *NBEAL1*,
		7:12292293	1.88E-05	*PDE1A*	*IDH1*
		7:13285939	6.29E-06	–	*CWC22*, *ZNF385B*, *SESTD1*, *CCDC141*
		7:25004119	9.48E-05	*RBMS1*	*LY75*, *PLA2R1*, *ITGB6*
		7:3916979	9.88E-05	–	*AGAP1*, *GBX2*, *ASB18*, *IQCA1*, *ACKR3*
8	2	8:17793153	9.35E-05	*PTPRF*	*IPO13*, *KDM4A*, *ST3GAL3*
		8:17793157	9.35E-05	–	
17	2	17:4080998	4.98E-05	*SH3GLB2*	*SPTAN1*, *TBC1D13*, *ENDOG*, *LRRC8A*, *PHYHD1*, *NUP188*, *TBC1D13*, *PTPA*, *NTMT1*
		17:3724688	1.71E-05	*SLC27A4*	*NAIF1*, *EEIG1*, *SH2D3C*, *DPM2*, *AK1*
19	1	19:601237	1.42E-05	–	*CLDN4*, *LIMK1*, *CALN1*, *MTMR4*, *ABHD11*, *METTL27*, *SBDS*, *GALNT17*
21	1	21:5674628	8.43E-05	*TRIM62*	*WNT4*, *P3H1*, *ZMYND12*, *PHC2*, *USP48*, *ECE1*, *EIF4G3*, *CDC42*, *C1orf50*, *PPIH*, *SLC2A1*, *ALPL*
24	1	24:2070512	4.36E-05	*IGSF9B*	*OPCML*, *NCAPD3*, *THYN1*, *B3GAT1*, *JAM3*, *VPS26B*, *ACAD8*
28	2	28:2442148	4.46E-05	*USHBP1*	*REEP6*, *THOP1*, *GADD45B*, *ABHD8*,
		28:2436106	1.79E-06	*NR2F6*	*ANO8*, *YJEFN3*, *MAU2*, *NCAN*

N o t e. Dashes in the table mean that no genes are located in the position of identified SNPs.

For other candidate genes we discovered, a connection was established with meat productivity and meat quality prameters in other farm animals and poultry. In particular, the *CNTN5* gene has been reported to make association with feed efficiency in Peking ducks up to 42 days of age [42], which may indicate the effect of this gene on the feeding behavior of the bird and, therefore, on the accumulation of intramuscular and abdominal fat. The effect of the *CNTN5* gene on the pH of the *longissimus dorsi* muscle in F_2 sheep of the resource population Texel × Altai breed has also been shown [43]. A relationship was found between the *PTPRF* gene and bodyweight of Holstein cows [44].

Thus, GWAS analysis for abdominal fat deposition in quails identifies with high confidence ($p < 0.00001$) 29 SNPs on chromosomes 1, 2, 7, 8, 17, 19, 21, 24 and 28. In the regions of the novel SNPs, 11 candidate genes are identified that are significantly associated with abdominal fat deposition in quails at the age of 8 weeks. The functional annotation showed the involvement

of seven identified genes in lipid metabolism in other species of farm animals. The data obtained allow for searching associations of identified mutations with other indicators of breeding value.

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THE RESULTS OF PRODUCTION AND TRANSPLANTATION OF IVEP EMBRYOS IN SHEEP (*Ovis aries*)

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Abstract

In vitro embryo production (IVEP) in sheep is necessary to develop because of its use in breeding and conservation of valuable animals and possible creation of new genotypes by genomic editing. In the present work for the first time in national practice, full-fledged ovine embryos were produced in vitro and live lambs were born after their transplantation to recipient ewes. The aim of this work was to model the main steps of IVEP technology in this species, and to evaluate its efficiency in vitro and in vivo. Female germ cells were obtained post mortem from the ovaries of sexually mature ewes and sheep of various breeds and ages after slaughtering. Cumulus-oocyte complexes (COCs) (n = 1028) were retrieved by dissecting of visible follicles and only high-quality COCs (n = 620) were cultured for 24 h, 25-35 COCs per 500 ml of TC-199 medium supplemented by 10 % fetal calf serum, 10 μg/ml of FSH and 10 μg/ml LH, 10 ng/ml of epidermal growth factor. A part of mature oocytes (n = 96) was used for cytological analysis of nuclear maturation rate, and other oocytes (n = 524) were transferred to BO-IVF medium (IVF Bioscience, UK) for in vitro fertilization. The granules of Katadin breed ram frozen semen were thawed and treated by "swim-up" method in Sperm-TALP medium (G.N. Singina, 2019). Mature oocytes were co-cultured with ram sperm in BO-IVF for 15-16 hours and then were transferred to BO-IVS medium (IVF Bioscience, UK) for in vitro embryo development. At day 2 of culture, cleavage rate was evaluated and a part of cleaved embryos was transplanted to recipient animals; at day 7, development to blastocyst occurred. Two-day embryos were transplanted synchronously to cycling Romanov breed ewes (n = 6) by endoscopic surgical method (V.A. Lukanina et al., 2023) using two-port laparoscopy under local anesthesia. After 35-42 days of transplantation, recipient ewes were examined for pregnancy, and fetus development was monitored until live lamb birth. According to cytological analysis, oocyte nuclear maturation rate was 77.1 % (74/96), 316 out of 524 mature and fertilized oocytes were cleaved (60.3 %), and 92 cleaved embryos were transplanted to recipient animals. Remaining early embryos (n = 224) continued in vitro development and 34.8 % reached blastocyst stage. According to ultrasound diagnostics after embryo transplantation, pregnancy rate was 50 % (3/6), and 33.3 % (2/6) transplantations resulted in live offspring. Thus, reported data demonstrated efficiency of IVEP technology in sheep: produced embryos were full-fledged and capable to develop to viable offspring. There is a good reason to believe that proposed technology of in vitro embryo production and transplantation to recipient ewes can be applied to reproduction technologies and gene editing in ovine.

Keywords: *Ovis arie*, domestic sheep, oocytes, in vitro maturation, in vitro fertilization, embryos, IVEP, transplantation

Assisted reproductive technology (ART) in husbandry uses mature and in vitro fertilized female gametes (oocytes) of domestic animals to produce embryos outside the body and their transplantation to recipients. The ART improvement

and practice may provide preservation and replication of valuable genotypes and the creation of new genotypes by genomic editing [1, 2]. According to statistics, in vitro embryo production (IVEP) from farm animals in the world increases every year [3]. The leader remains cattle with approximately 50% of such embryos out of the total number produced, and a significant part of them is intended for commercial use [4].

In small ruminants, particularly sheep, IVEP has so far had limited commercial use compared to cattle, although interest in IVEP technology is also growing [5]. In sheep, this approach is an alternative to multiple ovulation and transfer embryo production (MOET) technology. Its applicability in breeding is limited by the high cost and the unpredictability of the result [1, 5] because of the dependence on the reproductive status of the donor [6], significantly fluctuated superovulatory response to hormonal stimulation [7], insemination efficiency [8], and early luteal regression [6]. In addition, both embryo retrieval and transplantation require surgical manipulations [5, 6]. Recently, a serious impetus is the increased interest in creation of modified animals using a simple and effective CRISPR/Cas9 based genome editing system [9, 10]. Its application most often involves introduction into the cytoplasm of oocytes fertilized in vitro [11, 12]. Finally, for sheep as a farm animal, there is an easy and affordable way to retrieve oocytes from the ovaries post mortem. This expands the use of IVEP as a model in reproductive biology, including improving assisted reproductive technologies that are used to solve some human fertility problems and to conserve endangered animal species [13].

IVEP includes several main stages. These are extraction of female germ cells (post mortem or from living donors) from ovarian follicles, culture of isolated oocytes for in vitro maturation (IVM), in vitro fertilization (IVF) of mature oocytes using fresh or frozen semen, as well as IVF oocyte culture to produce embryos of different developmental stages (early or blastocyst) [13]. The first fact of IVEP for sheep embryos was reported in Cambridge (UK) in 1986 [14]. Due to the work of scientists from different countries, individual stages of IVEP were improved and, as evidenced by a number of reviews [2, 5, 13], this provides a significant increase of the efficiency of the method, but still its indicators, with rare exceptions, are inferior to those of cattle [2, 5]. Therefore, there is a need to continue such experiments. The overall performance of IVEP in terms of the number of embryos and their development to the blastocyst stage ranges from 18 to 89% and from 7 to 79%, respectively. However, there are significant differences between experiments due to particular protocols, the germ cell origin, the age, physiological status and genetic background of donors [2, 5, 13].

In domestic practice, the need to develop IVEP technology in sheep is especially acute. We have not found a single work that contains information about obtaining viable embryos from ewe oocytes that have matured and been fertilized outside the body.

Here, we report for the first time the IVEP production of high-quality sheep embryos after transplantation of which live offspring were born.

The aim of our study was to develop IVEP technology in sheep and evaluate its effectiveness in vitro and in vivo.

Materials and methods. In all experiments, except for specially indicated cases, reagents from Sigma-Aldrich (USA) were used.

Obtaining IVEP embryos. The ovaries of sexually mature ewes of different breeds and ages were collected post mortem at a meat processing plant and delivered to the laboratory within 2-4 h, where they were freed from excess tissue and washed several times in a physiological solution containing 100 IU/ml penicillin and 100 μg/ml streptomycin (OOO BioPharmGarant, Russia). To isolate cumulus-oocyte complexes (COCs), the ovaries were alternately placed in a

Petri dish (OOO Biomedical, Russia) with a diameter of 100 mm with TS-199 medium containing 2% fetal bovine serum (FBS), 10 μg/ml heparin and 50 μg/ml gentamicin (TS-199M) and fixed using surgical tweezers. The walls of the visible ovarian follicles were dissected with a blade held in surgical tweezers or a scalpel. After dissecting a batch of 5-7 ovaries, the Petri dish was examined under an SMZ stereomicroscope (Nikon, Japan) and the COCs were selected and transferred into fresh TS-199M medium. After completing a similar treatment for all ovaries delivered to the laboratory, the total pool of collected COCs was washed 3 more times. During the last washing, suitable for culture COCs of oocytes with homogeneous cytoplasm surrounded by at least one layer of cumulus cells (CC) were selected [15].

To mature in vitro, COCs were cultured in TC-199 medium containing HEPES (25 mM), Na-pyruvate (0.5 mM), follicle-stimulating and luteinizing hormones (10 μg/ml), epidermal growth factor (10 ng/ml) (Thermo Fisher Scientific, USA), FBS (10%) and gentamicin (50 μg/ml). Incubation was carried out in 4-well plates (OOO Biomedical, Russia) in groups of 25-35 COCs in 500 μl drops of medium. After 24 h of in vitro maturation (IVM), part of the COCs was used for cytological analysis of nuclear maturation [16], the remaining part was transferred to BO-IVF medium (IVF Bioscience, UK) for in vitro fertilization (IVF).

Oocytes were fertilized using the active sperm fraction obtained by the swim-up method according to a previously described protocol with minor modifications [16]. Granules of frozen sperm of a Katahdin ram were thawed in Sperm-TALP medium [17], 200 μl were transferred to the bottom of tubes containing 1 ml of same medium, and incubated for 1 h. After incubation, the top 700 μl layer was transferred to another tube with Sperm-TALP medium and centrifuged. The resulting sediment, containing motile sperm, was added to the BO-IVF medium with previously matured COCs placed there, the sperm concentration was 1×10^6/ml of fertilization medium. Oocytes and spermatozoa were incubated together for 15-16 h, then female germ cells were carefully released in fresh drops of BO-IVF from CC, dead and adherent spermatozoa, and transferred to embryo development medium.

Embryos were cultured in drops of BO-IVC medium under a layer of mineral oil in an Embryovisor plate incubator (Westtrade LTD, Russia) at 38.5 °C and a three gas mixture of 5% CO_2, 5% O_2 and 90% N_2. After 2 days of culture, a morphological assessment of the fertilized oocyte cleavage into blastomeres was carried out and a part of early embryos, having two or more blastomeres, was selected for transplantation into recipients. The remaining portion was transferred to fresh BO-IVC medium and cultured for another 5 days under similar conditions until the blastocyst stage to assess viability in vitro. Morphological assessment of embryo development was carried out according to common criteria [18].

The isolated COCs and the embryos that derived after in vitro fertilization were photographed using an Eclipse Ti-U inverted microscope (Nikon, Japan).

Embryo transplantation. The embryos were transplanted into sexually mature Romanov ewes aged 17-18 months, the animals had previously gone into natural heat (the physiological yard of the Ernst Research Center — VIZh, October 2022). The number of estrus was not taken into account before use. Recipient ewes were preliminarily stimulated to estrus by intramuscular injection of 125 μg of cloprestenol (Bioveta, Czech Republic) 13 and 2 days before the expected estrus [19]. Animals with signs of estrus were detected using a sample ram. On day 2 after heat detection, 2 day IVEP embryos were endosurgically transplanted (n = 6) using two-port laparoscopy under local anesthesia, as described previously [19].

Embryos were transferred into the uterine lumen (ipsilateral to the ovary

containing at least one functional corpus luteum, CL) using a 10 mm (outer diameter) capillary pipette connected to a 1 ml syringe. The capillary was filled as follows: 25-30 mm medium for embryo transfer, 2-3 mm air, 30-40 mm medium containing embryos, 2-3 mm air, 25-30 mm medium. The medium used was TS-199 containing HEPES (25 mM), gentamicin (50 μg/ml) and 10% FBS. The number of corpora lutea per recipient averaged 2.2, varying from 1 to 4.

35-42 days after embryo transfer, recipient ewes were examined for pregnancy. The development of fetuses was monitored until the birth of live offspring. Diagnosis in both cases was carried out using a portable ultrasound scanner Draminski 4Vet Slim (DRAMIŃSKI S.A., Poland), equipped with a convex sensor with the main frequency 2-8 MHz. Lambs born were examined for general health, identified by sex, and weighed.

The reliability of the origin of the surrogate lambs was assessed using microsatellite markers (short tandem repeats, STR) [20]. Mothers and offspring were studied for 11 STRs comprising 2 multiplex panels, the INRA005, INRA23, MAF65, McM527, SPS113, INRA063 (panel 1), and HSC, MAF214, OarAE129, OarCP49, OarFCB11 (panel 2). Genomic DNA of lambs and recipient ewes was isolated from ear tissue using the DNA-Extran kit (ZAO Synthol, Russia) and the perchlorate method. Amplification was run in a 15 μl final volume in PCR buffer containing 1.5 mM MgCl*2*, 200 μM dNTP, 0.5 mM primer mixture, 1 unit Taq polymerase (JSC Dialat Ltd, Russia) and 10-100 ng of genomic DNA. Conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 20 s; 63 °C (panel 1) and 55 °C (panel 2) for 1 min; 72 °C for 1 min; final elongation at 72 °C for 10 min. Amplification fragments were identified (an ABI Prism 3130xl genetic analyzer, Applied Biosystems, USA). Primary STR data were processed using the GeneMapper 4 program (Applied Biosystems, USA). The probability of exclusion of relationship between animals was assessed using the GenAlEx 6.5 program [20]. The calculated parameters were PI (probability of genotype matching), PX1 (exclusion of one parent in a pair), PX2 (exclusion of relationship when one parent is known, but the other genotype is not available), PX3 (exclusion of the relationship of the alleged parent couple).

Results. A total of 174 post mortem ovaries were delivered to the laboratory (Fig. 1, A), from which we isolated 1028 COCs, on average 5.9 per ovary. Based on the morphological assessment, 620 COCs (60.3%) were suitable for in vitro maturation, on average 3.56 per ovary (see Fig. 1, B) and cultured in IVM medium.

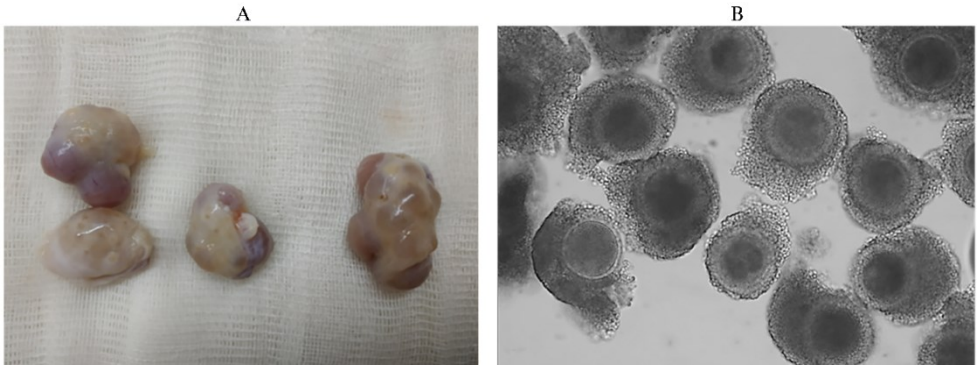

Fig. 1. Ewe ovaries used to isolate oocytes (A)**, and a micrograph of cumulus-oocyte complexes isolated for cultivation** (B)**.** A microphotography was made on an inverted microscope Eclipse Ti-U (Nikon, Japan), magnification ×100 (Ernst Federal Research Center — VIZh, Moscow Province, 2022).

After 24 h of incubation, 96 COCs were selected for cytological assessment

of nuclear maturation in oocytes, and 524 COCs were subjected to in vitro fertilization followed by assessment of the in vitro development to blastocysts (7 day culture). In addition, the viability of some 2 day embryos after transplantation into recipient animals was studied.

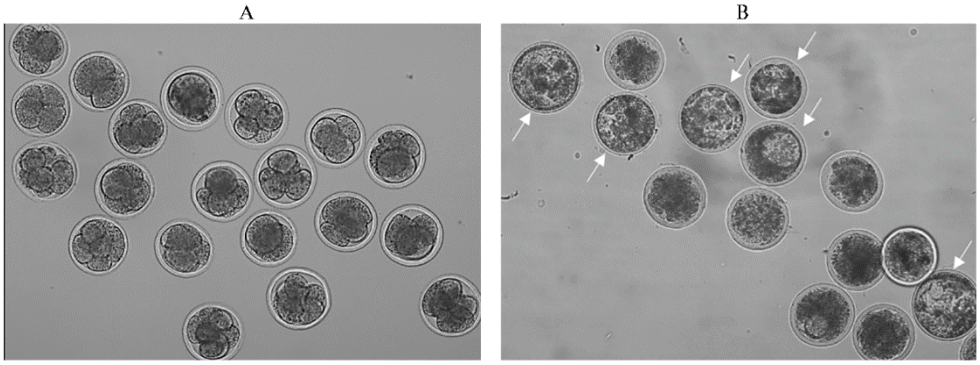

Fig. 2. Microphotographs of embryos developed from ewe oocytes matured and fertilized in vitro: A — day 2 cleaved embryos; B — 7 day embryos, including those that have reached the blastocyst stage (indicated by an arrow; an inverted microscope Eclipse Ti-U, Nikon, Japan, magnification ×100) (Ernst Federal Research Center — VIZh, Moscow Province, 2022).

Cytological analysis confirmed that the proportion of maturation, that is, the percentage of oocytes at metaphase II of the meiotic division from the initial number of cultured COCs, was acceptable and averaged 77.1% (74 out of 96). Of the 524 mature and fertilized oocytes, 316 oocytes (60.3%, Fig. 2, A) passed the first cleavage division; 92 cleaved embryos were transplanted. Of the rest 224 cleaved embryos that continued to develop in vitro, 78, or 34.8%, reached the blastocyst stage (see Fig. 2, B) In general, the percentage of blastocyst development vs. the number of COCs set for maturation was 21.0%.

1. The effectiveness of IVEP embryo transplantation to recipient Romanov ewes (Ernst Federal Research Center — VIZh, Moscow Province, **2023)**

Recipient number	Transplanted emvryos, *n*	Ultrasound examination results	Lambs born, *n*	
			total	alive
062	14	No	0	0
195	14	Yes	3	2
5750	19	No	0	0
039	13	No	0	0
005	15	Yes	0	0
26	17	Yes	1	1

N o t e. The pregnancy (yes/no) was assessed by an ultrasound examination on days 35-42 after embryo transfer.

Table 1 shows the results of transplantation of in vitro obtained embryos. Ultrasound diagnostics confirmed that after embryo transplantation, pregnancy occurred in 3 out of 6 recipients (50%) (Fig. 3, A). The proportion of transplants that resulted in the birth of live offspring (see Fig. 3, B) was 33.3% (2 out of 6). One ewe (recipient No. 195) naturally gave birth to three lambs, two females and one male, two of which (a male and a female) were alive and healthy (see Fig. 3, B), and one lamb was born dead, but full-term. The second ewe (recipient No. 26) similarly gave birth to one healthy ram. In the first case, the viability of the transplanted embryos was 21.4% (3 out of 14), in the second 5.9% (1 out of 17). In recipient No. 195, the duration of the gestation period from the date of embryo transfer was 147 days, the average weight of the born lambs was 2.97 kg, in recipient No. 26 it was 145 days and 3.0 kg, respectively.

To control for origin, we determined microsatellite profiles of animals (Table 2). This method is generally accepted for confirming/excluding kinship in a mother—offspring pair [20].

Fig. 3. **In vivo development of IVEP sheep embryos after transplantation:** on the left, ultrasound diagnostics (ultrasound scanner Draminski 4Vet Slim, DRAMI SKI S.A., Poland), 42 days after embryo transplantation, recipient No. 195; on the right, ewe No. 195 with two newborn lambs 3 h after lambing (March 17, 2023, Ernst Federal Research Center — VIZh, Moscow Province).

2. Microsatellite profiles of recipient Romanov ewes and surrogate lambs and STR panel indicators (Ernst Federal Research Center — VIZh, Moscow Province, 2023)

STR	Family 1			Family 2	
	M195	O1	O2	M26	O
INRA005	131/131	129/131	129/131	131/131	147/149[a]
SPS113	140/152	140/148	148/148[a]	140/148	140/152
McM527	169/169	169/171	173/175[a]	169/183	169/177
INRA23	201/205	205/223	203/203[a]	205/221	203/203[a]
MAF65	125/125	131/137[a]	131/135[a]	135/135	125/125[a]
INRA063	181/181	181/187	189/205[a]	173/183	177/181[a]
HSC	276/286	276/282	276/286	286/286	272/276[a]
OarCP49	106/108	94/102[a]	96/100[a]	100/106	98/106
OarAE129	147/149	147/149	139/139[a]	149/149	147/147[a]
MAF214	190/200	188/192[a]	190/192	192/192	190/190[a]
OarFCB11	131/135	123/135	127/139[a]	131/139	131/145
Parameter	Family 1			Family 2	
PI	8.58×10^{-10}			1.07×10^{-7}	
PX1	99.92			98.96	
PX2	98.31			92.25	
PX3	99.999			99.928	

N o t e. Family 1 — mother (M) recipient No. 195, offspring (O) 1 and 2, family 2 — mother (M) recipient No. 26, offspring (O); the letter ([a]) indicates microsatellite alleles in the offspring profile that do not coincide with the maternal.

The 11 STR based assessment of the origin of the surrogate lambs born by recipient No. 195 revealed the probability of genotype matching (PI) with a lower threshold of 8.58×10^{-10}. The accuracy of confirming the origin for two (PX1) and one (PX2) parents was 99.92 and 98.31%, respectively, the accuracy of excluding parents (PX3) was 99.99%. A comparison of allelic profiles in female and male offspring demonstrated their discrepancy with the surrogate mother at 3 and 8 microsatellite loci, respectively, and between each other at 6 loci, which excludes any probability of consanguinity. The lack of relationship with the surrogate mother (PX3) was also confirmed in the offspring born by recipient No. 26 for 7 microsatellite loci with an exclusion accuracy of 99.93%. A combined analysis for two surrogate families showed the accuracy of excluding consanguinity for two parents (PX3) to 99.9999%.

In sheep, due to the high cost and high invasiveness of the procedure for obtaining female germ cells from live animals, oocytes are usually removed from the ovaries post mortem [2], which involves either dissection of the follicle walls or aspiration of their contents [21]. The latter approach is considered simpler, but it does not allow full use of the generative potential of the original biological

material, and also, according to some reports, negatively affects the quality of the isolated COCs [22]. Careful dissection of the ovarian follicle walls with a cutting instrument increases the number of COCs recovered and better preserves their structure [5]. Using this technique, we recovered an average of 5.9 COCs from one ovary, of which 60% were suitable for obtaining embryos in vitro. Other researchers have reported similar results when choosing this method [5, 21, 22].

Oocytes isolated from follicles, in order to become suitable for fertilization, must mature in vitro, that is, must reach the metaphase II of the meiotic division. They must undergo the necessary cytoplasmic transformations [4]. It is well known that in vitro maturation is a key step in obtaining good quality oocytes, determining their competence for embryonic development. In sheep, a recent analytical review showed that 70-90% of oocytes mature in vitro [2]. In this case, we are talking about both donor (obtained from living animals) and post mortem oocytes. However, there are studies that report lower values of this parameter [23, 24] (Table 3). In our study, the maturation percentage of oocytes isolated from follicles post mortem is comparable to higher values and amounted to 77%.

3. The effectiveness of the in vitro production embryos from ewe oocytes isolated post mortem (according to the literature)

Country	Breed	Semen	IVM, %	Embryo development, %			Reference
				of total number of mature oocytes	up to the blastocyst stage		
					1	2	
Australia	Merino	Frozen	93.1	73.7	41.8	56.7	[15]
Brazil	No data	Frozen	88.0	68.0	33.8	48.9	[25]
Great Britain	Texel[б]	Frozen	63.2	89.7	40.4	45.1	[23]
Iran	No data	Frozen	92.0	63.3	20.0	31.7	[26]
Iran	Lori-Bakhtiari	Fresh	No data	81.6	31.7	38.7	[27]
Iran	Sajabi[б]	Fresh	No data	66.2	20.6	31.0	[28]
Spain	Rasa Aragonesa[б]	Fresh	No data	74.3	30.0	40.0	[29]
Spain	No data	Frozen	No data	60.4	4.0	6.7	[30]
Italy	Sarda sheep	Fresh	No data	80.0	30.0	38.0	[31]
Italy	Sarda sheep[б]	Frozen	92.0	74.5	22.4	59.2	[32]
Italy	Sarda sheep	Fresh	73.2	58.6	11.6	19.8	[33]
China	No data	Fresh	48.4	72.3	29.9	41.4	[24]
Mexico	Rideau Arcott[a]	Fresh	д/о	76.5	21.3	27.7	[34]
Poland	No data	Frozen	д/о	57.9	21.9	36.7	[35]
Portugal	Merino	Fresh	87	41.9	15.6	37.4	[36]
		Frozen		45.1	19.5	42.8	
Saudi Arabia	Naimi	Fresh	No data	29.9	7.1	23.5	[37]
		Frozen		18.8	1.3	7.0	
Saudi Arabia	Najdi	Frozen	No data	35.0	5.9	17.2	[37]
				18.98	0.7	4.1	
Uruguay	No data	Frozen	No data	69.8	35.2	50.5	[38]

Т щ е е. IVM — in vitro maturation; 1 — percentage of the number of mature oocytes, 2 — percentage of the number of cleaved embryos; [a] and [b] — information about the breed for sperm or oocytes, respectively.

The source of sperm for in vitro fertilization of mature oocytes can be either fresh or frozen semen (see Table 3). Here, we use the latter. This is more practical, in addition, there are few reportws about a decrease in the fertility of cryopreserved vs. native semen in vitro [30, 37]. Active sperm are obtained either by the swim-up method or by density gradient centrifugation, e.g., in Percoll. The question of which techniques are more effective in case of sheep semen remains open [5], so we preferred the swim-up method which we use for IVEP in cattle [16].

For the success of IVF, it is important that the germ cell co-incubation environment is capable of providing sperm with ideal conditions for their penetration into the oocytes. Most laboratories use synthetic oviduct fluid, supplemented with the necessary capacitating agents [1]. We set out to simplify and make this IVEP step more reproducible, so commercial BO-IVF medium (IVF Bioscience, UK) was used for co-incubation of oocytes and sperm. The proportion

of oocytes that proceeded to cleavage which is a criterion for assessing the frequency of fertilization. In our work, this estimate was 60.3%, being comparable with the values established by other authors [26, 30, 33, 35] (see Table 3), and in some cases exceeding these values [36, 37].

In our study, to determine the competence of fertilized oocytes to embryonic development, they were either cultured for 7 days until the blastocyst stage, or transplanted into the uterine horn of recipient ewe after 2 days of incubation and cleavage. The yield of embryos at the blastocyst stage was 21.0% from the COCs subjected to maturation, and 34.8% from the number of cleaved oocytes. If compare these values with those presented in Table 3, we can see that in general they are consistent with the results of other authors [26, 32, 35, 26], but there are also more successful [15, 23, 25] and less successful [30, 37] works. The discrepancies can be explained by peculiar protocols used, differences in the quality of COCs, and the influence of the physiological state and genetic background of germ cell donors [2, 5, 13].

In our study, after transplantation of IVEP embryos, pregnant ewes accounted for 50% (3/6), and 33.3% (2/6) of transplantations resulted in the birth of lambs. The offspring (three lambs, two rams and one ewe) are the first in domestic practice; currently the animals are healthy and their development is being monitored.

Given the proportion of oocyrw cleaved after fertilization to the Bl stage in vitro (34.8%), the described positive effect of group embryo transplantation [5] and the multiple pregnancy of the Romanov ewes, we transplanted from 13 to 19 2 day embryos per recipient. Approximately that number of embryos has been reported by other authors [39, 40].

Thus, the results we obtained under in vitro maturation of sheep oocytes (77%), in vitro fertilization (60%) and in vitro development of embryos (34%) indicate the compliance of the IVEP technology we used with world analogues. The obtained embryos could develop into viable offspring. There is every reason to believe that the proposed IVEP technology with embryo transplantation into recipient animals can be practiced during reproduction and genome editing in sheep.

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PRESENCE OF FOLLICULAR FLUID EXTRACELLULAR VESICLES DURING in vitro MATURATION OF DONOR COW (*Bos taurus)* OOCYTES INCREASES THEIR ABILITY TO in vitro EMBRYO DEVELOPMENT

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Abstract

In vitro embryo production (IVEP) technology from the oocytes isolated by transvaginal follicle puncture (ovum-pickup, OPU) is widely used to generate a number of descendants from the most valuable donor cows and becomes a routine in cattle breeding programs to replicate and conserve precious genotypes. To increase the efficiency of OPU/IVEP-technology, in this work for the first time, bovine OPU-oocytes were matured in the presence of extracellular vesicles (EVs) from follicular fluid (FF), and the ability of treated oocytes to IVEP after in vitro fertilization was investigated. The aim was to study the effects of FF EVs on oocyte maturation and capacity to develop up to blastocyst of OPU-oocytes, as well as to resistance of such blastocysts to freezing. EVs were obtained from FF by differential centrifugations followed by ultracentrifugation at 100,000 g. The preparations were analyzed by transmission electron microscopy that confirmed the presence of exosome-size EVs. Oocyte donors were sexually mature Yaroslavl breed heifers (n = 6) following natural cycle. OPU was performed twice a week. The isolated oocytes were in vitro matured in TC-199 medium, supplemented by fetal bull serum (10 %), follicle stimulating and luteinizing (10 μg/ml) hormones, epidermal growth factor (10 ng/ml) in absence (control) or presence of EVs (experiment). Vesicular preparations were added to the in vitro maturation (IVM) medium in the physiological concentration (EVs isolated from 1 ml of FF per 1 ml of medium). After 24 hours of IVM, the oocytes were subjected to in-vitro fertilization and in vitro embryo culture. At day 3 after fertilization, oocyte morphology was checked, and at day 7 of culture, a number of embryos developed to blastocyst (Bl) was determined. The resulting Bl were frozen and stored for some time at –196 °C. Then the blastocysts were thawed and in vitro cultured until hatching to determine their viability. A total of 10 independent experiments were performed, 57 and 56 oocytes were analyzed in control and treatment, respectively. No impact of experimental conditions on nuclear maturation rate was evidenced. The mature oocytes were similar in control and EVs-treated groups and accounted for 90.4±5.6 % and 94.3±3.1 %, respectively. In addition, the presence of EVs during IVM did not change oocyte cleavage rate after vitro fertilization, 78.6±7.3 % and 86.7±4.9 % in control and EV-treated groups, respectively. However, beneficial effect of EVs on blastocyst rate was found. In control, 26.6±5.8 % of OPU oocytes developed to Bl. EVs added during IVM increased blastocyst rate to 41.2±3.2 % ($p < 0.05$). EVs also tended to increase cryoresistance of resulting blastocysts. In control, blastocyst hatching rate after thawing and short-term culture was 29.1±8.8 % and increased to 53.3±9.2% in the EVs group. Thus, addition of EVs from cow follicular fluid during IVM culture increases oocyte quality and, consequently, their competence to embryo development after in vitro fertilization. Therefore, using of follicular fluid EVs during in

vitro maturation of OPU oocytes can improve the efficiency of OPU/IVEP technologies in cattle.

Keywords: extracellular vesicles, bovine follicular fluid, bovine oocytes, in vitro maturation, oocyte aging, embryo development

Biotechnologies aimed at obtaining embryos from living donors and their transplantation into recipient animals are widely used in livestock breeding to increase the number of offspring from the best mothers in order to more fully realize their genetic potential in generations and accelerate genetic progress in cattle breeding [1, 2].

Embryos are obtained either in vivo using Multiple Ovulation and Embryo Transfer (MOET) technology or in vitro. In MOET, the essence of the technique is to induce superovulation in donor cows through hormonal treatment followed by artificial insemination, embryo flushing and transplantation of fresh or frozen-thawed embryos into recipients [1]. The technology for obtaining embryos in vitro (in vitro embryo production, IVEP), in turn, includes the isolation of female germ cells (oocytes), their maturation and fertilization in vitro, followed by in vitro culture of the resulting zygotes to embryonic stages suitable for freezing and transplantation [2]. Oocytes are recovered from the ovarian follicles of living cows and heifers most often by transvaginal follicle puncture (ovum pick up, OPU). Unlike MOET, OPU can be performed repeatedly and over relatively long periods of time [3, 4]. In addition, to isolate oocytes from follicles, it is not necessary to perform hormonal stimulation of the ovaries, and therefore, the procedure can be repeated at shorter intervals, (usually twice or once a week, compared to obtaining embryos in vivo, no more than once every 2 months. According to statistics of recent years, the number of in vivo embryos used in practice for transplantation into recipients is approximately at the same level [5, 6], and those obtained in vitro (in vitro production, IVP) continue to increase with an average annual growth rate of 12% [7]. This is due, first of all, to the fact that IVEP technology, which involves the use of donor OPU oocytes (OPU/IVEP technology), has now become an alternative to the traditional program for obtaining embryos in vivo [6] and is increasingly used for commercial purposes in many countries [7, 8].

An important condition for the practical use of OPU/IVEP technology remains its efficiency, which, undoubtedly, when compared in retrospect, has increased significantly in recent years, but is nevertheless inferior in a number of MOET indicators. First of all, we are talking about a decrease in the quality of embryos obtained using OPU oocytes and the resistance of OPU/IVEP embryos to freezing, as well as a decrease in their viability after transplantation compared to those obtained in vivo [9-11]. Continuing research on optimizing the stages of OPU/IVEP technology and identifying factors affecting its effectiveness will solve this problem.

As is known, the effectiveness of OPU/IVEP technology is primarily determined by the quality of oocytes before the in vitro fertilization procedure, which they acquire during in vitro maturation (IVM) [12, 13]. However, IVM conditions still remain suboptimal and require improvement.

In routine practices of in vitro bovine reproductive biotechnologies, maturation of bovine oocytes is carried out in optimized media containing, at a minimum, growth factors such as EGF and bovine fetal serum [14], and commercial serum-free media are also used. When developing serum-free media which were initially less effective, various components were used (hormones, amino acids, antioxidants, fatty acids, vitamins, metal ions and various biological preparations). When added, these components could have a positive effect on the cytoplasmic maturation of the oocytes and increase in vitro the number and quality of blastocysts capable of developing into a viable fetus [15, 16]. However, the quality of

OPU oocytes maturing in vitro, usually in small groups, is usually lower than in culture with a large number of cumulus-oocyte complexes (COCs), usually from 25 to 50 in 0.5 ml of medium. OPU oocytes obtained from some cows are often surrounded by fewer cumulus cells (CCs) which can reduce the results of in vitro maturation, primarily cytoplasmic. The addition of 5% follicular fluid (FF) to the IVM medium, which is the optimal medium for oocyte maturation, increases their competence to embryonic development in vitro, especially in the culture of individual oocytes [17]. In addition to the hormonal and steroid components of FF, secretion products of follicular cells and plasma derivatives are contained in extracellular vesicles (EVs), in particular in nanovesicles with a diameter of 30-150 nm called exosomes. Such EVs contain various regulatory factors involved in the molecular dialogue of the oocyte with follicular cells, and primarily with cumulus cells. EVs concentrated in follicular fluid are taken up by target cells via intercellular connections and transzonal projections between the oocyte and the surrounding CCs [18, 19]. Bovine FF EVs contain various proteins [20], lipids [21] and nucleic acids, including microRNAs that regulate gene expression in target cells, in particular in the oocyte [22, 23]. These components are necessary for the development of the oocyte and the formation of its competence to embryonic development. FF vesicular factors are involved in the regulation of signaling pathways that control the development of the follicle and oocyte in it, serve as mediators of cell responses to hormonal and environmental stress, and influence oocyte maturation. In vitro, EVs from FFs enhance the proliferation of follicular granulosa cells, increase their steroid synthesis, cumulus expansion, reduce apoptosis in CCs and oocytes, and influence the activation of various signaling pathways in CCs [22]. FF EVs, when added to IVM medium, improve IVEP efficiency and embryo quality in vitro in cows [24, 25] and positively influence the development and survival of embryos under thermal stress in cows [25]. Although the mechanisms of these effects are far from fully understood, they involve regulation by microRNAs of the functions of specific proteins and lipids that mediate molecular signals in oocytes and CCs and thus influence the oocyte maturation.

In the presented work, we for the first time studied the effect of FF EVs added to the maturation medium on the competence of bovine OPU oocytes to embryonic development in vitro.

The purpose of the work was to study the effect of cow FF EVs on the maturation of OPU oocytes during IVM and on their ability to develop in vitro to the blastocyst stage. The influence of the tested conditions on the resistance of the resulting blastocysts to freezing was also assessed.

Materials and methods. Reagents from Sigma-Aldrich (USA) were used in the work except for specially indicated cases.

To isolate EVs, cow ovaries obtained from a meat processing plant were transported to the laboratory on ice. Ovaries were freed from surrounding tissues and washed in a sterile physiological solution which was pre-cooled to +4 °C and contained antibiotics (100 IU/ml penicillin and 50 μg/ml streptomycin; BioPharmGarant, Russia). To obtain EVs, FFs were aspirated from antral follicles of 3-6 mm diameter and centrifuged for 15 min at +20 °C and 300 g to remove somatic cells. All subsequent centrifugations were also carried out at +20 °C. The supernatant was re-centrifuged for 15 min at 2000 g to remove apoptotic bodies 1-5 μm in size. A third centrifugation (15 min at 12,000 g) was carried out to remove large microvesicles measuring 200-1000 nm. After this preliminary purification, the EVs were pelleted by ultracentrifugation for 90 min at 100,000 g (CS 150 NX centrifuge, Hitachi, Japan). The supernatant was diluted with sterile phosphate-buffered

saline (PBS, pH 7.4) and re-centrifuged for 90 min at 100,000 g for more complete isolation of EVs. The pellets from the two ultracentrifugations were combined and dissolved in PBS. Two 5 μl aliquots were taken from the obtained samples. One of the aliquots was used to determine the number of EVs by protein concentration on a Qubit™ 4 Fluorometer using a Qubit Protein Assay Kit (Thermo Fisher Scientific, USA) and a Qubit protein standard 0.125 to 5 mg/ml. The second aliquot was used for ultrastructural analysis of the particle preparation using transmission electron microscopy (TEM), for which it was mixed with an equal volume of 2% glutaraldehyde (electron microscopy quality, EM) in PBS (Agar Scientific, Ltd., UK) and fixed for 1 h at room temperature. 2 μl of fixed EVs samples were applied to nickel EM grids (Agar Scientific, Ltd., UK) coated with carbon-coated Formvar film and incubated for 60 min in a humid chamber. The grids with EVs deposited on the film surface were washed with distilled water to remove PBS salts. To do this, a drop of 10 μl was applied to the film surface 3 times; after 10 s, it was removed by touching the filter paper with the edge of the mesh. Negative contrast with a 2% aqueous solution of uranyl acetate (Electron Microscopy Science, USA) was carried out in a similar way (3 times for 10 s), removing a drop of the solution by touching the edge of the filter paper mesh. After removing the last drop, the mesh was air dried. Ultrastructural studies were performed usinga JEOL 1011 transmission electron microscope (JEOL, Ltd., Japan). The samples were photographed with a GATAN RIO 9 digital camera using the DigitalMicrograph3 program (Gatan, Inc., USA). Based on the electron microscopy analysis, the EVs in the obtained samples were detected and their morphology was assessed.

The concentration of EVs was measured (a ZetaView nanoparticle analysis apparatus, Particle-Metrix, Germany), calibrated with control 100 nm particles. EVs preparations from cow FFs were diluted in a ratio from 1:1000 to 1:5000 in sterile PBS (0.1 μm filter) and examined in laser (λ = 488 nm) measurement mode using 1 ml of the diluted preparation. The concentration was calculated (specialized program ZetaView, version 8.05.14 SP7).

When obtaining IVEP embryos using OPU technology, oocyte donors were sexually mature, clinically healthy Yaroslavl heifers (*Bos taurus*) (n = 6) aged 3.2-3.5 years. The donors' diets were balanced in terms of energy, nutrients and biologically active substances in accordance with the animals' needs. OPU was performed 2 times a week with an interval of 3 or 4 days.

Transvaginal puncture of the follicles was performed using an OPU system, which included a Versana Active ultrasound scanner with a convection broadband probe (frequency 5 MHz), a probe holder (GE HealthCare, USA), and a vacuum pump (Mini-tube, Germany). Animals were individually fixed in a pen and epidural anesthesia was performed with 2% novocaine. Visible follicles were aspirated with a needle connected by silicone tubing to a 50-ml centrifuge tube. Dulbecco modified medium added with the of 10% fetal bovine serum (FBS) (Biolot, Russia) and heparin (18 IU/ml) was used as aspiration medium. Aspirates from each donor (1 OPU session) were filtered individually, washed with PBS supplemented with 1% PBS (Biolot, Russia). Oocytes were looked for under a stereomicroscope (Nikon, Japan) and transferred to Petri dishes with TC-199 medium, containing 5% FBS and 50 μg/ml gentamicin (TC-199M).

The total pool of oocytes collected from each donor was washed three times in TC-199M and divided according to morphological criteria into three quality categories, good, medium and poor. Oocytes with homogeneous cytoplasm surrounded by more than one layer of compact cumulus were assigned to good quality group; oocytes with homogeneous or moderately heterogeneous cytoplasm, with one layer of cumulus cells (CC) or partially surrounded by CC were assigned

to medium quality group. Oocytes with heterogeneous cytoplasm with signs of granulation or lysis, naked cells, and mature cumulus-oocyte complexes (COCs) were classified as poor. Only oocytes of good and medium quality were considered suitable for further work.

Selected oocytes of good and medium quality were pooled and cultured in groups (4-8 oocytes) in 200 μl drops of medium applied to the bottom of a well of a 4-well plate (Biomedical, Russia) and completely covered with mineral oil. As a control, TC-199 with FBS (10%), sodium pyruvate (0.5 mM), follicle-stimulating hormone (10 μg/ml), luteonizing hormone (10 μg/ml), epidermal growth factor (20 ng/ml) and gentamicin (50 μg/ml) was used as an IVM medium. In the experiment, EVs were added to this medium in a physiological concentration, that is, vesicular protein isolated from 1 ml of follicular fluid was added to 1 ml of IVM medium. Oocytes were cultured at 38.5 °C and 5% CO_2 (an incubator MCO-18AIC, Sanyo, Japan). After 24 h maturation, the oocytes were subjected to in vitro fertilization as described previously [26].

For in vitro fertilization, straws with frozen sperm of a Yaroslavl bull were thawed, and active sperm were obtained by the swim-up method using the Sperm-TALP medium [27] as described [28]. The isolated spermatozoa were added to the fertilization medium with the COCs previously transferred, so that the sperm concentration was 1.5×10^6/ml.

Germ cells were co-cultured for 10-11 h, and then the oocytes were separated from CC and adherent sperm and assessed morphologically. The percentage of maturation was fetermined as the proportion of oocytes with polar bodies (PB) to the total number of oocytes. Purified fertilized oocytes were transferred to 100 μl drops of BO-IVC medium (IVF Bioscience, UK) in the wells of a 4-well plate (Nunc, Denmark) for embryo development (an incubator (MCO-50M-PE, Sanyo, Japan, 38.5 °C and 5% CO_2, 5% O_2 and 90% N_2). Three days after fertilization, the medium was replaed, and the cleaved zygotes were morphologically assessed. On day 7 of incubation, the number of embryos that had developed to the blastocyst stage was counted.

The resulting blastocysts were frozen using an automated freezer CL-8800 (Cryologic, Australia) in a 1.5 M ethylene glycol solution (IVM Technologies, France), and then stored in liquid nitrogen vapor for at least 1 month. To assess viability, embryos were thawed and cultured in BO-IVC medium (IVF Bioscience, UK) supplemented with 5% FBS for 3 days until the hatching stage.

Cleavage, development to the blastocysts and hatched blastocysts were evaluated under a stereomicroscope (Nikon, Japan). In some cases, IVEP embryos were photographed (an Eclipse Ti-U microscope, Nikon, Japan).

Statistical processing of the data was performed using one-way analysis of variance in the SigmaStat program (Systat Software, Inc., USA). Data are expressed as means (*M*) and standard errors of the means (±SEM). The significance of the differences in the compared means was assessed using the Tukey test (p not more than 0.05).

Results. Preparations enriched with extracellular nanovesicles were obtained by successive centrifugations of FF from antral follicles measuring 3-6 mm. Isolation was completed by ultracentrifugation at 100,000 g and washing of excess protein with PBS. Such preparations, diluted in phosphate buffer, contained EVs similar in size to exosomes [29, 30], as well as clusters of lipoproteins (Fig. 1, A). In preparations, the total protein content was 31.18±4.4 μg/ml of FF. The EVs concentration of per 1 ml of FF was $2.4\text{-}4.5\times10^{12}$ with an average particle size of 132.4-135.9 nm (see Fig. 1, B).

A total of 321 ultrasound-visible follicles were aspirated in 34 OPU sessions, and 156 oocytes of varying quality were isolated from these follicles. After

morphological assessment, oocytes of good and moderate quality were cultured for maturation in either control IVM medium or medium containing EVs. Table 1 shows that the groups had comparable indicators both for the OPU effectiveness and in the quality of oocytes used for research. The first parameter was assessed by the number of aspirated follicles per OPU session and by the proportion of retrieved oocytes from the number of aspirated follicles. The second parameter, was assessed by the proportion of oocytes of good and average quality suitable for cultivation from the total number of isolated oocytes.

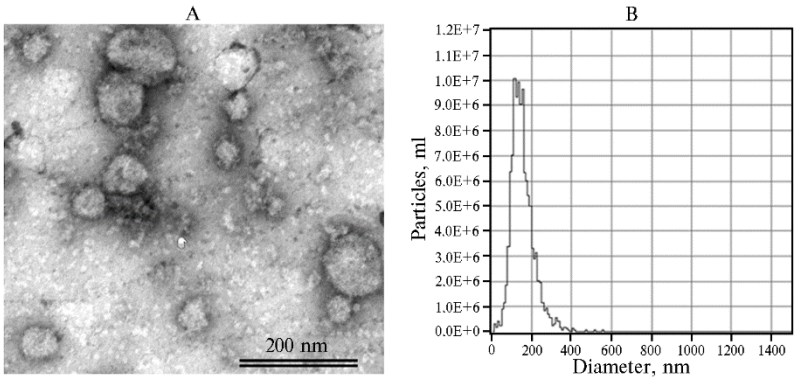

Fig. 1. Analysis of extracellular vesicles (EVs) **from the follicular fluid** (FF) **of the antral follicles of cows:** A — preparation of EVs (transmission electron microscopy, JEOL 1011, JEOL, Ltd., Japan); B — graph of size (diameter) distribution and concentration of particles in a diluted preparation of EVs from the FF of cow ovaries (Laboratory of Cell Biology and Electron Microscopy, Faculty of Medicine, University of Tours, 2022).

1. Isolation and quality assessment of OPU oocytes from Yaroslavl heifers (*Bos taurus*) in the control and test (EVs) **groups** (Ernst Federal Research Center — VIZh, Moscow Province, 2023)

Parameter	Control	EVs
Number of OPU sessions, *N*	18	16
Aspirated follicles, *n*	164	157
Number of aspirated follicles per OPU session, *n*	9.11	9.81
Total number of isolated oocytes, *n*	80	76
Proportion of oocyte recovery from the number of aspirated follicles, %	55.7±7.2	53.3±7.3
Proportion of good quality oocytes from the total number of isolated oocytes, %	33.3±7.3	32.5±8.7
Proportion of oocytes of average quality from the total number of isolated oocytes, %	39.6±5.2	46.7±8.8
Proportion of oocytes suitable for cultivation from the total number of isolated ones, %	72.9±4.3	79.3±5.5

N o t e. In vitro maturation mediumwithout or with extracellular vesicles from the follicular fluid of cows mean control and treatment (EVs) groups, respectively.

Table 2 shows the results of maturation and embryonic development of OPU oocytes.

2. Competence to embryonic development after in vitro fertilization of OPU oocytes of Yaroslavl heifers (*Bos taurus*) as influenced by extracellular vesicles (EVs) **from the follicular fluid in the maturation medium** (Ernst Federal Research Center — VIZh, Moscow Province, 2023)

Parameter	Control	EVs
Tepetitions, *N*	10	10
Number of oocytes, *n*	57	56
Proportion of mature oocytes, %	90.4±5.6	94.3±3.1
Proportion of fragmented oocytes, %	78.6±7.3	86.7±4.9
Proportion of oocytes that have developed to the blastocyst stage, %	26.6±5.8	41.2±3.2*

N o t e. Control means medium for in votro maturation IVM.
* Differences with control are statistically significant at $p < 0.05$.

The EVs in the IVM medium did not affect the nuclear maturation of OPU oocytes. The percentage of maturation as proportion of oocytes with PB was equally high, more than 90%, in both groups. We also did not reveal the effect of

vesicles on the ability of oocytes to enter the first cleavage division (Fig. 2, A). The proportion of cleaved oocytes after in vitro fertilization was approximately 70%. Nevertheless, a positive effect of EVs from FF on the development of mature and in vitro fertilized oocytes to the blastocyst stage was found (see Fig. 2, B). When culturing OPU oocytes in the control IVM medium, the blastocyst yield was 26.6±5.8%. EVs added to the IVM medium increased the blastocyst yield 1.5-fold ($p < 0.05$), up to 1.37 blastocysts per OPU session vs. 0.77 blastocysts per OPU session in the control.

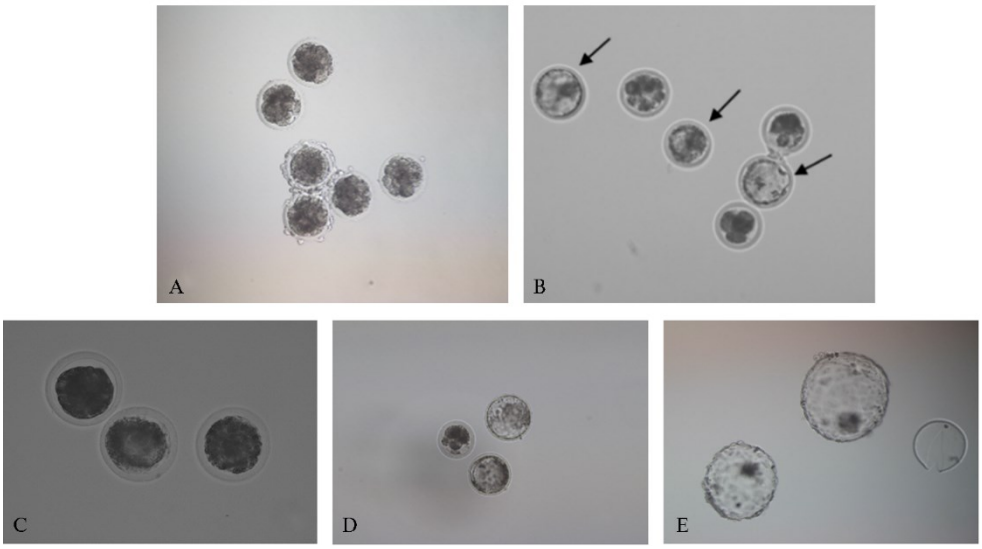

Fig. 2. Embryos of Yaroslavl cattle (*Bos taurus*) developed after in vitro fertilization of OPU oocytes: A — cleaved oocytes; B — embryos that have developed to the blastocyst stage (Bl, marked by arrows); C — frozen Bl right after the defrosting procedure (magnification ×200); D — thawed BL after 24 h culture; E — thawed Bl that reached the hatching stage after 3 days of culture. Microscope Eclipse Ti-U, Nikon, Japan (magnification ×100 except as indicated for B) (Ernst Federal Research Center — VIZh, Moscow Province, 2023).

3. The influence of extracellular vesicles (EVs) **from follicular fluid in the maturation medium of OPU oocytes of Yaroslavl heifers (*Bos taurus*) on the viability of frozen-thawed blastocysts after short-term incubation in vitro** (Ernst Federal Research Center — VIZh, Moscow Province, 2023)

Group	Number		Hatched blastocyst, %
	repetitions, *N*	blastocysts, *n*	
Control (in vitro maturation medium IVM)	10	14	29.1±8.8
EVs (IVM + EVs)	10	22	53.3±9.2*

* Differences with control at $p = 0.081$.

We also determined the long-term effects of FF EVs in the oocyte maturation medium using a test for the freezing resistance of developing blastocysts. When studying the survival of blastocysts after the freezing-thawing procedure, a positive effect of vesicle preparations on maturing oocytes was discovered (Table 3). Exposure to EVs during the maturation of OPU oocytes was responsible for an increase, although not significant, in the proportion of hatched blastocysts (see Fig. 2, E) which likely resulted from the increase in their viability and/or developmental potential before freezing.

Extracellular vesicles are membrane-coated secretory granules that have been found in all types of biological fluids [18], including fluid from female ovarian follicles, which is the natural environment of oocytes during their development in vivo. It has been shown that EVs are involved in intercellular communications within the follicle, since they are secreted by cells and are able to transfer their

contents to other cells due to the ability of the latter to absorb them [18, 19]. In this regard, EVs are being actively studied, including in cows, as potential regulators of oocyte quality and competence to embryonic development in vitro [24, 25, 31-33].

To date, it has been shown that EVs added to IVM medium for post mortem bovine oocytes increases the yield of blastocysts from 26% (control) to 37% [32], and improves the quality of embryos [24]. In addition, it has been found that EVs can improve performance of mature oocytes, increasing their resistance to age-related transformations [34] and protecting from stress [25].

In the present work, we used cow oocytes isolated from the ovarian follicles of individual donors using the OPU method. To our knowledge, this is the first study in which OPU oocytes were cultured to mature in the absence (control) or presence of EVs, then fertilized in vitro and cultured to the blastocyst stage. Moreover, we investigated both the effect of EVs on the maturation and development of OPU oocytes to the blastocyst stages and the resistance of these blastocysts to freezing and thawing. EVs from the FFs of antral follicles (diameter 3-6 mm), according to the generally accepted classification [29, 30], were exosomes in size (see Fig. 1, A, B). Their presence in the maturation medium did not affect the nuclear maturation of OPU oocytes and their ability to enter the first cleavage division after fertilization (see Table 2). However, we have shown that EVs from bovine ovarian fluid, when added to the medium during oocyte maturation, can improve embryo development in vitro. When exposed to EVs, the proportion of oocytes that reached the BL stage increased compared to the control from 26 to 42%, which was even slightly higher than other researcher reported for post mortem cow oocytes [32]. In addition, the presence of EVs in the IVM medium may have a long-term effect, as there was a tendency for the viability of the resulting BLs to increase after the freeze-thaw procedure. In our opinion, the statistical uncertainty in determining the influence of EVs in the IVM medium on the cryostability of Bl may be due to the fetal bovine serum in the medium. The negative effects of some PBS components have previously been reported in similar studies on EVs isolated from media conditioned by oviductal epithelial cells [35].

Thus, the use of extracellular vesicles EVs from the follicular fluid (FF) of bovine ovaries in the in vitro maturation (IVM) procedure improves the quality of oocytes and, theretore, their competence to embryonic development after in vitro fertilization. Preparations enriched with extracellular vesicles from cow FFs can be used during extracorporeal oocyte maturation to more effectively produce high-quality cattle embryos using OPU/IVEP technology.

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EXTRACELLULAR VESICLES INCLUDING EXOSOMES FROM ANIMAL MESENCHYMAL STEM/STROMAL CELLS

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Abstract

Mammalian mesenchymal stem/stromal cells (MSCs) produce extracellular vesicles (EVs) associated with the plasma cell membrane, which may contain growth factors, chemokines, cytokines, and microRNAs. Currently, EVs are widely used to develop new regenerative strategies in the treatment of numerous diseases, since they convey most of the therapeutic properties of MSCs. This work shows for the first time that EVs enriched with exosomes can be isolated from conditioned media (CM) of MSCs from five different animal species using the method of differential centrifugation (DC) followed by ultracentrifugation (UC). The purpose of the work is to obtain EVs from conditioned media (CM) of MSCs of bone marrow (BM), umbilical cord blood (UCB) and adipose tissue (AT) of agricultural (cattle, sheep, horses) and small domestic animals (dogs, cats). We used MSCs that were previously obtained from the bovine and ovine BM, equine UCB, ovine, bovine, equine, canine and feline AT. MSCs were thawed and seeded into 25 cm^2 growth area flasks, after 48 hours they were reseeded into 175 cm^2 growth area flasks in a ratio 1:7 and incubated for 10 days until the cells reached a complete monolayer. Then the CM from all MSC samples was poured into 50 ml sterile centrifuge tubes and EVs were isolated. For this purpose, we used the method of DC followed by UC. In all samples, electron microscopy revealed round or irregularly shaped microparticles of different sizes. The diameters of individual EVs did not differ statistically between different animal species ($p = 0.1$). When comparing the number of particles isolated from 50 ml of CM from MSCs of different animal species, no statistically significant differences were detected ($p = 0.1$). Thus, on one mesh of bovine MSC(BM) and MSC(AT), 3±0.1 and 6±0.07 particles with a size of 50-100 nm, 7±0.02 and 4±0.03 particles of 100- 150 nm, as well as 3±0.4 and 2±0.06 particles larger than 150 nm. EVs from CM of canine MSC(AT) were the most homogeneous in both shape (round) and size, and the main part was found in the range of 50-100 nm (12±0.02 particles). In samples isolated from the CM of ovine, bovine, equine MSC(AT) and equine MSC(UCB), the number of particles with a diameter of 50-100 nm was 7±0.2; 7±0.01; 5±0.7 and 8±0.02. Analysis of the obtained electron diffraction patterns showed that more than 70 % of EVs had a diameter from 50 to 100 nm, that is, they were classified as exosomes. EVs isolated from CM canine MSC were positively stained with antibodies against the TSG101 antigen (cytoplasmic protein, exosome marker). The results obtained demonstrate that the CM of animals MSCs, isolated from BM, AT and UCB contains EVs, including exosomes. The DC method does not exclude the possibility that other particles are present in the preparation, so we propose to designate the result obtained as micro explosives enriched with exosomes. Obtaining EVs of a certain composition from the CM of agricultural and domestic animal MSCs opens up broad prospects for the use of exosomes in the diagnosis of diseases and treatment of agricultural and domestic animals.

Keywords: mesenchymal stem/stromal cells, bone marrow, adipose tissue, umbilical cord blood, horses, cattle, sheep, dogs, cats, extracellular vesicular, exosomes, isolation, differential centrifugation, identification

Animal мesenchymal stem/stromal cells (MSCs) have been attracting

attention for several decades as a promising cellular material in regenerative medicine due to their in vitro adaptation and proliferation, differentiation ability, and powerful immunomodulatory and anti-inflammatory properties [1, 2]. It is possible that the regenerative abilities of MSCs are due to pleiotropic effects mediated by the secretion of soluble paracrine factors and extracellular vesicles (EVs) [3, 4]. EVs are naturally produced nanoparticles. They are an important component of the secretomes of various cells, provide complex transfer of biologically active molecules and horizontal transfer of genetic information [5, 6]. EVs are present in all biological fluids of the body, consist of double layers of phospholipids and differ in origin, composition and functions. Depending on how extracellular vesicles are formed and their size, they are called apoptotic bodies, ectosomes, or exosomes.

Apoptotic bodies, also known as apothecs, are cell fragments bounded by the plasma membrane that are formed as a result of apoptosis. They are approximately 50-5000 nm in diameter and may contain organelles or even fragments of the nucleus of a dead cell. Ectosomes, or budding microvesicles, are formed by protrusion of the plasma membrane from the cell outward; the bulge is detached from the cell membrane and turns into vesicles ranging from 50 to 200 nmm sometimes up to 1000 nm, in diameter. Exosomes are small vesicles, usually 40 to 100 nm in size that appear inside the cell and bud into a cavity called an endosome. When a sufficiently large number of exosomes accumulate in this cavity, its further fate depends on what lipids its membrane consists of. If the endosome is labeled with lysobisphosphatidylic acid (phosphatidylinositol-3-phosphate) and contains ubiquitinated proteins, its contents will be destroyed, it will fuse with the lysosome, a membrane vesicle filled with enzymes that break down proteins, carbohydrates, lipids and nucleic acids. If the endosome membrane contains ceramides, the endosome fuses with the cell surface membrane, and many exosomes are pushed out into the extracellular environment [7].

Exosomes secreted by MSCs are of special interest [3, 8]. Currently, they are widely used to develop new regenerative strategies in the treatment of numerous diseases, since they convey most of the therapeutic properties of these cells. The use of MSC-derived exosomes serves as an alternative to cell therapy and may minimize safety concerns when administering viable cells [9].

Exosome function is easily regulated by pretreatment of MSC culture, for example by adding chemical factors or cytokines, creating hypoxic conditions, and introducing gene modifications [10]. However, the mechanisms of the regenerative abilities of MSC exosomes in target cells are still not well understood. In addition, there are several unresolved issues, namely the lack of standards and guidelines regarding the size of EVs, their purity, the expression of certain biomarkers (e.g., CD9, CD63, CD81) on their surface and acceptable levels of contamination for identification and quality control of isolated exosomes [11]. The use of MSC exosomes in clinical settings is also limited due to the lack of standardized cell culture conditions and optimal uniform protocols for isolation and storage of exosomes, optimal therapeutic dose and administration schedule, and reliable assays to evaluate the effectiveness of EVs therapy.

Improving methods for obtaining EVs of a certain composition opens up broad prospects for their use in the clinical practice of veterinary medicine [12]. Infectious diseases and zoonoses are among the main problems in keeping farm animals. Modern understanding of the biology of infectious agents is critical to ensuring the health and welfare of animals, maintaining their high productivity performance and food safety. It is believed that human EVs can carry viral elements, participating in both the spread of infectious diseases and the induction of an immune response against the infectious agent, and represent promising material

as diagnostic biomarkers for a better understanding of the disease pathogenic mechanisms [13]. EVs that are released by HeLa cells infected with Newcastle disease virus (NDV) or Madin-Darby bovine kidney cells (MDBK) infected with caprine parainfluenza virus type 3 (CPV3) carry microRNA, RNA or proteins that enhance the cytopathic activity of the virus. In addition, EVs were able to suppress the expression of the interferon gene (*IFN-β*) in HeLa cells after NDV infection and inhibited autophagy during CPIV3 infections, suggesting a significant role for EVs in viral spread [14, 15]. EVs are secreted by various cells and, upon adaptation to in vitro conditions, are released into the growth medium, reaching a maximum amount when the medium becomes conditioned.

This work shows for the first time that using differential centrifugation (DC) followed by ultracentrifugation (UC), EVs enriched in exosomes can be isolated from conditioned media (CM) of MSCs from five animal species.

The purpose of the work is to obtain extracellular vesicles from conditioned media of mesenchymal stem/stromal cells of bone marrow, umbilical cord blood and adipose tissue of cattle, sheep, horses and small domestic animals (dogs and cats).

Magterials and methods. MSCs from the bone marrow (BM) of cattle and sheep, umbilical cord blood (UC) of horses, adipose tissue (AT) of sheep, cattle, horses, dogs and cats were isolated by us previously [16-19] and deposited in the Specialized Collection of Continuous Transplantable Somatic Cultures of Agricultural and Game Animals RKKK (P) (SKhZh RAS, FSC ARRIEVM RAS) after identification and standardization. In the experiment of 2023, primary MSCs were thawed and plated into flasks with 25 cm^2 avaible growth area; after 48 h culture, they were re-plated (1:7) into flasks with 175 cm^2 growth area and cultured for 10 days.

The main culture medium for MSCs was DMEM with a low glucose content (1 g/l) (PanEco, Russia) supplemented with 10% bovin fetal blood serum (FBS) (HyClone, USA) and 1× solution of essential amino acids and antibiotics (PanEco, Russia). The final concentration of streptomycin was 50 μg/ml, penicillin 50 U/ml. Before use, FBS was preliminarily purified from exosomes (a L7 Ultracentrifuge, Beckman, USA; 100,000 g for 60 min).

Conditioned media (CM) were collected from MSCs cultures after they reached 100% monolayer in flasks with 175 cm^2 surface area, the CM volume per flask was 50 ml. Morphological analysis of cells was performed visually (a phase-contrast microscope with AxioVision Rel software for measurements. 4.8, Carl Zeiss, Germany).

EVs were isolated by differential centrifugation (DC). First, CM were collected in 50 ml sterile tubes (SPL Life Sciences Co, Korea) and subjected to low-speed centrifugation (500 g, 20 min, a CM-6M centrifuge, SIA ELMI, Latvia) to remove dead cells, decayed cell components and other extracellular matrix particles in the CM that sediment. The supernatant was poured into another sterile tube and filtered (filter pore size 0.22 μm; GE Osmonics, USA). The filtered CM was again centrifuged (CM-6M, SIA ELMI, Latvia) at 2300 g for 30 min to remove apoptotic bodies from the sediment, and the supernatant was subjected to high-speed centrifugation at 13000 g for 30 min (an Eppendorf 5804, Eppendorf, Germany). Finally, EVs were recovered by ultracentrifugation at 100,000 g for 60 min (a L7 Ultracentrifuge, Beckman, USA). The supernatant was removed and 100 μl phospate-buffered saline (PBS) (PanEco, Russia) was added to the sediment and carefully resuspended by pipetting.

The resulting EVs were examined using the negative contrast method [20]. A 30-μl EVs suspension in PBS was applied to a 150 mesh copper grid coated with butvar film; after 1 min, excess liquid was removed by touching grid edge

with filter paper. Next, 30 μl of a 2% phosphotungstic acid neutralized with a 1 M NaOH to pH 6.5, was applied to the grid. Excess liquid was removed after 10 s in a similar manner. The grids were air-dried for 10 min and examined (an electron microscope JEM-100CXII, JEOL, Japan; instrumental magnifications ×27000, ×40000 and ×50000). The exosomes collected from 50 ml suspension of animal MSCs were assessed by the size (diameter) and counted at least in 10 fields of view per preparation in triplicate.

Samples of EVs from CM of canine MSCs (AT), mouse embryonic fibroblasts STO line, and bovine MSCs (BM) (negative control) were lysed (RIPA buffer, Wuhan Servicebio Technology, Ltd., China). The protein concentration in the lysates was measured using a protein assay kit (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Inc., USA). A 5× buffer (10% SDS, 250 mM Tris-HCl, pH 6.8, 0.5% bromophenol blue, 50% glycerol, 5% β-mercaptoethanol) was added to equal volumes of lysates at 1:4 and heated for 10 min at 95 °C for reduction and denaturation. Proteins were sedeparated electrophoretiaacally in 10% polyacrylamide gel with 1% SDS (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Merck Millipore, USA). Membranes were blocked with 5% skim milk in PBS with 0.1% Tween-20 for 1 h at room temperature. Next, they were incubated at the same temperature for 1 h with primary antibodies (AB) to TSG101 (sc-7964, 1, Santa Cruz Biotenology, USA; dilution 1:1000) followed by 1 h incubation at room temperature with secondary goat AB to mouse antigens (AG) labeled with horseradish peroxidase (Axioma BIO, Russia).

Data statistical processing was carried out using Microsoft Excel.Arithmetic means (*M*) and standard errors of the means (±SEM) were calculated. The significance of differences was assessed using Student's *t*-test at $p < 0.05$.

Results. EVs were isolated from the CMs of MSCs of five animal species, for cattle and sheep the cell populations were MSCs (AT) and MSCs (BM). The cells had similar properties in vitro.

Figure 1 shows the fibroblast-like morphology of MSCs isolated from the BM of cattle (a) and sheep (g), from AT of cattle (d) and dogs (m), and UC of horses (k). Due to the lack of specific antibodies to AG of farm animals, the MSC phenotype was confirmed functionally by the ability to generate adipose and bone tissue cells upon induction. After induction by an adipogenic medium for 21 days, MSCs gradually transformed from fibroblast-like cells into flattened cells, and lipid droplets of different sizes appeared in the cytoplasm, which were stained red with fat red O (see Fig. 1, b, e, h, k, n). After 21-day incubation in an osteogenic medium, morphological changes appeared in all MSC populations, and silver-rhenium staining revealed phosphates and carbonates that turned black (see Fig. 1, c, f, i, l, o).

Each sample of isolated EVs was resuspended in 100 μl of PBS (assigned in triplicate). Electron microscopic analysis revealed round or irregularly shaped microparticles of different size and clusters of fibrous structures in all samples. The latter were probably dried substances contained in part of the EVs which were destroyed during preparation, since they were not fixed.

Analysis of the electron diffraction patterns showed that in all samples more than 70% of the preserved EVs had sizes from 50 to 100 nm, that is, they were classified as exosomes (Fig. 2). The diameters of the EVs did not differ statistically between different animal species ($p = 0.1$). When comparing the number of particles isolated from 50 ml CM of MSCs from different animal species, no statistically significant differences were found ($p = 0.1$). Thus, the EVs number per grid for bovine MSCs (BM) and MSCs (AT) were 3±0.1 and 6±0.07 of 50-100 nm particles, 7±0.02 and 4±0.03 of 100-150 nm particles, and 3±0.4 and

2±0.06 particles larger than 150 nm, respectively. EVs from CM of MSCs (AT) of dogs were the most homogeneous both in shape (round) and in size, the main part (12±0.02) were 50-100 nm particles. In samples isolated from CMs of sheep, cat, horse MSCs (AT) and horse MSCs (UC), the number of 50-100 nm particles was 7±0.2, 7±0.01; 5±0.7 and 8±0.02, respectively. From CM of sheep MSCs (BM) we isolated 2±0.1 particles up to 50 nm in size, 5±0.6 particles of 50-100 nm, 1±0.03 particles of 100-150 nm, 5±0.7 particles of 150-200 nm, 3±0.01 particles larger than 200 nm, two of which had an irregular shape, similar to ectosomes, budding microvesicles.

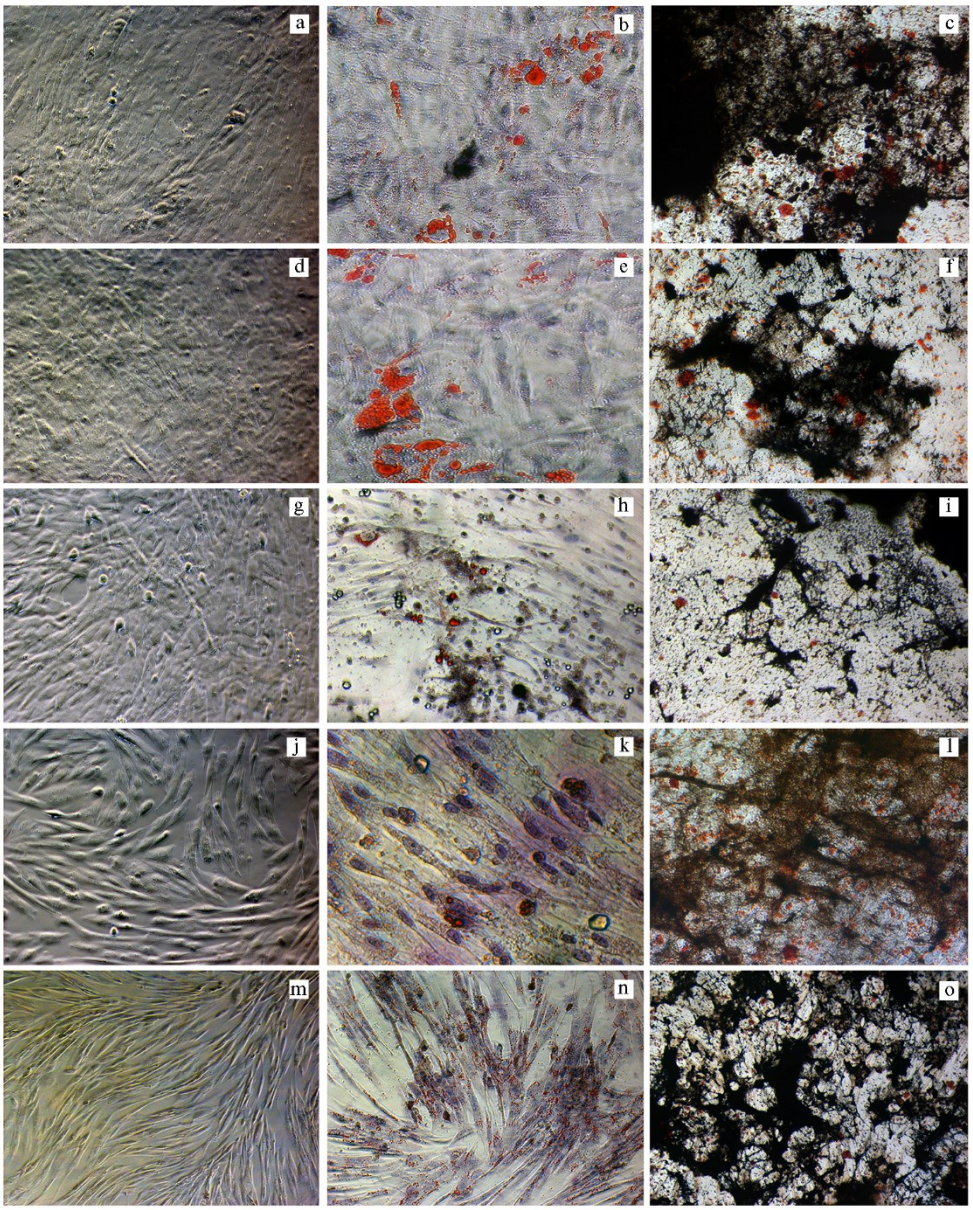

Fig. 1. Microphotographs of mesenchymal stem/stromal cells (MSCs) before collecting the conditioned medium for isolating exosomes (left, monolayer) **and their competence to adipo-** (fat red O staining, center) **and osteogenic** (silver-rhenium staining according to von Kossa, right) **differentiation upon induction:** a, b, c — MSCs from cattle bone marrow (BM), d, e, f — MSCs from cattle adipose tissue (AT), g, h, i — MSCs (BM) of sheep, j, k, l — MSCs from horse umbilical cord blood; m n, o — MSCs (AT) of a dog (microscope Carl Zeiss, Germany, magnification ×100; for b, e, j, k, ×200).

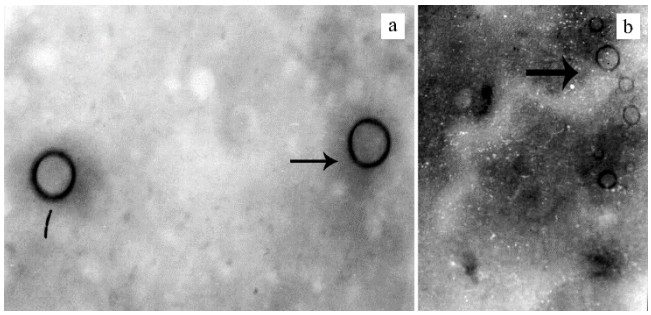

Fig. 2. Micrograph of extracellular vesicles from conditioned media of mesenchymal stem/stromal cells of cattle (a) **and sheep** (b) **bone marrow.** Arrows indicate exosomes (electronogram, JEM-100CXII microscope, JEOL, Japan, a — magnification ×54000, b — magnification ×50000).

To identify the phenotype of exosomes, EVs isolated from MSC CM of canine MSCs were used. This choice was due to the availability of mouse monoclonal antibodies to TSG101 recommended for the detection of TSG101 of mouse, rat, human and canine origin. Lysates of dog MSCs (AT) from which EVs were isolated, mouse embryonic fibroblasts STO line, and bovine MSCs (BM) (negative control) were used as controls. To identify exosomes, we used a protein marker for cellular exosomes and Western blot (immunoblot), an analytical method for determining specific proteins in a sample. Western blot is often used as a control test to confirm exosome isolation [7]. Surface proteins CD9, CD 63, CD81 can serve as markers of exosomes. We chose to detect the cytoplasmic protein TSG101 due to availability of the corresponding antibodies. As a result, in all samples, except for the negative control, 50-55 kDa bands stained with the anti-AG TSG101 antibody were found (Fig. 3).

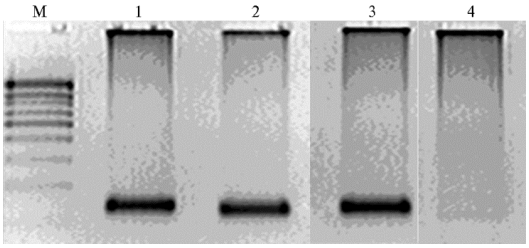

Fig. 3. TSG101 protein (Western blot) in samples isolated from canine adipose tissue (AT) **mesenchymal stem/stromal cell lysate** (1)**, extracellular vesicles from conditioned media of canine adipose tissue** (AT) **mesenchymal stromal cells** (2)**, mouse embryonic fibroblast STO lysate** (3)**, negative control** (4)**.** M — molecular weight marker, 8-195 kDa (Wuhan Servicebio Technology, Ltd., China).

In our work, we demonstrated that cells with the MSC phenotype from agricultural (cattle, sheep, horse) and small domestic (dog, cat) animals produce EVs which accumulate in the CM and can be extracted. Previously, exosomes were obtained from MSCs of horses [21], dogs [22], and cats [23]. Many methods are used to isolate EVs from the CV of MSCs [24-26] among which we can highlight differential centrifugation based on the difference in size and density between the EVs and other substances in the CM. This method is often used to extract exosomes due to the simplicity and stability of the result [27]. By DC method, unnecessary material is gradually removed from the CM. At the final stage, the purified CM is subjected to UC. UC is the most widely used method for exosome isolation and was once called the “gold standard” for exosome production [28].

We also used the DC method to obtain exosomes from animal MSC CM. In our study, we assessed the ultrastructure and particle size by electron microscopy. The results showed that EVs isolated from the CMs of MSCs (BM) and MSCs (AT) of all animal species are round or elliptical vesicles with membrane structures around the vesicles that are similar in shape. More than 70% of particles isolated from the CM of all MSCs had a size of 50-100 nm.

Exosomes can contain various proteins, the composition of which depends

on the host cell and is additionally modulated by its state (for example, stress or activation or inhibition of specific signaling pathways). Tetraspanins (CD9, CD63, and CD81) are the most common canonical exosome marker proteins present on the surface of vesicles. The surface localization of tetraspanin AG makes them suitable candidate targets for immunolabeling and purification of exosomes from biological samples. It should be noted that the difficulty in characterizing animal EVs compared to humans is due to the lack of specific EV related or non-related markers that can be used to characterize EVs across species. In addition, there are no available tissue- or cell-specific markers for isolating EV subpopulations and marker genes that could be used in the study of nucleic acids associated with EVs. Exosomes are enriched with Tsg101 and Alix proteins which are components of the endosomal sorting complex necessary for their transport. The cytoplasmic protein Tsg101 (Tumor Susceptibility Gene 101), involved in the formation of multivesicular exosome bodies and considered another important exosome marker, was identified as a marker of exosomes in the isolated samples [29]. Western blotting showed that all samples isolated from dog MSCs (AT) were positive for this protein.

Thus, conditioned media of mesenchymal stem/stromal cells from bone marrow, adipose tissue and umbilical cord blood of agricultural (cattle, sheep, horse) and small domestic (dog, cat) animals contain extracellular vesicles, including exosomes. Since the size and density of most EVs and other cellular components overlap to some extent, it can be assumed that the differential centrifugation method does not isolate only exosomes, but rather results in an enrichment of the sample in exosomes. In our work, we described a DC-based method for isolating micro-EVs containing exosomes from five animal species. This will provide the basis for the use of exosomes in disease diagnosis and treatment of farm and domestic animals in the future.

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BACTERIAL LUMINESCENCE OF MANGANESE- AND COBALT-CONTAINING ULTRAFINE PARTICLES (Mn_2O_3 and Co_3O_4) IN THE RUMEN FLUID

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Abstract

Along with the main nutrients, proteins, fats and carbohydrates, mineral elements are important in feeding farm animals, including cattle and poultry (D.V. Mashnin et al., 2022; T.M. Okolelova et al., 2018). Their inorganic or organic forms are components of premixes (M.Y. Mishanin et al., 2021; O.S. Koschaeva 2018). However, nanocompositions are more promising, the properties of which can be modeled by changing the shape, synthesis paths and size of ultrafine particles (UFP) (S. Miroshnikov et al., 2019). However, the use of UFPs has a number of limitations related to their potential toxicity (E. Rusakova et al., 2015). It is also known that the symbiotic microflora forms a multicomponent suspension of organic substances, intermediate and final metabolites of the microbiome, capable of interacting with UFP (B.S. Nurzhanov et al., 2019). In this work, the dynamics of luminescence of a bacterial test object was established for the first time when a complex of UFPs and rumen fluid was introduced into the nutrient medium. This combination has been shown to neutralize the toxicity of nano-structures. The purpose of our work was to evaluate the properties of ultrafine particles using the example of various concentrations of manganese and cobalt oxide in the biochemical environment of the ruminal community based on the method of inhibition of bacterial luminescence. The study was conducted on the basis of the center Nanotechnology in Agriculture of the FRC BST RAS (Orenburg) in 2022. Chemically pure manganese oxides Mn_2O_3 and cobalt Co_3O_4 (99 %) for analysis in the amount of 157.8 and 240.7 mg were dispersed by ultrasound at a frequency of 35 kHz in 1 ml of distilled water for 30 minutes at 25 ºC. The rumen fluid (RF) was collected through a chronic rumen fistula (d = 80 mm, ANKOM Technology Corporation, USA) 3 hours after feeding in a Kazakh white-headed bull, whose main diet was 30 % concentrates and 70 % coarse feed without the addition of UFP. The luminescent bacterial test Ecolum (a lyophilized culture of *Escherichia coli* microorganisms carrying a hybrid plasmid pUC19 with *lux*CDABE cloned *P. leiognathi* 54D10 genes, SIS IMMUNOTECH, Russia) was used. In a bioluminescent plan, a series of double dilutions of the UFP and RF suspension was prepared starting from 50 μl Mn_2O_3 (1 mol/l) + 50 μl RF; 50 μl Co_3O_4 (1 mol/l) + 50 μl RF; 50 μl Mn_2O_3 (1 mol/l) + 50 μl distilled water; 50 μl Co_3O_4 (1 mol/l) + 50 μl distilled water; 100 μl RF; 100 μl distilled water (control). Then 100 μl of the Ecolume test system were added to each cell to a total concentrations of UFPs from 0.25 to 0.00025 mol/l and dilution of RF from 1_2 to 1:2048 in a pure test and from 1:4 to 1:4096 in the test with UFPs. The toxicity of the studied samples was determined on a multifunctional micro-lancet reader TECAN Infinite F200 (Tecan Austria GmbH, Austria), fixing the luminescence value of the bacterial strain *E. coli* K12 TG1 at different concentrations of ultrafine particles and rumen fluid for 3 hours with a period of 5 minutes. Based on the obtained data, graphs reflecting the dynamics of bioluminescence inhibition were constructed and the toxicity index (T) and the relative value of bioluminescence (A) was calculated. It was found that UFPs in their pure form cause dose-dependent inhibition of bacterial luminescence, suppressing over 50 % of the luminescence (EC_{50}) even when diluted by 2048 times (0.00025 mol/l). The values of the toxicity index, when calculating which the control is taken as 100 %, clearly indicate a decrease in the toxic properties of suspensions with a decrease in the proportion of UFPs in them. For

Mn_2O_3, this value ranged from 89.76 % at a concentration of 0.25 mol/l to 38.57 % at 0.00025 mol/l at the 1st minute of the exposure and from 95.16 to 52.85 % at the end of the 3rd hour; for Co_3O_4 — 99.44 and 32.80 %, respectively, at the 1st minute, and 99.43 and 54.72 % at the end of the 3rd hour. Similar indicators in the experiment with rumen fluid appeared only in the first minutes of exposure, after which the luminosity increased significantly, reaching 769.10 % to the control at 64-fold dilution. When combining rumen fluid with UFPs, a regression of the toxic properties of the latter was observed, although the maximum luminosity in combination with Mn_2O_3 was only 43.28 % of those for native RF, in combination with Co_3O_4 36.44 %. The observed changes in luminescence were divided into three types. The first type is control (luminescence changes in proportion to the growth phases of the bacterial culture; without additives). The second type corresponds to deep changes (suppression of luminescence throughout the entire exposure period; with the addition of UFPs), and the third type is competitive (increase in luminescence from the beginning to the end of the experiment; with the addition of RF or a complex of RF+UFP). Thus, the combination of rumen fluid with metal oxide particles leads to an inhibition of their toxicity to the test object.

Keywords: ultrafine particles, bacterial cells, bioluminescence, manganese oxide, cobalt oxide, rumen fluid

Along with proteins, fats and carbohydrates as the main nutrients, mineral elements are important in feeding farm animals, including cattle and poultry [1, 2]. Mineral elements are components of accessory substances, e.g., respiratory pigments, vitamins, hormones, enzymes and coenzymes, and influence a variety of physiological processes which affects growth and ontogeny [3, 4]. Optimal supply of microelements with feed is a factor mediating high animal productivity pweformace [5, 6]. Therefore, improving various formulants of mineral-containing premixes is relevant.

Traditionally, inorganic and organic compounds are used in premixes [7, 8], but chelates and nanoforms of essential elements are more promising [9-11]. The properties of the latter can be modeled by changing the shape, synthetic rections and particle size [12]. Howevern this cheaper and highly cost-effective method of fortifying feed with micronutrients [13, 14] has some limitations. Ultrafine particles (UFPs) impose risks of potential toxicity [15]. Thus, though stable metal oxides are absolutely inert, metal UFPs with redox potential can be geno- and cytotoxic [16] due to high bioavailability, a lsynergistic effect with other pollutants that may occure [17] and wide variability of properties depending on the nature, size, concentration, ζ-potential, shape and reaction medium.

The latter is especially relevant for ruminants, in which the ruminal contents, in fact, is an integral ecosystem with many connections [18]. Symbiont microbiota also needs macro- and microelements to maintain normal metabolism and peoduces a multicomponent suspension of organic substances, the intermediate and final metabolites of the microbiome that can interact with UFPs [19]. This should be accounted when determining the maximum permissible doses of dietary UFPs for animals.

This work is the first report on the pattern of in vivo luminescence bacterium test when a UFP complex and rumen fluid were added to the nutrient medium. It has been shown that this combination neutralizes the toxicity of nanostructures.

Our goal was to evaluate the properties of ultrafine particles using the example of different concentrations of manganese and cobalt oxide in the biochemical environment of the ruminal community based on the method of inhibiting bacterial luminescence.

Materials and methods. The study was carried out in 2022 (the Federal Scientific Center BST RAS, the Center Nanotechnologies in Agriculture, Orenburg). UFPs of manganese oxide Mn_2O_3 and cobalt oxide Co_3O_4 chemically pure for analysis (grade 99%, IP Khisamutdinov R.A., Russia) were used (157.8 and 240.7 mg. respectively; laboratory scales VLA-225M, accuracy class I, permissible error ±0.5 mg, Gosmetr, Russia). Oxides were ultrasonically dispersed by

(35 kHz) in 1 ml of distilled water for 30 min at 25 °C.

Three hours after feeding, rumen fluid (RF) was collected through a chronic rumen fistula (d = 80 mm, ANKOM Technology Corporation, USA) of a Kazakh white-headed bull (250 kg weight, 10 month age). The basal diet was 30% concentrates and 70% roughage without the addition of UFPs. RF was delivered within 30 min at 38.5-39.5 °C. Before use, the RF was thoroughly shaken and filtered.

The luminescent bacterial test Ecolum (NVO IMMUNOTECH, Russia), consisting of a lyophilized *Escherichia coli* carrying the hybrid plasmid pUC19 with the cloned *lux*CDABE genes of P. *leiognathi* 54D10, was prepared according to the method by E.S. Aleshina et al. [20]. Distilled water cooled to 4 °C (10 ml) was added to the strain, and the suspension was allowed for 30 min at the same temperature.

Serial two-fold dilutions were performed in a bioluminescent plate. The starting samples were as follows: 50 μl Mn_2O_3 (1 mol/l) + 50 μl RF for line A, 50 μl Co_3O_4 (1 mol/l) + 50 μl RF for line B, 50 μl Mn_2O_3 (1 mol/l) + 50 μl distilled water for line C, 50 μl Co_3O_4 (1 mol/l) + 50 μl distilled water for line D; 100 μl RF for line E, 100 μl distilled water for line F (control). To each cell, 100 μl of the Ekolum test system was added to final UFPs concentrations from 0.25 to 0.00025 mol/l and RF dilutions from 1:2 to 1:2048 in the test without UFPs and from 1:4 to 1:4096 with UFPs.

The toxicity of the samples was determined (a multifunctional microplate reader TECAN Infinite F200, Tecan Austria GmbH, Austria), recording the luminescence of the bacterial strain *E. coli* K12 TG1 (Ecolum) in a medium with different concentration of ultrafine particles and rumen fluid during 3 h each 5 min.

Based on these data, graphs were constructed of the dynamics of bioluminescence inhibition, and the toxicity index (T) was calculated:

$$T = (Ic - It)/Ic \times 100\%,$$

where Ic is luminosity of the control sample, It is the luminosity of the test sample.

This indicator was used to assess how strong the negative impact of the factor under study is. However, if the latter has a positive component and intensifies the luminosity, it is more rational to calculate the relative value of bioluminescence (A) in order to avoid negative values:

$$A = Ic/It \times 100\%.$$

In essence, this value is the inverse of the toxicity index, reflecting the difference between the luminescence intensity of the control and test samples.

Statistical processing of the results was carried out using the Statistica 12 software (StatSoft Inc., USA) and Microsoft Excel package (Microsoft, USA). The significance of the differences between the absolute values of luminescence was determined assessed using the Studen's *t*-test with the required significance level $p \leq 0.01$. The tables indicate relative values corresponding to this threshold.

Results. Manganese and cobalt, along with other transition metals (Fe, Ni, Cu, Zn), are essential microelements involved in a number of biochemical transformations in bacteria [21, 22], plants [23-25], protozoa [26, 27], invertebrate [28, 29[and vertebrate [30, 31] animals. Specifically, manganese is responsible for macronutrient metabolism, bone formation, free radical defense systems, ammonia clearance, and neurotransmitter synthesis in the brain. It is a cofactor for enzymes that degrade reactive oxygen species, mainly Mn-superoxide dismutase (SOD) and Mn-cofactored catalases and peroxidase [32, 33]. In addition, Mn replaces iron in some Fe-mononuclear enzymes in *E. coli* under oxidative stress, protecting them from Fenton reaction-mediated oxidative damage while main-

taining catalytic activity [34, 35]. To maintain optimal concentrations of metal ions, bacteria use molecular importers and exporters [36, 37].

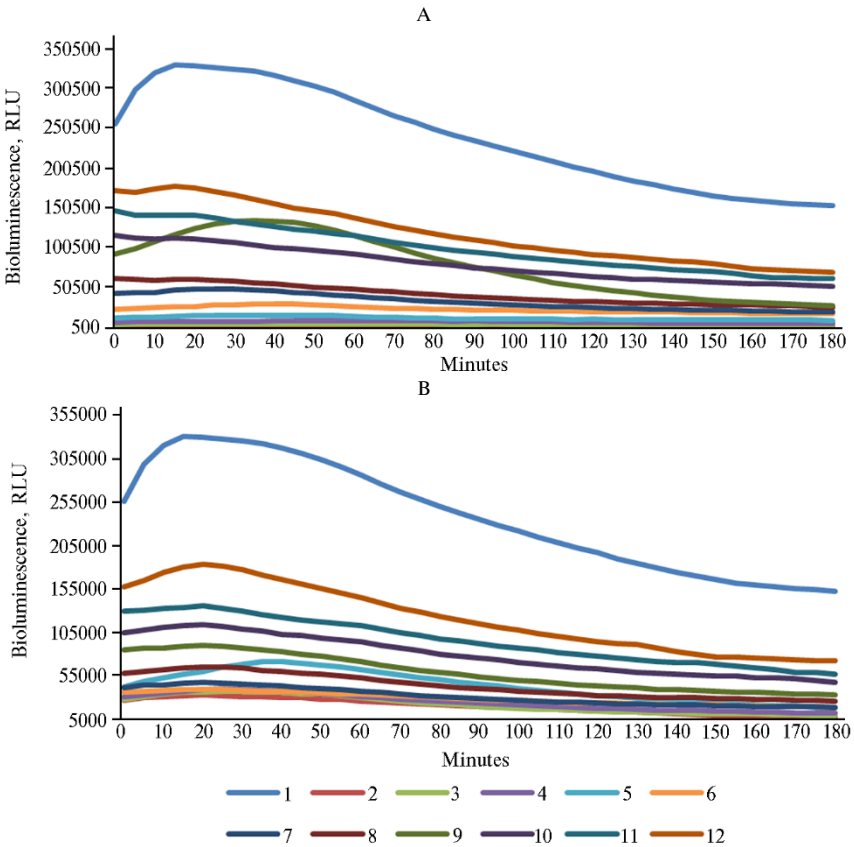

Fig. 1. Bioluminescence of *Escherichia coli* K12TG1 with a suspension of ultrafine particles (UFPs) **Co_3O_4** (A) **and Mn_2O3** (B) **depending on concentrations:** 1 — 0 mol/l (control), 2 — 0.25 mol/l, 3 — 0.125 mol/l, 4 — 0.063 mol/l, 5 — 0.031 mol/l, 6 — 0.016 mol/l, 7 — 0.008 mol/l, 8 — 0.004 mol/l, 9 — 0.002 mol/l, 10 — 0.001 mol/l, 11 — 0,.0005 mol/l, 12 — 0.00025 mol/l.

Mn is essential component in dozens of proteins and enzymes found in all tissues, especially those rich in mitochondria and melanin. Normally, conmaximum Mn concentrations are characteristic of the liver and pancreas [38]. With its excess or deficiency in the diet, neurodegenerative disorders most often occur [39-41].

Cobalt is used by bacteria to synthesize the tetrapyrrole ring known as corrin of vitamin B_{12} ($C_{63}H_{88}O_{14}N_{14}PCo$) [21, 42] the chemical structure and properties of which have been described in detail by D. Osman et al. [43]. Cobalt is involved in nitrogen fixation in plants, serves as a cofactor for adenosylcobalamin-dependent isomerases, ethanolamine ammonia lyase, methylcobalamin-dependent methyltransferase, ribonucleotide reductase, and number of metalloproteins in animals, bacteria and yeast [24]. In humans, Co is a component of ethylmalonyl-CoA mutase (MCM) and methionine synthase the deficiency of which leads to methylmalonic aciduria and megaloblastic anemia [44], neural tube defects, stroke and dementia, and retarded intellectual development in children [45]. In excess, Co has genotoxic and carcinogenic properties [46].

The bioluminescence of samples with different UFP concentrations of manganese and cobalt oxides without RF was inversely proportional to their concentration (Fig. 1). At the beginning of the experiment, the luminosity of the

control sample due to intensive consumption of the nutrient substrate increased by 22.5% in 15 min and then gradually decreased to 59.6% of the initial value. At the largest dilution of 0.00025 mol/l, the luminosity of Mn_2O_3 and Co_3O_4 suspensions vs. the control sample in the first second was 61.4 and 67.2%, respectively, whereas at 0.25 mol/l dilution only 10.2 and 0.5%.

1. Toxicity index (T, %) **of different concentrations of Co_3O_4 ultrafine particles** (UFPs) **suspensions at assessed by co-exposition with the luminescent strain *Escherichia coli* K12TG1 in distilled water**

Minutes	UFP concentration, mol×10^{-3}/l										
	250	125	63	31	16	8	4	2	1	0.5	0.25
0	99.4/A	99.0/A	97.8/A	95.6/A	91.5/A	83.5/A	76.1/A	64.0/B	54.8/B	43.0/C	32.8/C
30	99.7/A	99.0/A	97.8/A	95.5/A	91.3/A	85.3/A	82.3/A	59.1/B	67.2/B	58.9/B	49.0/C
60	99.7/A	99.0/A	97.3/A	95.3/A	91.0/A	86.5/A	83.5/A	59.8/B	67.9/B	59.7/B	51.8/B
90	99.7/A	98.8/A	96.9/A	95.6/A	90.8/A	87.4/A	84.0/A	68.0/B	68.2/B	60.1/B	53.4/B
120	99.6/A	98.6/A	97.1/A	95.2/A	90.3/A	87.4/A	83.7/A	75.1/A	67.5/B	59.6/B	53.6/B
150	99.5/A	98.5/A	97.2/A	94.6/A	89.1/A	87.0/A	83.1/A	79.5/A	65.8/B	57.6/B	51.9/B
180	99.4/A	98.4/A	97.3/A	94.6/A	89.3/A	87.8/A	83.8/A	82.7/A	66.7/B	60.2/B	54.7/B

N o t e. A — EC_{70}, B — EC_{50}, C — EC_{20}, that is, UFP concentrations that cause over 75, 50 and 20% quenching of the biosensor compared to the control.

The values of the T index to calculate which we take the control as 100%, clearly indicate a decrease in the toxic properties of the suspension with a decrease in the UFP concentration. For Mn_2O_3, the T value ranged from 89.76% at 0.25 mol/l to 38.57% at 0.00025 mol/l during the 1st minute of the exposition and from 95.16 to 52.85% at the end of the 3rd hour of the exposition (Table 1). For Co_3O_4, the T index was 99.44 and 32.80% at the 1st minute and 99.43 and 54.72% at the end of the 3rd hour (Table 2).

2. Toxicity index (T, %) **of different concentrations of Mn_2O_3 ultrafine particles** (UFPs) **suspensions assessed by co-exposition with the luminescent strain *Escherichia coli* K12TG1 in distilled water**

Minutes	UFP concentration, mol×10^{-3}/l										
	250	125	63	31	16	8	4	2	1	0.5	0.25
0	89.8/A	89.0/A	87.9/A	83.7/A	85.9/A	83.8/A	77.3/A	66.7/B	59.0/B	49.3/B	38.6/C
30	90.4/A	88.7/A	88.0/A	79.0/A	88.3/A	86.1/A	80.5/A	73.3/B	66.4/B	60.2/B	45.5/B
60	91.0/A	89.6/A	89.1/A	78.5/A	88.2/A	86.9/A	81.7/A	75.0/B	67.1/B	60.7/B	49.4/B
90	91.7/A	91.6/A	90.1/A	80.9/A	88.3/A	87.7/A	83.1/A	77.0/A	68.1/B	61.0/B	51.0/B
120	92.5/A	92.7/A	91.0/A	83.7/A	88.1/A	88.0/A	83.6/A	78.0/A	68.1/B	60.7/B	52.2/B
150	94.3/A	93.4/A	91.3/A	86.0/A	87.4/A	87.4/A	82.6/A	77.5/A	66.8/B	59.0/B	53.6/B
180	95.2/A	94.0/A	92.1/A	88.3/A	87.8/A	87.9/A	83.4/A	78.5/A	68.8/B	62.5/B	52.9/B

N o t e. A — EC_{70}, B — EC_{50}, C — EC_{20}, that is, UFP concentrations that cause over 75, 50 and 20% quenching of the biosensor compared to the control.

In the test with ruminal fluid without UFPs, the relative bioluminescence ranged from 25.29 to 769.10% with a tendency to increase. Thus, the first test with RF during the first 30 min showed EC_{70} and EC_{30}, that is, inhibition of 70 and 30% of the luminescence of the luminescent strain, respectively. However, starting from the 2nd hour, the sample could not be assessed as toxic; moreover, its indicators by the end of the experiment were 3.5 times higher than the control (Fig. 2). In the dilution 1:4, the intensity of bioluminescence in the first second corresponded to the EC_{50} and then increased 6.8 times, or 5.27 times compzred to control. In general, the luminosity of the samples was inversely proportional to the concentration of rumen fluid up to 1:32-1:64 dilutions after which the trend changed. Moreover, within one concentration, the bioluminescence increased within 3 hours until the 1:128 dilution, then (1:256-1:2048) in the first 30 min an increase in bioluminescence was recorded followed by a drop below the initial level. The overall dynamics demonstrated a sharp increase in biolu-

minescence in the first 20-30 min followed by a slowdown (for 4-128-fold dilutions) or a slow decrease in the bioluminescence intencity (for 256-2048-fold dilutions) (Table 3).

When rumen fluid was co-incubated with ultrafine particles of manganese and cobalt oxides, the toxic properties of the latter regressed. As a result, the graphs of bioluminescence dynamics approached that of rumen fluid (Fig. 3), although the maximum luminescence in combination with Mn_2O_3 were only 43.28% of that for native RF, with Co_3O_4 36.44%. For Mn_2O_3, samples with UFP concentrations of 0.00025 and 0.0005 mol/l followed the changes in the control, but exceeded in the luminescence level. All other samples were initially inferior in bioluminescence to the suspension without UFPs, but overtook it at various time intervals, exceeding by 2.32-3.22 times at the end of the test. For Co_3O_4, the dynamics were the same as in the control, but luminescence did not exceed the values of the sample with UFP concentrations of 0.004 and 0.002 mol/l. On the contrary, dilutions of 0.001-0.0002 mol/l, having the same dynamics, exceeded the control values, 0.25-0.031 mol/l did not reach them, and 0.016 and 0.008 mol/l exceeded them by only 1.14-1.55 times.

3. Relative bioluminescence (A, %) **depending on the dilution of a Kazakh white-headed bull ruminal fluid** (RF) **assessed by co-exposition with the luminescent strain *Escherichia coli* K12TG1**

Minutes	RF dilutions										
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
0	25.2/A	45.9/B	75.19/C	91.3/D	118.4/E	138.1/E	134.4/E	132.0/E	130.7/E	121.1/E	118.7/E
30	69.6/C	126.9/E	189.3/E	207.7/E	228.4/E	232.2/E	207.3/E	191.3/E	179.9/E	178.6/E	145.8/E
60	98.5/D	160.7/E	235.2/E	269.6/E	298.6/E	292.2/E	237.0/E	206.4/E	184.3/E	178.4/E	144.3/E
90	140.6/E	208.6/E	303.3/E	366.0/E	369.0/E	366.7/E	264.6/E	224.4/E	187.4/E	177.9/E	136.9/E
120	233.4/E	294.9/E	397.5/E	459.8/E	453.8/E	459.8/E	300.4/E	226.0/E	176.7/E	167.5/E	128.2/E
150	309.6/E	423.41/E	491.7/E	538.0/E	592.6/E	622.1/E	324.9/E	216.6/E	160.1/E	156.4/E	124.6/E
180	368.7/E	526.73/E	603.2/E	636.7/E	670.1/E	769.1/E	349.8/E	193.7/E	139.0/E	144.2/E	117.2/E

N o t e. A — EC_{70}, B — EC_{50}, B — EC_{20}, B — NTOX, E — NTOX+, that is, UFP concentrations that cause over 75, 50 and 20% quenching of the biosensor compared to the control, non-toxic and luminescence-intensifying concentrations.

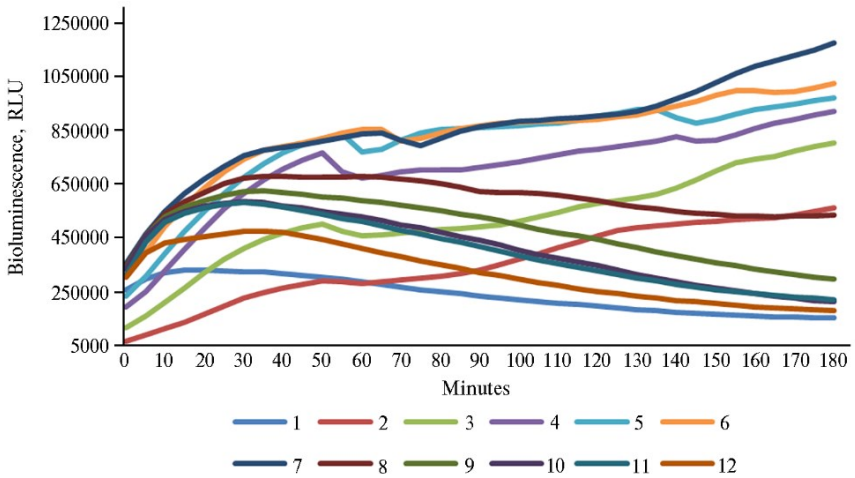

Fig. 2. Bioluminescence of *Escherichia coli* K12TG1 in the rumen fluid of a Kazakh white-headed bull depending on the dilution: 1 — control (distilled water), 2 — 2-fold, 3 — 4-fold, 4 — 8-fold, 5 — 16-fold, 6 — 32-fold, 7 — 64-fold, 8 — 128-fold, 9 — 256-fold, 10 — 512-fold, 11 — 1024-fold, 12 — 2048-fold.

These data indicate the greater toxicity of cobalt oxide compared to manganese oxide. Calculated relative bioluminescence parameters confirmed the con-

clusion (Tables 4, 5). In general, these data implied UFP subtoxic concentrations which are the amounts that did not cause more than 20% quenching of bioluminescence throughout the entire experiment. For Mn_2O_3 and Co_3O_4, these were 0.001 mol/l concentrations and a 1024-fold dilution of rumen fluid.

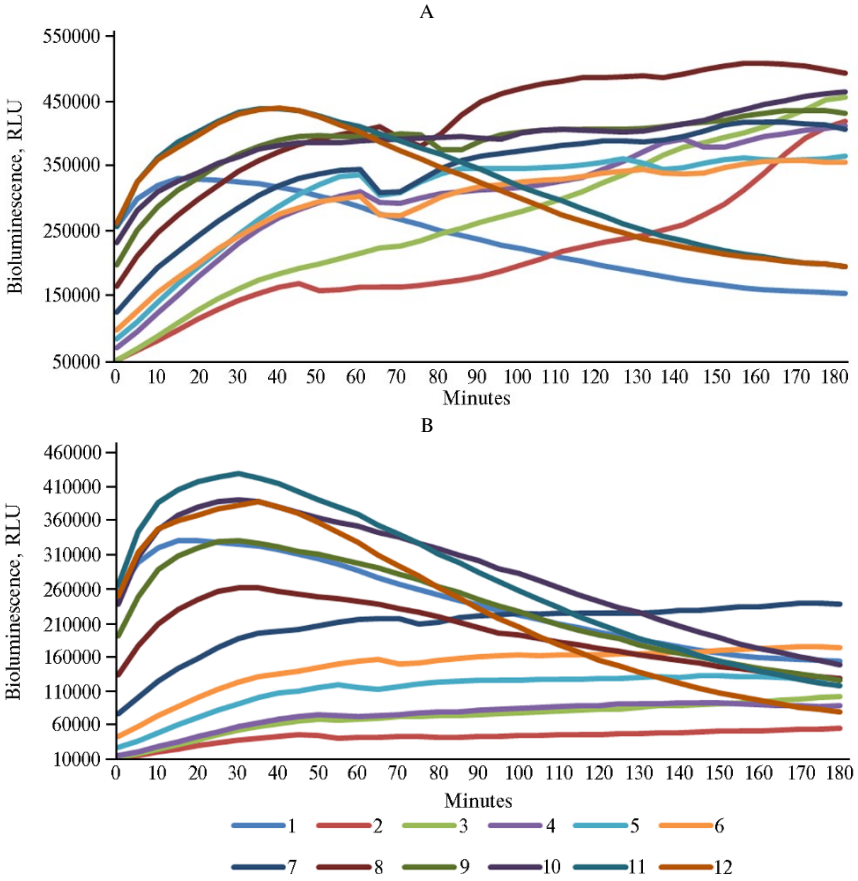

Fig. 3. Bioluminescence of the *Escherichia coli* K12TG1 depending ob the dilution of a Kazakh white-headed bull rumen fluid (RF) **and UFP Mn_2O_3** (A) **and Co_3O_4** (B) **concentrations:** 1 — 0 mol/l (distilled water, control), 2 — 0.25 mol/l (2-fold dilution), 3 — 0.125 mol/l (4-fold), 4 — 0.063 mol/l (8-fold), 5 — 0.031 mol/l (16-fold), 6 — 0.016 mol/l (32-fold), 7 — 0.008 mol/l (64-fold), 8 — 0.004 mol/l (128-fold), 9 — 0.002 mol/l (256-fold), 10 — 0.001 mol/l (512-fold), 11 — 0.0005 mol/l (1024-fold), 12 — 0.00025 mol/l (2048-fold).

4. Relative bioluminescence (A, %) **depending on a Kazakh white-headed bull ruminal fluid** (RF) **dilutions, Mn_2O_3 ultrafine particles** (UFPs) **concentrations and the time of co-exposition with the luminescent strain *Escherichia coli* K12TG1**

Minutes	UFP concentration, mol×10^{-3}/l (RF dilution)										
	250 (1:2)	125 (1:4)	63 (1:8)	31 (1:16)	16 (1:32)	8 (1:64)	4 (1:128)	2 (1:256)	1 (1:512)	0.5 (1:1024)	0.25 (1:2048)
0	19.6/A	19.6/A	27.1/A	32.1/B	37.6/B	48.4/B	64.3/B	77.1/B	90.2/D	99.9/D	102.4/D
30	43.6/B	49.4/B	70.6/C	74.9/C	74.0/C	87.7/D	105.1/D	113.1/D	112.0/D	132.8/E	132.0/E
60	56.8/C	75.2/C	108.3/D	117.4/D	106.0/D	120.5/E	141.0/E	138.0/E	136.0/E	143.3/E	140.6/E
90	76.4/C	111.8/D	132.7/E	146.9/E	135.6/E	154.9/E	191.0/E	165.4/E	166.7/E	146.5/E	137.6/E
120	117.6/D	164.6/E	175.2/E	181.0/E	172.2/E	197.9/E	247.6/E	206.6/E	205.7/E	138.7/E	129.8/E
150	175.5/E	238.0/E	228.8/E	217.7/E	210.1/E	249.6/E	304.7/E	254.2/E	259.6/E	132.6/E	129.3/E
180	273.7/E	298.4/E	269.5/E	238.5/E	232.8/E	265.8/E	322.5/E	281.9/E	304.0/E	127.5/E	127.7/E

N o t e. A — EC_{70}, B — EC_{50}, C — EC_{20}, D — NTOX, E — NTOX+, that is, UDP concentrations that cause over 75, 50 and 20% quenching of the biosensor compared to the control, non-toxic and luminescence-intensifying concentrations.

5. Relative bioluminescence (A, %) **depending on a Kazakh white-headed bull ruminal fluid** (RF) **dilutions, Co_3O_4 ultrafine particles** (UFPs) **concentrations and the time of co-exposition with the luminescent strain *Escherichia coli* K12TG1**

Minutes	UFP concentration, mol×10^{-3}/l (RF dilution)										
	250 (1:2)	125 (1:4)	63 (1:8)	31 (1:16)	16 (1:32)	8 (1:64)	4 (1:128)	2 (1:256)	1 (1:512)	0,5 (1:1024)	0,25 (1:2048)
0	4.5/A	5.0/A	5.9/A	10.3/A	17.0/A	29.5/Б	51.9/B	74.5/B	92.8/D	103.7/D	97.1/D
30	11.5/A	16.0/A	17.5/A	27.9/Б	37.8/Б	57.2/B	80.5/D	101.6/D	120.1/Д	131.7/Д	117.6/D
60	14.4/A	24.0/Б	25.1/Б	40.2/Б	53.5/B	75.0/B	84.4/D	103.9/D	122.8/Д	128.8/Д	114.5/D

Continued Table 5

90	18.2/A	32.0/Б	34.3/Б	53.2/B	68.2/B	93.6/D	86.2/D	103.8/D	127.7/Д	120.6/Д	98.0/D
120	23.5/Б	41.8/Б	44.9/Б	65.2/B	82.9/D	114.2/D	87.6/D	97.7/D	122.5/Д	105.9/D	79.0/B
150	30.6/Б	54.9/B	55.5/B	79.5/B	102.3/D	139.1/Д	88.1/D	92.8/D	113.6/D	93.1/D	64.6/B
180	35.7/Б	66.0/B	57.3/B	83.4/D	113.8/D	155.1/Д	83.7/D	82.4/D	97.1/D	76.6/B	51.5/B

N o t e. A — EC_{70}, B — EC_{50}, C — EC_{20}, D — NTOX, E — NTOX+, that is, UDP concentrations that cause over 75, 50 and 20% quenching of the biosensor compared to the control, non-toxic and luminescence-intensifying concentrations.

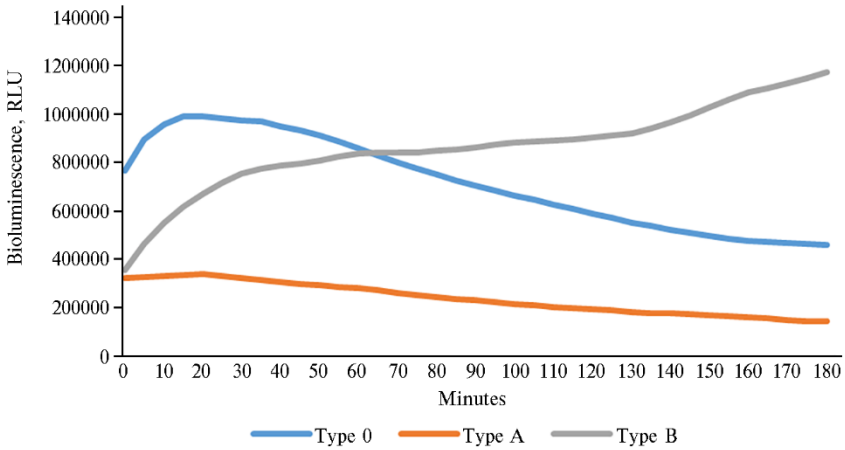

Fig. 4. Bioluminescence of *Escherichia coli* K12TG1 exposed to ruminal fluid (RF)**, ultrafine particles** (UFPs) **or RF + UFPs:** type 0 — control (luminescence dynamics corresponds to the growth phases of the bacterial culture), type A — deep (extinguishing the glow by ultrafine particles throughout the entire experiment), type B — competitive (luminescence intensifies, changing from negative to positive values as influenced by RF of a Kazakh white-headed bull or a RF + UFPs complex throughout the entire experiment).

According to the data we obtained, all dynamic processes could be divided into several types (Fig. 4).

Type 0 (control) means that in the absence of UFPs and RF in the medium, the luminosity of *E. coli* K12TG1 was proportional to the culture growth rate and corresponded to the phases of logarithmic growth, negative acceleration, stationary phase and death phase. The lag phase and positive acceleration occurred during the sample preparation for analysis.

Type A (deep) means that in the presence of UFPs of manganese and cobalt oxides, the bioluminescence decreased throughout the entire exposure period. This illustrates a deep degenerative effect, the degree of which decreased with the concentration of UFPs. In the experiment, it did not reach acceptable values (T < 20%) even when UFPs were diluted 1024 times (0.00025 mol/l) because of the reactivity and physicochemical dynamics of UFPs [47] and high cyto- and genotoxicity which has been confirmed in a number of works. F. Ameen et al. [48], using soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Azotobacter*, *Bacillus*, *Thiobacillus*, *Pseudomonas*, *Azospirillum* and mycorrhizal fungi, pointed out the negative effects of metal UFPs, inclusing induction of apoptosis, structural damage to the cell wall, inhibited nitrogen fixation and nitrification, suppression

of urease, N-acetylglucosaminidase, glycine aminopeptidase, arylsulfatase, polyphenol oxidase and peroxidase, which destabilize the soil community. As for manganese and cobalt itself, their toxic properties have been assessed in mammals [49, 50], plants [51-53], fish [29], invertebrates [54] and protozoans, including ciliated of rumens [26] and parasitic *Leishmania* [55]. S. Rana et al. [56], studying a consortium of algae and bacteria, identified the following stages of the impact of UFPs: changes in membrane permeability, surface adsorption, damage to transport proteins; penetration into a bacterial cell; interaction with cellular organelles; generation of reactive oxygen species (ROS); the beginning of degenerative changes. A similar effect is associated with the ability of metals exhibiting variable valence to performe lipid peroxidation and generate substances that block the active centers of enzymes [57], including luciferase which is responsible for bioluminescence [58].

However, most researchers agree that the cause of UFP toxicity is oxidative stress, leading to the production of ROS, decreased reduced glutathione levels, increased lipid peroxidation, and the release of metal ions causing protein coagulation and bacterial aggregation. Another mechanism of toxicity may be the UFPs immobilization on the cell plasmalemma, described, in particular, by T.P. Dasari et al. [59] on *E. coli* as an example. Moreover, in their work, the UFPs were arranged according to the degree of toxicity as $ZnO < CuO < Co_3O_4 < TiO_2$ in the dark and $ZnO < CuO < TiO_2 < Co_3O_4$ in the light.

Type B (competitive) means that in samples with RF without and with UFPs, the luminescence intencity was minimum at the beginning of the experiment with further growth. This dynamics can be explained based on the RF composition the main component of which is the symbiont microbial mass. It mainly consists of bacteria, protozoa, fungi, archaea and a small proportion of phages, which are in dynamic equilibrium [60]. Essentially, this is a separate ecosystem with its own consumers and decomposers, each playing a specific role, e.g., the destruction of plant cell walls, the fermentation of organic components and the utilization of metabolic products.

Bacteria are represented mainly by anaerobic lignocellulolytic *Prevotella*, *Butyrivibrio*, *Ruminococcus*, *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales* and *Clostridiales* [61], archaea by methanogenic *Methanobrevibacter*, *Methanosphaera* and *Methamassilicoccus* [62], fungi by *Neocallimastix*, *Caecomyces*, *Piromyces*, *Anaeromyces*, *Oorpinomyces* and *Cyllamyces* [63], ciliated and flagellated protozoa by *Entodinium* and *Epidinium* [64]. Various microorganisms secrete digestive enzymes such as cellulases, hemicellulases and ligninases. With their help, rumen microorganisms convert cellulose, hemicellulose and lignins into monosaccharides which can be further converted into volatile fatty acids, CH_4 and other products [18, 65].

Assessing the influence of the rumen fluid composition on the glow of the biosensor, E.A. Drozdova et al. [57] suggested that the reduced luminosity is due to free hydrogen ions, enzymes and ammonia in the RF. In addition, they studied the effects of glucose, propionic, lactic, succinic, acetic acid and a 10% ammonia solution. As a result, an intensification of luminescence was revealed in the first case, its slight suppression in the second-fourth cases and strong suppression in the last two cases. This is consistent with an ecological model of dynamic processes in the rumen we submit hereinbelow.

During the first minutes of the experiment, the low luminosity of the bioluminescent strain can be explained by its adaptation to the RF chemical composition and competition with anaerobic microorganisms the proportion of which will certainly decrease in air. However, fermentation processes and, as a result, the synthesis of short-chain fatty acids, ammonia, hydrogen and methane slow down. In other words, a series of negative factors that inhibit *E. coli* are excluded

while quite a lot of glucose remains in the medium. The only threat to the *E. coli* K12TG1 growth are ciliates, and the structure of the native community alters.

Similar conclusions are confirmed by our data (see Fig. 2, Table 4), therefore, it is possible to postulate an ecological model of the bioluminescence dynamics in ruminal contents (Table 6). Based on this model, the "ecooptimum" for the ratio (v/v) of the bioluminescent strain culture to diluted RF is 100 μl bacterial suspension per 100 μl of RF in a dilution of 1:64 when the luminosity of the biosensor increases throughout the test (criterion B > 1) and exceeds control values (criterion A = max).

6. Ecological model of the interaction of a Kazakh White Head Tall bull ruminal fluid with bioluminescent strain *Escherichia coli* K12TG1

Factor	Stage	Dilution										
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Negative	S											
	F											
Positive	S											
	F											
BL	S											
	F											
	M	↑	↑	↑	↑	↑	↑	con	con	↓	↓	↓
A		3.69	5.27	6.03	6.37	6.70	7.69	3.50	1.94	1.39	1.44	1,17
B		8.70	6.84	4.79	4.16	3.38	3.32	1.55	0.88	0.63	0.71	0,59

N a t e. Negative means negative factors (H^+, VFA, NH_3, CH_4, predatory protozoa, competitive anaerobes), positive means nutrient substrates (including D-lucose). BL is bioluminescence intensity, A is ratio of the luminosity of the test sample to the control at the end of the test, B is ratio of luminosity at the end of the test to the beginning of the test. S (start) is beginning of the test, F (finish) is end of the test, M (movement) — dynamics of bioluminescence; — low content, — high content, — intermediate value of the component in the medium; ↓ — decrease, ↑ — increase, con — maintenance of bioluminescence at the same level.

The last test in which we studied the interaction of UFPs and RF was essentially an averaging of tests 1 and 2 with dynamic types A and B. The released metal ions react with fatty acids to form non-toxic salts, e.g., acetate, propionate, etc. They can also be bound to RF proteins and form a protein corona [37, 66] or metabolized by rumen bacteria [67] and inactivated by fungi [68]. Thereof, the initially low relative bioluminescence at high UFPs concentrations exceeded the control by the end of the experiment (see Tables 4, 5). However, there were differences between the oxides of manganese and cobalt. For the first case, the reduction and increase in luminosity occurred much faster. This may be due to the chemical nature of the elements themselves [59], the degree of their dispersity, or the possibility to form large aggregates [69] and is consistent with the data of the first experiment.

Thus, pure ultrafine particles (UFPs) of Mn_2O_3 and Co_3O_4 inhibit the luminescence of *Escherichia coli* K12TG1 in a dose-dependent manner and proportionally to the exposure time, suppressing over 50% (EC_{50}) of luminescence when diluted even 2048 times (to 0.00025 mol/l). Therefore, the toxicity index for UFPs Mn_2O_3 ranged from 89.76% at 0.25 mol/l to 38.57% at 0.00025 mol/l during the 1st minute of the exposure and from 95.16 to 52.85% at the end of the 3rd hour. For UFP Co_3O_4, these values were from 99.44 to 32.80% during the 1st minute, and from 99.43 to 54.72% at the end of the 3rd hour. Rumen fluid (RF) of a Kazakh white-headed bull, in turn, suppresses the glow only in the first minutes of exposure which may be due to the presence of "competing" anaerobic microorganisms and gradually volatilizing toxic metabolic products, e.g., ammonia. After this, RF acts as an additional nutrient substrate, intensifying luminescence by more than 7-fold at dilution 1:64. The combination of RF with particles of metal oxides leads to inhibition of their toxicity, although the maximum values of relative luminescence are still inferior to those for native rumen fluid that indicates the mutual binding of the medium components with the UFPs. Based on

the results obtained, we can argue that the ultrafine particles as sources of microelements in feeding of ruminants is promising and should be further studied..

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MATHEMATICAL MODEL OF THE TRANSFER OF LEAD FROM PERIPHERAL BLOOD INTO THE ORGANS AND MUSCLE TISSUE OF SHEEP (*Ovis aries*)

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Abstract

To obtain livestock products that meet sanitary and hygienic standards for lead, it is necessary to establish the permissible limits of its daily intake by animals from the ration. In this work, based on the model we have developed, the parameters of lead transport between peripheral blood, organs and muscle tissue were determined for the first time, depending on the daily concentration of the metal in the ration and the duration of its entry into the body. Our aim was to develop and parametrize a chamber model of the transfer of lead from peripheral blood to the organs and muscle tissue of sheep during chronic dietary intake. The experiments were carried out on 27 Romanov sheep. The age of the animals is 1-1.5 years, body weight is 33.5±0.7 kg. Sheep were kept in boxes of 4-5 heads in the vivarium of the All-Russian Research Institute of Physiology, Biochemistry and Nutrition (BIFIP, Kaluga region, Borovsk). Feeding was carried out twice a day with free access to water. The animals were divided into four groups: group I (control) — 4 sheep, group II — 5 sheep, groups III and IV — 9 sheep each. The concentration of lead in the ration for group II was 5 mg · kg^{-1} (1 MPL), for group III — 25 mg · kg^{-1} (5 MPL), for group IV — 150 mg · kg^{-1} (30 MPL). Lead nitrate $Pb(NO_3)_2$ was added to compound feed once a day. The daily intake of metal for group II was 10 mg/head, group III — 50 mg/head, group IV — 300 mg/head, or 0.3, 1.5 and 9 mg · kg^{-1} body weight. Blood samples were taken before feeding from the jugular vein before the experiment, on days 30, 60 and 90. During the study period, animals were slaughtered, 1 sheep before the experiment, on days 30 and 60 1 sheep from group II and 3 sheep from groups III and IV; on day 90 — 3 sheep from each group. The patterns of distribution and accumulation of lead in the organs and tissues of sheep were analyzed using a mathematical model in which the liver, kidneys, spleen, lungs, heart and muscle tissue are represented as separate chambers physiologically interconnected by transport communications. Changes in the constants of the rate of transfer of lead from peripheral blood into different organs and muscle tissue of sheep, depending on the metal content in the ration and the duration of its intake, were established. The parameters characterizing the ratio of the constants of the rate of transfer of lead from the blood into the organs and back (from the organs into the blood) are determined. It is shown that the values of the parameters for the liver and kidneys as compared to other organs and tissues (spleen, lungs, heart and muscle tissue) are 10 and 100 times lower, respectively. Comparative analysis of experimental data and calculations on the model is carried out. The degree of coincidence of the results shows that the chamber model satisfactorily describes the transfer of lead from the peripheral blood into the organs and muscle tissue of sheep. The developed mathematical model is recommended for assessing and predicting the safety of sheep products.

Keywords: lead, chamber model, sheep, blood, liver, kidneys, spleen, lungs, heart, muscle tissue

Contamination of agricultural land with lead compounds increases the likelihood of its transfer into food (meat, milk, offal) via the trophic chain soil—

plant—animal [1-3]. Lead is highly cumulative and, depending on the dose and duration of exposure in mammals, exhibits high general toxicity, embryotoxicity, carcinogenicity and genotoxicity [4-6]. The World Health Organization (WHO) has approved the maximum permissible level of lead for humans at 25 $\mu g \cdot kg^{-1}$ body weight per week, or 3.6 $\mu g \cdot kg^{-1}$ body weight per day. However, in 2011, this threshold was canceled because it did not ensure the health safety of children and adults [7]. In the Russian Federation, permissible daily doses of lead for humans have not been established, although in the USSR the recommended dose was 4 $\mu g \cdot kg^{-1}$ body weight per day [8]. In the United States, the Food and Drug Administration (USFDA) in 2020 issued a temporary permissible daily intake of lead for children and adults of 3 and 12.5 μg, respectively [9]. In 2021, the US Centers for Disease Control and Prevention (CDC) updated the standard for lead concentrations in peripheral blood, and the temporary permissible daily intake of lead was reduced to 2.2 μg for children and 8.8 μg for women of childbearing age [10).

The toxic effect of lead on mammals is more correctly assessed not by daily intake, but by peripheral blood concentration which plays an important role in the transport and redistribution of the metal to organs and tissues. Determination of lead concentrations in peripheral blood eliminates the uncertainties caused by gastrointestinal (GI) absorption of the metal [11]. In the USA, the standard for the lead concentration in human peripheral blood was 60 $\mu g \cdot dl^{-1}$ in 1960-1970, 30 $\mu g \cdot dl^{-1}$ in 1970-1985, 25 $\mu g \cdot dl^{-1}$ in 1985-1991, in 10 $\mu g \cdot dl^{-1}$1991-2012, 5 $\mu g \cdot dl^{-1}$ in 2012-2021, 3.5 $\mu g \cdot dl^{-1}$ from 2021 to the present [9, 10, 12]. Biokinetic models were used to calculate the permissible amount of lead entering the body [13-15]. It should be noted that mathematical models for predicting has primarily been used to assess the risk of human exposure to lead [16-18] through food intake [19].

Currently, mathematical models for predicting the risk of lead exposure in farm animals have not been developed. Existing conceptual and chamber models assess the accumulation of radionuclides in animal products (meat, milk) [20] or in the organs and tissues of laboratory animals [21]. In 2023, a simulation model was presented to assess the permissible levels of cadmium, lead, mercury and arsenic in the diets of cows and sheep for food production (meat and milk) that meet the requirements of SanPiN 2.3.2.1078-01 [22]. It must be emphasized that with chronic intake of lead, the kidneys and liver of farm animals are most severely affected. Producing meat and milk that meets sanitary and hygienic standards does not fully guarantee the safety of livestock products. The functional activity of the organs of the detoxification (liver) and excretory (kidney) systems can have a negative impact on the animal metabolism and healt. Therefore, when exposed to lead, the safety assessment of meat, milk and by-products must be carried out.

An analysis of scientific information on the lead effects in mammals suggests that research on farm animals is fragmented. The main attention was paid to livestock farming under lead contamination of territories and to obtaining food products (meat, milk) that meet sanitary and hygienic standards [23-25]. Animal model studies have assessed exposure to lead in food-producing animals at concentrations that significantly exceeded maximum permissible levels (MPLs) in feed [2, 26, 27].

Lifetime assessment of lead content in the organs and tissues of farm animals can predict the degree of contamination of livestock products. To predict the lead content in meat and milk, transition coefficients (CT) are mainly used. Thus, with chronic intake of lead in sheep, CTs from the diet were established for the peripheral blood, liver, kidneys and spleen [28, 29] and a method was proposed

for estimating the amount of metal in muscle tissue based on the concentration in wool and feces [30]. Note that the high variability of CTs does not allow us to correctly predict the lead content in organs and tissues. Therefore, the alternative methods for intravital assessment of the lead accumulation is still relevant.

Previously, a conceptual scheme of the lead distribution in ruminants [31] and a chamber model of the transition of the metal from different parts of the gastrointestinal tract into the peripheral blood of sheep [32] were reported.

In this work, based on the model we developed, we assessed for the first time the parameters of lead transport between peripheral blood, organs and muscle tissue depending on the daily dietary concentration of the metal and the duration of its entry into the body.

Our goal was to develop and parameterize a chamber model of the lead transition from peripheral blood to the organs and muscle tissue of sheep during chronic intake of the metal in the diet.

Materials and methods. The model used was developed based on our own experimental data [28-30]. The experiments were carried out on 27 Romanov sheep (*Ovis aries)* aged 1-1.5 years, live weight 33.5±0.7 kg. Animals were kept in boxes, 4-5 sheep per each (the vivarium of the All-Russian Research Institute of Physiology, Biochemistry and Nutrition, ARRIPB&N, Borovsk, Kaluga Province). Sheep were fed twice a day with free access to water. The animals were divided into four groups: group I was 4 control animals, group II contained 5 animals, groups III and IV 9 animals each. The dietary lead was fed at 1 MPL (5 mg · kg^{-1}) to group II, at 5 MPL (25 mg · kg^{-1}) to group III, and at 30 MPL (150 mg · kg^{-1}) to group IV. Lead nitrate Pb(NO3)2 was fed with mixed feed once a day, given on average 2 kg of feed entering the gastrointestinal tract. To do this, 100 g of feed was mixed with 50 ml of a Pb(NO3)2 solution of a certain concentration. The daily Pb intake in group II was 10 mg per sheep, in group III 50 mg per sheep, in group IV 300 mg per sheep, or 0.3, 1.5 and 9 mg kg^{-1} bodyweight.

To collect organs and muscle tissue, animals were slaughtered before priming (one sheep), on days 30 and 60 (at each term, one sheep of group II and three sheep of groups III and IV), and on day 90 (three sheep of each group). Blood was sampled from the jugular vein before feeding (at proming as the basal level, on days 30, 60 and 90). The lead content in samples (blood, liver, kidneys, spleen, lungs, heart, and muscle tissue) was measured by the atomic emission method on a Liberty AX Sequential ICP-AES spectrometer (Varian, Austria) after dissolving the ash sediment.

Given that changes of the lead concentrations in organs and tissues depend on the intensity of their blood supply and the peripheral blood concetration of the metal, the lead transition from peripheral blood to the liver, kidneys, spleen, lungs, heart, and muscle tissue of sheep was describrd as a system of differential equations:

$$\begin{aligned} dq_1/dt &= k_{bl1} \cdot q_{bl} - k_{1bl} \cdot q_1; \\ dq_2/dt &= k_{bl2} \cdot q_{bl} - k_{2bl} \cdot q_2; \\ dq_3/dt &= k_{bl3} \cdot q_{bl} - k_{3bl} \cdot q_3; \\ dq_4/dt &= k_{bl4} \cdot q_{bl} - k_{4bl} \cdot q_4; \\ dq_5/dt &= k_{bl5} \cdot q_{bl} - k_{5U} \cdot q_5 - k_{5bl} \cdot q_5; \\ dq_6/dt &= k_{bl6} \cdot q_{bl} - k_{6U} \cdot q_6 - k_{6bl} \cdot q_6, \end{aligned} \quad (1)$$

where q_1, q_2, q_3, q_4, q_5, q_6 and q_{bl} are the lead concentration (mg · kg^{-1}) in the heart, spleen, lungs, muscle tissue, liver, kidneys and peripheral blood, resprctively; k_{bl1}, k_{bl2}, k_{bl3}, k_{bl4}, k_{bl5}, k_{bl6} are rate constants (day^{-1}) for the transition of lead from peripheral blood to the heart, spleen, lungs, muscle tissue, liver, kidneys; k_{1bl}, k_{2bl}, k_{3bl}, k_{4bl}, k_{5bl}, k_{6bl} are rate constants (day^{-1}) for the transition of lead

from the heart, spleen, lungs, muscle tissue, liver and kidneys to peripheral blood; k_{5U} and k_{6U} are rate constants (day^{-1}) for lead excretion from the liver and kidneys; t — days of observation.

The lead concentration in the peripheral blood of sheep depended on the dietary metal content, the duration of its entry into the body [32] and was determined by the formula:

$$q_{\text{bl}} = \frac{\sum_{j=1}^{6} k_{j,bl} \cdot q_j - 0.00002 \cdot d}{k_{blU}}, \quad (2)$$

where q_{bl} is the lead concentration in peripheral blood, mg · kg^{-1}; d is daily intake of dietary lead, mg · kg^{-1} · day^{-1}; $k_{j,\text{bl}}$ is the rate constant of transition from the j-th section of the gastrointestinal tract to the blood, day^{-1}; q_j is the lead concentration in the j-th section of the gastrointestinal tract, mg · kg^{-1}; $k_{\text{bl}U}$ is the rate constant for the removal of lead from the blood, day^{-1}.

The elimination of lead from the sheep liver and kidneys was described by the equations

$$\begin{aligned} dU_1/dt &= k_{5U} \cdot q_5; \\ dU_2/dt &= k_{6U} \cdot q_6, \end{aligned} \quad (3)$$

where U_1 and U_2 are the lead concentration in feces and urine, mg · kg^{-1}; $q5$ and $q6$ are lead concentrations in the liver and kidneys, mg · kg^{-1}; k_{5U} and k_{6U} are the rate constants for lead excretion from the liver and kidneys, day^{-1}; t — days of observation.

Statistical processing of the data was carried out by the variation statistics method using the Excel 2013 and Mathcad application package. The article presents the mean values of the indicators (M) and standard errors of the means (±SEM).

Results. The patterns of lead distribution and accumulation in the organs and tissues of sheep during chronic dietary intake of lead were analyzed using a model in which organs and tissues are conidered as separate chambers [33, 34], physiologically interconnected by transport communications (Fig. 1).

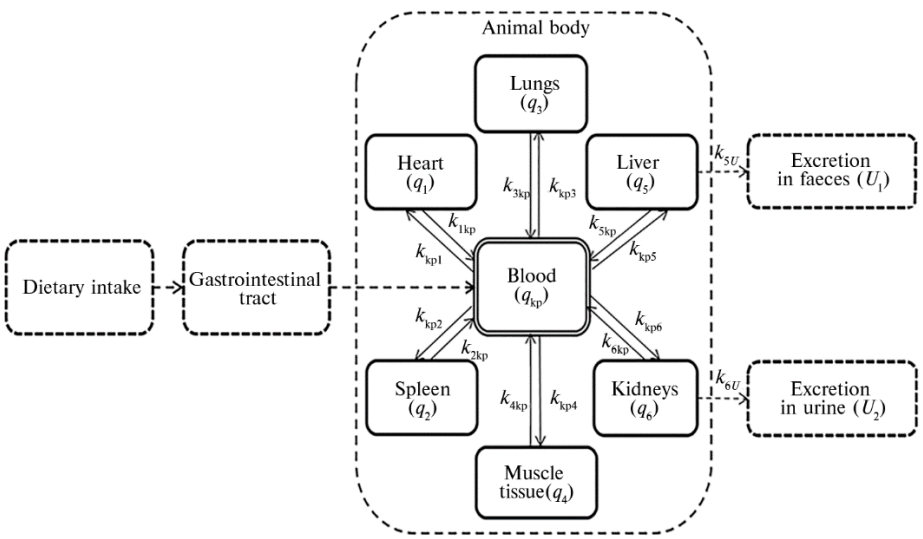

Fig. 1. Conceptual scheme of the lead transition from the diet into the sheep (*Ovis aries*) organs and tissues:: q_1, q_2, q_3, q_4, q_5, q_6 and q_{bl} — the concentration of lead in the heart, spleen, lungs, muscle tissue, liver, kidneys and peripheral blood, respectively, mg · kg^{-1}; $k_{\text{bl}1}$, $k_{\text{bl}2}$, $k_{\text{bl}3}$, $k_{\text{bl}4}$, $k_{\text{bl}5}$, $k_{\text{bl}6}$ — rate constants for the transition of lead from peripheral blood to the heart, spleen, lungs, muscle tissue, liver and kidneys, day^{-1}; $k_{1\text{bl}}$, $k_{2\text{bl}}$, $k_{3\text{bl}}$, $k_{4\text{bl}}$, $k_{5\text{bl}}$, $k_{6\text{bl}}$ — rate constants for the transition of lead from the heart, spleen, lungs, muscle tissue, liver and kidneys to peripheral blood, day^{-1}; k_{5U} and k_{6U} — rate constants for lead elimination from the liver and kidneys, day^{-1}.

Solving a system of differential equations (1) resulted in a formula that allows us to assess the change of the lead concentration in the liver, kidneys, spleen, lungs, heart and muscle tissue of sheep depending on the lead content in the peripheral blood and daily intake from feed:

$$q_i = \frac{1}{f_i} \cdot \left(q_{bl} - \frac{a_i \cdot d}{k_{bli}}\right), \quad (4)$$

where q_i is the lead concentration in the i-th organ or muscle tissue, mg · kg^{-1}; q_{bl} is the lead concentration in peripheral blood, mg · kg^{-1}; d is daily intake ofdietary lead, mg · kg^{-1} · day^{-1}; k_{bli} is the rate constant for the lead transition from peripheral blood to organs, day^{-1}; f_i (i = 1.6) stands for parameters characterizing the of the rate constant ratio for lead transition from organs and muscle tissue to peripheral blood k_{bli} and from peripheral blood to organs and muscle tissue k_{bli}; a_i (I = 1.6) are calculated constants (for kidneys $25.2 \cdot 10^{-3}$, for liver $18.4 \cdot 10^{-3}$, for spleen $8.0 \cdot 10^{-5}$, for lungs $2.0 \cdot 10^{-5}$, for the heart $16.0 \cdot 10^{-6}$, for muscle tissue $12.0 \cdot 10^{-6}$).

The lead transport from peripheral blood to organs and back depends on the physiological processes occurring in the animal's body. Based on experimental data, the rate constants for the lead transition from organs and muscle tissue to peripheral blood (k_{ibl}) and from peripheral blood to organs and muscle tissue (k_{bli}) were calculated. For the liver, kidneys, spleen, lungs, heart and muscle tissue of sheep, acceptable values of the parameters f_i were determined, characterizing the ratio of k_{ibl} to k_{bli}. Analysis of f_i parameters revealed the following descending order: heart (< 0.57) > muscle tissue (< 0.49) > lungs (< 0.34) > spleen (< 0.23) > liver (< 0.042) > kidneys (<0.0032).

Given the uncertainties in the lead transition from the blood to organs and tissues, f_i values were established that are most suitable for describing the accumulation of the toxicant in the sheep liver, kidneys, spleen, lungs, heart and muscle tissue. Below there are formulas for calculating the rate constants for the lead transition from peripheral blood to organs and tissues, depending on the daily dietary concentration and the duration of entry into the body:

heart (f_i = 0.1) — $k_{bl1} = \frac{d}{3380.625+1.15 \cdot d \cdot t}$;

spleen (f_i = 0.1) — $k_{bl2} = \frac{d}{632.75+0.15 \cdot d \cdot t}$;

lungs (f_i = 0.1) — $k_{bl3} = \frac{d}{2334.5+0.9 \cdot d \cdot t}$;

muscle tissue (f_i = 0.1) — $k_{bl4} = \frac{d}{4428.35+1.57 \cdot d \cdot t}$;

liver (f_i = 0.01) — $k_{bl5} = \frac{100 \cdot d}{320.842+0.08695 \cdot d \cdot t}$;

kidneys (f_i = 0.001) — $k_{bl6} = \frac{1000 \cdot d}{2488.467+0.587 \cdot d \cdot t}$.

The maximum rate constants for the lead transition from the blood to organs and muscle tissue, regardless of daily intake occurred on day 1 of intoxication (Table, Fig. 2). In next periods, there was a decrease in the values of indicators. On day 90 of intoxication, changes in the transition rate constants were weakly expressed.

Values of the rate constants for the lead transition from the blood into the organs and tissues of Romanov sheep (*Ovis aries*) depending on the metal content in the diet and duration of its intake (ARRIPBF, Kaluga Province, Borovsk)

Constant	Lead content in the diet, mg · kg^{-1}											
	5				25				150			
	Days											
	1	30	60	90	1	30	60	90	1	30	60	90
K_{bl1}	0.0015	0.0014	0.0013	0.0013	0.0073	0.0059	0.0049	0.0042	0.0422	0.0175	0.0109	0.0079
k_{kbl2}	0.0079	0.0076	0.0074	0.0071	0.0393	0.0335	0.0291	0.0258	0.2289	0.1147	0.0757	0.0564
K_{bl3}	0.0021	0.0020	0.0019	0.0018	0.0106	0.0083	0.0068	0.0057	0.0607	0.0235	0.0144	0.0104

Continued Table

K_{bl4}	0.0011	0.0011	0.0010	0.0010	0.0056	0.0045	0.0037	0.0031	0.0322	0.0131	0.0081	0.0059
K_{bl5}	1.5563	1.4975	1.4412	1.3890	7.7395	6.4758	5.5400	4.8405	44.9256	21.0639	13.5944	10.0357
k_{kbl6}	2.0069	1.9406	1.8765	1.8165	9.9874	8.5362	7.4207	6.5630	58.2181	29.2400	19.3014	14.4051

N o t e. k_{bl1}, k_{bl2}, k_{bl3}, k_{bl4}, k_{bl5}, k_{bl6} — rate constants for the transition of lead from peripheral blood to the heart, spleen, lungs, muscle tissue, liver and kidneys, day^{-1}.

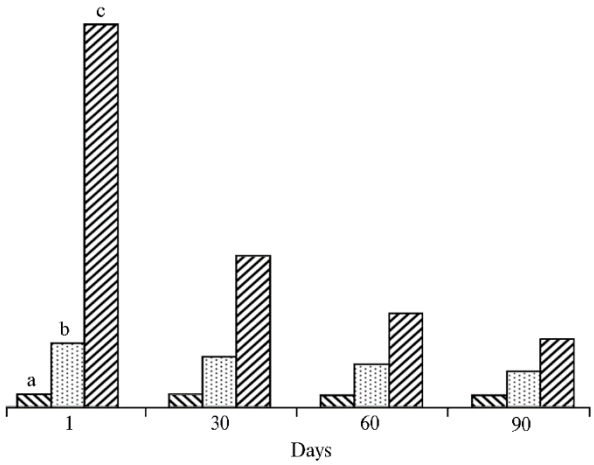

Fig. 2. Relative values of rate constants for the transition of lead from peripheral blood to the body of Romanov sheep (*Ovis aries*) depending on the metal content in the diet and duration of its intake: a — 5 mg · kg^{-1}, b — 25 mg · kg^{-1}, c — 150 mg · kg^{-1} feed (ARRIPBF, Kaluga Province, Borovsk). The diagram shows constant values in proportion to those at minimum lead level (5 mg · kg^{-1}, a). The patterns of constant changes are similar for all studied organs (heart, spleen, lungs, liver, kidneys) and muscle tissue.

We calculated the rate constants for the lead transition from the *i*-th organ and muscle tissue into the peripheral blood ($k_{i\text{bl}}$, $i = 1.6$):

$$k_{i\text{kbl}} = f_i \cdot k_{\text{bl}i}. \quad (5)$$

According to formula (5), changes in the rate constants for the lead transition to peripheral blood are of a similar nature.

The simulation quality was assessed by a comparative analysis of calculated and experimental data using the Theil test (discrepancy index). The discrepancy index shows the degree of similarity, the closer it is to zero, the closer the compared series are [35]. In our experiment, the Theil test value ranged from 0.058 to 0.186 (Fig. 3).

Mathematical models as a tool to assess the parameters of lead accumulation in mammals are of scientific and practical interest. Currently developed mathematical models are mainly aimed at assessing the lead transfer from the food into human peripheral blood [34, 36] or from the feed into livestock products (milk, meat) [22]. This approach does not take into account the fact that the critical organs for lead exposure are the liver and kidneys [29].

The construction of a logically sound and consistent conceptual scheme underlies the development of mathematical models of the transition of lead into the organs and tissues of productive animals. Previously, based on the analysis of lead metabolism in the body, we presented a conceptual scheme for the transition of the metal into the organs and tissues of ruminants [31]. Considering the complexity of the mathematical description of lead metabolism in sheep, we decided to develop two independent but interrelated models. One is a chamber model of the lead transition of from the rumen, mesh, book, abomasum, small and large intestines into the peripheral blood [32], another is a chamber model of the lead transition from peripheral blood to organs and muscle tissue.

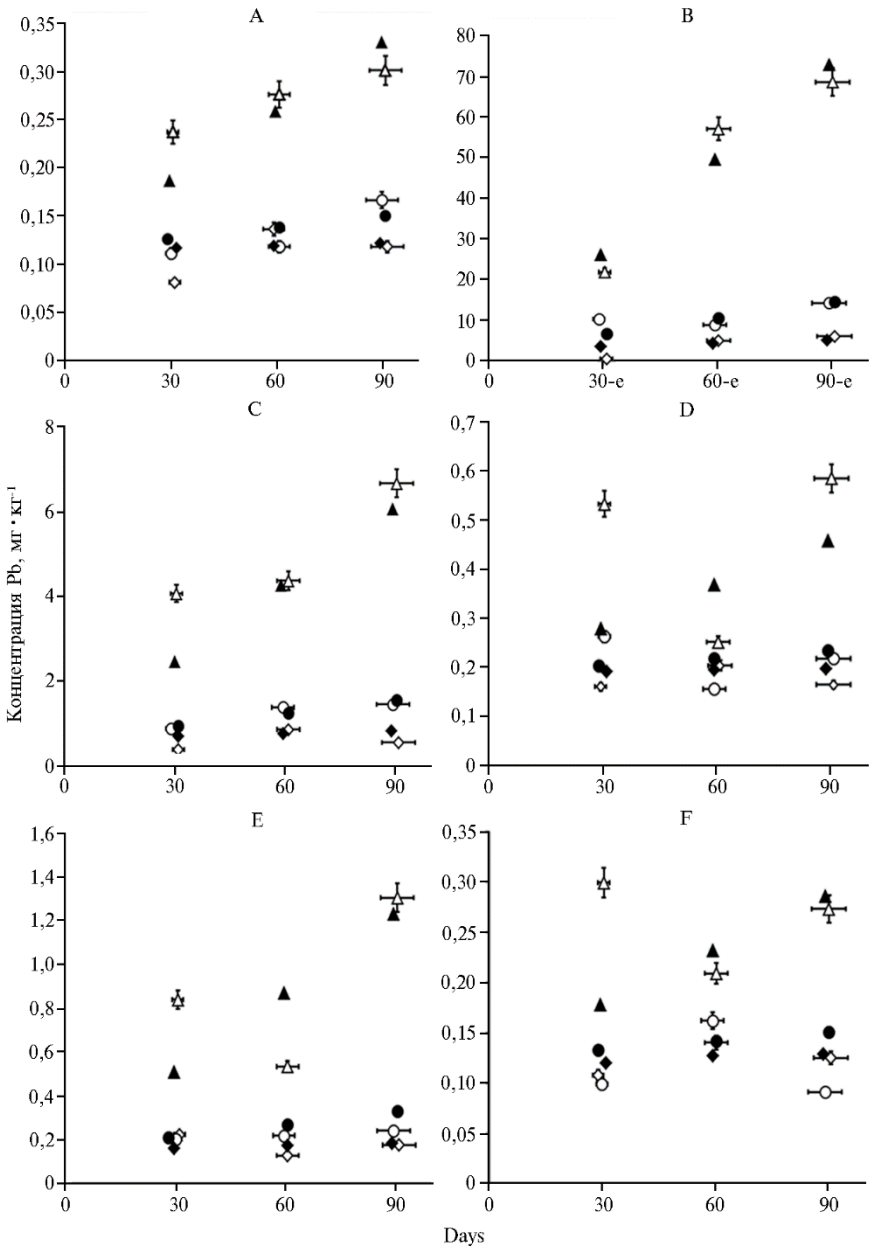

Fig. 3. Concentration of lead in organs and muscle tissue of Romanov sheep (*Ovis aries*) depending on the metal content in the diet and duration of its intake: A — heart, B — kidneys, C — liver, D — lungs, E — spleen, F — muscle tissue; ◊ and ♦ —observed and calculated data, respectively, for 5 mg · kg^{-1} dietary lead, ○ and ● — 25 mg · kg^{-1}, Δ and ▲ — 150 mg · kg^{-1} feed (M±SEM; ARRIPBF, Kaluga Province, Borovsk). For sample sizes depending on the dietary lead concentration for each observation period, see the Materials and methods section.

In the presented work, changes in the rate constants for the lead transition from peripheral blood to organs and muscle tissue and back, depending on the amount of lead in the diet and the duration of its entry into the body of sheep, were established for the first time. It was shown that on day 90 of intoxication, the transition rate constants practically do not change and reach a plateau, which suggests the onset of an equilibrium state. This can be supported by data that when a drug is administered orally to mammals, the time to reach its maximum concentration in the peripheral blood (approximately 97% of its steady-state level) is

approximately five half-lives [37]. Since the half-life of lead in soft tissues and peripheral blood is 24-40 days [38], the equilibrium state between the entry of the metal into the organs and its excretion should be expected on days 120-200 of intoxication.

It has been shown that the ratio of the rate constants for the lead transition from the blood to the liver and kidneys and back to the blood is 10 and 100 times lower, respectively, than for the spleen, lungs, heart and muscle tissue of sheep. It is likely that the lead content in the peripheral blood entering the liver and kidney tissues is significantly higher than in the outflowing blood. It is assumed that low parameters for the liver and kidneys characterize, on the one hand, the detoxification and excretory functions of the organs, and on the other hand, the level of lead accumulation. This is supported by data on the content of lead and metallothioneins in the liver and kidney tissues of sheep [29, 39]. It is not possible to experimentally confirm or refute the obtained research results because of the complexity of the blood supply system to organs and tissues.

A comparative analysis of experimental data and model calculations using the Theil test showed that the chamber model satisfactorily describes the lead transition from peripheral blood to organs and muscle tissue. The degree of agreement between the results allows us to recommend a mathematical model for determining the concentration of lead in the liver, kidneys, spleen, lungs, heart and muscle tissue of sheep during chronic dietary intake. The chamber model we developed can be used to estimate the permissible daily intake of dietary lead and forecast the safety of sheep products. This will optimize sheep feeding technologies through feed formulaying.

Thus, based on our own experimental data, a mathematical model of the lead transition from peripheral blood to different organs and muscle tissue of sheep has been developed. The model predicts the concentration of lead in the liver, kidneys, spleen, lungs, heart and muscle tissue of sheep, depending on the amount of metal in the diet and the duration of its intake. Changes in the rate constants for the lead transition from peripheral blood to various organs and muscle tissue of Romanov sheep have been established. The minimum transition rate constants were on day 90 of the examination. Parameters have been determined that characterize the ratio of the rate constants for the lead transition from the blood to the organs and back from the organs to the blood. It has been shown that the parameter values for the liver and kidneys are 10 and 100 times lower than for the spleen, lungs, heart and muscle tissue.

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PATHOMORPHOLOGICAL CHARACTERIZATION OF LENS CAPSULE TISSUES IN ANIMALS AFTER PREVENTION OF SECONDARY CATARACTS: AN EXPERIMENTAL STUDY

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Abstract

One of the current problems of veterinary science is the treatment and prevention of pathologies that reduce the working and productive qualities of especially valuable animals. In veterinary ophthalmology, it remains an important goal to find ways to prevent secondary cataract after ultrasound phacoemulsification. Different methods are used for this purpose, but the questions about pathomorphosis of secondary cataract and scientific basis of effective methods of its prevention remain open. In the present work morphologic changes in the lens capsule of rabbits 2 and 6 months after phacoemulsification under different methods of secondary cataract prophylaxis were identified for the first time. On the basis of clinical picture together with morphological changes the most effective method of prophylaxis - capsulorhexis of the posterior lens capsule - was established. The aim of the work was to study the features of morphological changes occurring in the lens capsule in rabbits after phacoemulsification combined with intraoperative techniques of secondary cataract prophylaxis. The study was carried out in 2020-2021 at the Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology named and at Dr. A.G. Shilkin Veterinary Ophthalmology Center on 18 crossbred rabbits (*Oryctolagus cuniculus domesticus*) of both sexes, aged 4 months, without ophthalmic pathologies. The animals were divided into three groups (6 animals in each group). Ultrasound phacoemulsification was performed in all groups according to the generally accepted technique. Animals of group 1 were implanted with intraocular lens (AquaFree Y-PL, hydrophobic, Overal Dia 13.0 mm, Rumex, USA), group 2 — with intracapsular ring (polymer ophthalmic intracapsular ring for lens bag spreading and lens capsule tensioning, KPV-"ETP-MG" ring No. 3, 13.0 mm, Federal State University Cross-Sectoral Research and Technology Centre, Acad. Fedorov Eye Microsurgery Rosmedtechnologiya-ETP-MG, Russia). In group 3 posterior capsulorhexis was performed. In the postoperative period, the rabbits received general (enrofloxacin, 5 mg/kg body weight, 2 times per day; ketoprofen, 3 mg/kg body weight, 1 time per day for 3 days) and topical treatment (atropine for 10 days; moxifloxacin, eye drops for 14 days; dexamethasone + neomycin + polymyxin B sulfate for 14 days; nepafenac for 28 days). The animals were under continuous monitoring, during which ophthalmologic examination and photographic fixation of the eye condition were performed. Signs of secondary cataract were identified by using slit-lamp biomicroscopy, the state of the ocular fundus was examined by ophthalmoscopy, and the transparency of the capsule was determined. The rabbits were subjected to drug euthanasia 2 and 6 months after the surgery, by 2 and 4 animals, respectively. Eyeballs were enucleated and placed in 10% neutralized formalin solution. For macroscopic evaluation of the state of the lens capsule, a slice was made in the area of the serrated edge of ora serrata with retinal capture. Most of the vitreous body was aspirated. After photography, a sagittal section of the eye was performed and subjected to histologic examination. It was found that within 2 months after performing the above techniques, proliferation of lens epithelium resumes in the capsule, lens fibers are formed (mainly in the area of the equator of the capsular bag), and adhesions with the iris may develop. Within 6 months, the changes progress, however, to a different degree depending on the technique used. It is shown that capsulorhexis of the

posterior lens capsule is the most effective technique for secondary cataract prophylaxis due to a limited impact on tissues in general, absence of alien objects after the surgery and, therefore, the least proliferation of pathological lens tissues with preservation of optical transparency of the central region of the capsular bag.

Keywords: lens, cataract, posterior capsulorhexis, capsule opacification, secondary cataract, phacoemulsification, secondary cataract prevention

The search for methods of treating animals with cataracts is an urgent problem in veterinary medicine, in particular in service dog breeding and sports horse breeding. The prevalence of lens pathologies in horses, especially thoroughbreds, is due to a significant incidence of uveitis (inflammation of the choroid); cataracts are recorded in 46-53% of all ophthalmopathies. Among lens pathologies in dogs, cataracts account for 90% [1]. This is one of the most common causes of decreased visual function in animals, leading to a decrease in the performance of dogs and their premature culling. An effective method for treating animals with cataracts is phacoemulsification which results in positive outcomeы in most cases. However, a common complication after surgery is secondary cataract which leads to a decrease in visual function [2] and, according to various sources, develops within 1-6 months after the intervention in 62% [3] and even in 100% of cases [4].

In human medicine, YAG laser discission successfully correct this condition, removing the optically opaque region of the capsule [5]. However, in veterinary medicine this technique is ineffective due to numerous complications. Thereof, methods of intraoperative prevention of secondary cataracts acquire particular importance [6, 7]. Among surgical techniques combined with phacoemulsification [8, 9], intraocular lens implantation has shown effectiveness in veterinary practice [10, 11]. There is limited information about the positive effects of mechanical polishing of the lens capsule and implantation of an intracapsular ring [12-15], and about capsulorhexis of the posterior lens capsule [16-21].

Discussing the pathogenesis of secondary cataracts, some authors [22, 23] describe the mechanism of fibrous metaplasia of lens epithelial cells. In their opinion, the cells of the lens epithelium in patients with cataracts underwent metaplasia with the formation of so-called plaques, consisting of connective tissue and ectopic basement membrane formed by epithelial cells. M.E. Bernays et al. [24] identified this condition in 7 of 25 cases studied. Despite the data obtained, the metaplasia of lens cells into fibroblasts followed by connective tissue formation is questionable. In addition, there has not yet been a comparative study of the changes, occurring in tissues after phacoemulsification combined with intraoperative techniques to prevent secondary cataracts. Rabbits are similar to horses and dogs in terms of inflammation and exudation types and can be used to develop methods for preventing secondary cataracts [25, 26].

In this paper, for the first time, morphological changes were identified in the lens capsule of rabbits 2 and 6 months after phacoemulsification with various methods of preventing secondary cataracts. Based on the clinical picture and morphological changes, the capsulorhexis of the lens posterior capsule has been established as the most effective method to prevent secondary cataracts.

The purpose of the work is to study morphological changes that occur in the lens capsule in rabbits after phacoemulsification combined with intraoperative manipulations for the prevention of secondary cataracts.

Materials and methods. Rabbits (*Oryctolagus cuniculus domesticus*) without ophthalmological pathology aged 4 months (n = 18, mixed breeds of both sexes) were used in the experiment (Scryabin Moscow State Acafemy of Veterinety Medicine and Biotechnology, the Center for Veterinary Ophthalmology of Dr. A.G. Shilkin, 2020-2021).

The rabbits were assigned to three groups, 6 animals in each. In all groups, ultrasound phacoemulsification was performed according to the generally accepted technique. Animals of group 1 were implanted with an intraocular lens (AquaFree Y-PL, hydrophobic, Overal Dia 13.0 mm, Rumex, USA), animals of group 2 were implanted with an intracapsular ring (a polymer ophthalmic intracapsular ring for straightening the lens bag and tensioning its capsule, CPV-ETP-MG ring No. 3, 13.0 mm, Fedorov MNTK Eye Microsurgery, Rosmedtekhnologii-ETP MG, Russia). In group 3, posterior capsulorhexis was performed. In the postoperative period, the rabbits received enrofloxacin (5 mg/kg bodyweight 2 times a day), ketoprofen, 3 mg/kg BW, 1 time a day for 3 days) and local application of atropine for 10 days, moxifloxacin eye drops for 14 days, dexamethasone + neomycin + polymyxin B sulfate for 14 days, and nepafenac for 28 days.

The animals were under observation during which an ophthalmological examination and photographic recording of the eye conditions were carried out (days 1, 3, 7, then once every 3 days during the first month after surgery, and after the first month once a month). During the examination, intraocular pressure was measured to compare to normal range of 10 to 24 mm Hg (Tonovet, iCare, Finland). Signs of secondary cataracts were revealed using slit biomicroscopy (SL 17, Kowa Company, Ltd., Japan), the condition of the fundus was studied ophthalmoscopically (Omega 500, Heine Optotechnik GmbH & Co KG, Germany), additionally determining the capsule transparency. Depending on the severity of the changes, secondary cataracts of grade I, II or III were diagnosed. In grade I, the fundus was well ophthalmoscoped, in grade II, local opacities appeared in the field of view; in grade III, fundus details were poorly visible or the fundus was not visualized.

Rabbits were removed from the experiment by medical euthanasia, 2 and 4 animals 2 and 6 months after surgery, respectively. The eyeballs were enucleated and placed in a 10% neutralized formaldehyde solution. For a macroscopic assessment of the condition of the lens capsule, a section was made in the area of the dentate line (ora serrata) involving the retina. Most of the vitreous was aspirated. After photographing (a Huawei P30 smartphone, Huawei Technologies Co., Ltd., China), a sagittal section of the eye was made and its histological examination was carried out. After fixation in a 10% formalin solution, the samples were embedded in paraffin according to the common technique. Sections were prepared (a rotary automated microtome HM-325, MICROM International GmbH, Germany) and stained with hematoxylin and eosin to identify the general morphology and by Van Gieson to identify connective tissue and lens components. To study histological sections, a Jenamed-2 light microscope (Carl Zeiss Jena, Germany) combined with an ImageScope C digital microscopy system (Systems for Microscopy and Analysis LLC, Russia) was used.

All manipulations were carried out in accordance with Directive 2010/63/EU of the European Parliament and the Council of the European Union for the protection of animals used for scientific purposes (Strasbourg, September 22, 2010).

Results. In group 1, on day 2 after surgery, intraocular pressure reduced to 8.5±1 mm Hg. Its normalization occurred on day 3. After 2 months, opacification of the capsule corresponded to grade I (83.3% of cases) and grade II (16.7% of cases) cataracts. Macroscopically, after opening the eyeball, the lens was clearly identified, the lens capsule had a typical anatomical position (Fig. 1, A), and the opacification was located mainly in the equatorial zone. Microscopically, in the area of opacification, we found a significant growth of the lens substance between the layers of the capsule (see Fig. 1, B). Signs of chronic inflammation were visualized in the ciliary body and iris, and the pigment tissue of the ciliary body adhered to the capsule, forming adhesion that spread over its surface (see Fig. 1,

C). Six months after surgery, fundus ophthalmoscopy was difficult in 25% of cases. The lens capsule retained its typical location; the intraocular lens was clearly visible (see Fig. 1, D). In the long-term period, more pronounced thickening, compaction and opacification of the capsule occurred. Turbidity occupied a significant part of the surface, more pronounced in the equatorial zone. Foci of thickening and accumulations of lens cells between the layers of the capsule were microscopically detected (see Fig. 1, E, F).

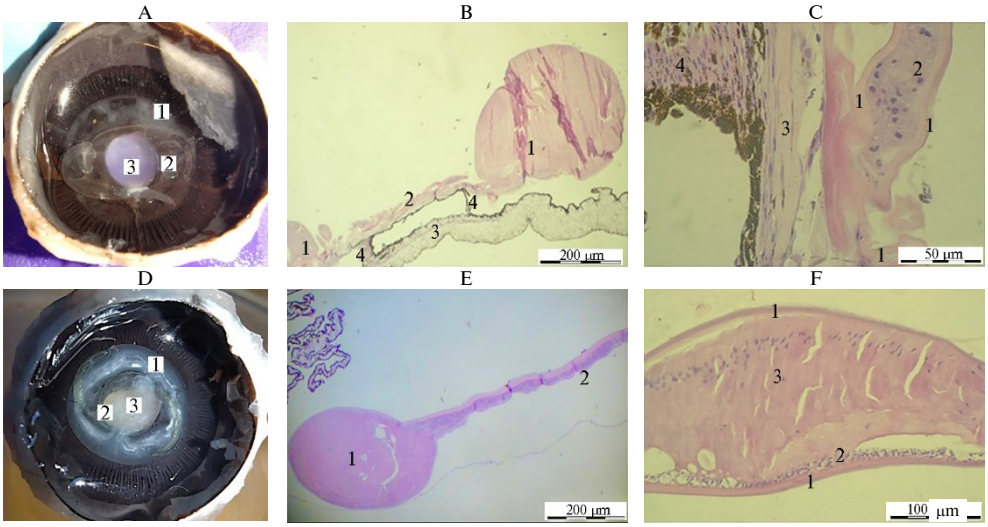

Fig. 1. The lens capsule of rabbits (*Oryctolagus cuniculus domesticus*) after phacoemulsification and implantation of an intraocular lens into the capsular bag: A — opened eyeball 2 months after surgery (1 — lens capsule with secondary cataract, 2 — intraocular lens, 3 — pupillary zone); B — microstructure of the lens capsule, general view (1 — area of thickening of the capsule, 2 — area of thinning with slight proliferation of lens epithelial cells between the layers of the capsule, 3 — iris, 4 — adhesions with the iris); C — fragment of the thickening focus (1 — capsule, 2 — epithelial cells of the lens, 3 — connective tissue capsule, 4 — pigment tissue of the iris); D — opened eyeball 6 months after surgery (1 — lens capsule with secondary cataract, 2 — intraocular lens, 3 — pupillary zone); E — microscopic changes in the lens capsule (1 — area of capsule thickening, 2 — area of thinning with accumulations of lens epithelial cells between the layers of the capsule); F — fragment of the thickening area (1 — capsule, 2 — proliferation of epithelial cells of the lens, 3 — lens fibers, signs of edema are observed) (hematoxylin and eosin staining, Jenamed-2 microscope, Carl Zeiss Jena, Germany; B, E: lens ×4, eyepiece ×10; B: objective ×40, eyepiece ×10; E: objective ×10, eyepiece ×10).

In group 2, during the postoperative period, intraocular pressure on day 2 reduced (8.6±1.2 mm Hg), in one case, ocular hypertension and iris bombardment were observed. Within 2 weeks, intraocular pressure returned to normal, but posterior synechiae of the iris remained. After 2 months, the secondary cataract (grade II) developed in 66.7% of cases, while it spread mainly in the equatorial zone (Fig. 2, A) and did not interfere with ophthalmoscopy. Microscopically, in the area of turbidity, numerous thickenings were revealed, formed by epithelial cells and lens fibers, in which signs of fragmentation, edema, and vacuolar degeneration of the lens epithelium were visible (see Fig. 2, B, C). In the ciliary body and iris, signs of chronic inflammation were identified as moderate marcrophage-lymphocytic infiltration. In the optically transparent area, only the lens capsule was observed in the form of acellular, weakly basophilic, fine-fibrous substance.

After 6 months, secondary cataracts were detected (see Fig. 2, D), complicating ophthalmoscopy in 50% of cases, in 25% the fundus was not visualized due to adhesions in the pupillary zone. Microscopic examination revealed an intracapsular ring; a small number of lens cells and lens fibers were observed between its material and the capsule (see Fig. 2, E). Nearby, encapsulated material of the

ring was revealed, and the capsule formed an adhesion with a thick layer of connective tissue (see Fig. 2, F). Signs of chronic inflammation were recorded in the iris and ciliary body: proliferation of connective tissue on the side of the iris, macrophage-lymphocytic infiltration, and edema.

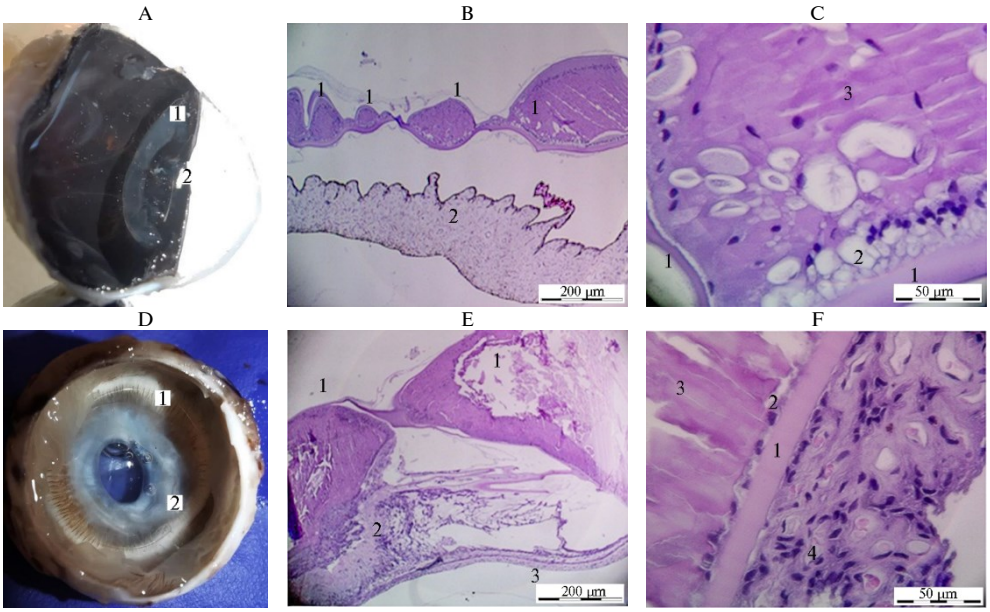

Fig. 2. The lens capsule of rabbits (*Oryctolagus cuniculus domesticus*) after phacoemulsification and implantation of an intracapsular ring: A — opened eyeball 2 months after surgery (1 — secondary cataract, 2 — pupillary zone); B — microstructure of the lens capsule, general view (1 — numerous thickenings of different sizes, 2 — iris); C — fragment of the thickening area (1 — capsule, 2 — epithelial cells of the lens, many in a state of hydrooptic dystrophy, 3 — lens fibers); D — opened eyeball 6 months after surgery (1 — intracapsular ring, displaced outside the capsular bag, 2 — equator of the capsular bag with secondary cataract); E — microscopic changes in the lens capsule (1 — areas of thickening in the lens capsule, 2 — connective tissue capsule formed around the ring, 3 — fibrous membrane, 4 — intracapsular ring); F — marginal zone of the thickening focus (1 — capsule, lens epithelium, 2 — lens fibers, 3 — connective tissue growing to the capsule from the side of the iris) (hematoxylin and eosin staining, Jenamed-2 microscope, Carl Zeiss Jena, Germany); C, F: objective ×4, eyepiece ×10; B, E: objective ×40, eyepiece ×10).

In group 3, intraocular pressure on day 2 was low (9.0±1.5 mm Hg) and normalized by day 5. After 2 months, the fundus of the eye was well visualized by ophthalmoscopy in all animals. At autopsy, secondary cataract grade I was revealed in the equatorial zone; an optically transparent "window" was preserved in the central zone in the area of the posterior capsulorhexis (Fig. 3, A). In the area of opacity, foci of thickening were identified, formed by lens epithelial cells and fibers inside the lens capsule (see Fig. 3, B, C). An optically transparent space was visualized in the pupil projection. The ciliary body and iris showed signs of chronic inflammation.

Six months after the surgery, all animals showed signs of secondary cataract, more pronounced in the equatorial zone, but it did not interfere with ophthalmoscopy, since the central zone where capsulorhexis was performed remained optically transparent (see Fig. 3, D). Microscopically, in the area of opacification, abundant deposition of lens fibers between the layers of the capsule was revealed (see Fig. 3, E). Signs of destruction, fragmentation, and edema were identified in the lens fibers, and the adjacent lens capsule locally formed folds (see Fig. 3, F). Signs of chronic inflammation were identified in the ciliary body and iris. The capsule structures were not identified in the pupil projection.

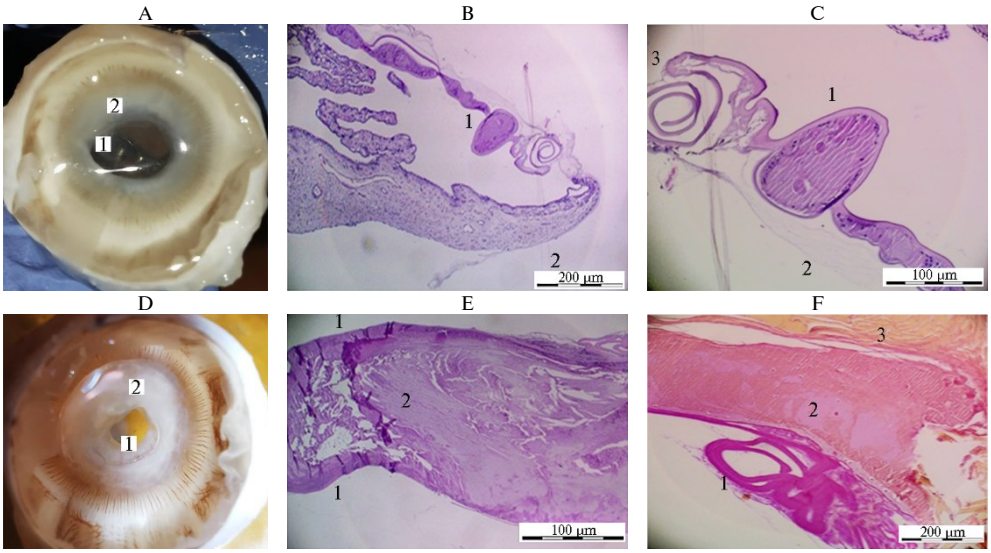

Fig. 3. The lens capsule of rabbits (*Oryctolagus cuniculus domesticus*) after phacoemulsification and capsulorhexis of the posterior capsule of the lens: A — opened eyeball 2 months after surgery (1 — optically transparent central part of the capsule, 2 — area of secondary cataract); B — microstructure of the capsule, general view (1 — numerous foci of thickening, 2 — iris); C — fragment of the cataract area (1 — thickening area, lens epithelium and fibers, inside the lens capsule, 2 — connective tissue capsule, 3 — capsule folds); D — opened eyeball 6 months after surgery (1 — optically transparent central part of the capsule, 2 — area of secondary cataract); E — area of turbidity (1 — capsule leaves, 2 — lens fibers); F — microscopic changes in the lens capsule (1 — folds of the capsule, 2 — lens fibers) (staining with hematoxylin and eosin, Jenamed-2 microscope, Carl Zeiss Jena, Germany; B, E: objective ×4, eyepiece ×10; C, F: objective ×10, eyepiece ×10).

Based on the studies, it was established that within 2 days after the sugery, all animals experienced intraocular inflammation, one of the characteristics of which was a decrease in intraocular pressure. During the postoperative period, secondary cataracts developed in all animals, which corresponds to the data of I.D. Bras et al. [4], however, its severity turned out to be different in the studied groups, for example, after capsulorhexis of the posterior surface of the lens capsule, it was minimal. Without intraoperative preventive measures, secondary cataracts of grades II-III develop in 16.7% rabbits within 2 months and in 100% rabbits within 6 months [1]. The technique of intraoperative prophylaxis changed the situation. According to our results, 2 months after phacoemulsification combined with implantation of an intracapsular ring, this complication occurred in 66.7% of cases, after implantation of an intraocular lens in 16.7%, and did not develop after capsulorhexis of the posterior capsule of the lens. After 6 months, the process progressed and was recorded in 75% rabbits after implantation of an intracapsular ring, in 25% after implantation of an intraocular lens, while did not occur after posterior capsulorhexis.

In morphological studies, we obtained results consistent with the data of A. Morales et al. [15] and indicating that secondary cataract is associated with the proliferation of epithelial cells and the formation of lens fibers. Moreover, our data show that the formation of fibrous tissue on the surface of the capsule is not associated with metaplasia of the lens epithelium, but with the involvement of the iris in inflammation. The iris adheres to the lens capsule and forms adhesions with it. This information complements modern ideas about the pathogenesis of secondary cataracts.

Our studies have shown that the most effective intraoperative method for the prevention of secondary cataracts, which is advisable to use in combination with phacoemulsification, is capsulorhexis of the posterior lens capsule (Table).

This is evidenced by the minimal, compared to other methods, severity of postoperative structural changes (e.g., proliferation of epithelial cells, formation of lens fibers, development of adhesions) and maintaining the optical transparency of the central zone of the capsule located in the projection of the pupil. Thereof, posterior capsulorhexis can restore visual functions due to a better clinical effect than with implantation of an intraocular lens and intracapsular ring.

Proportion (%) of rabbits (*Oryctolagus cuniculus domesticus*) with secondary cataracts upon intraoperative prevention ($n = 18$)

Group	2 months after surgery			6 months after surgery		
	grade I	grade II	grade III	grade I	grade II	grade III
1	83.3	16.7	–	75.0	25.0	–
2	33.3	66.7	–	25.0	50.0	25.0
3	100	–	–	100	–	–

N o t e. For a description of groups and sample sizes by group and observation period, see the Materials and methods section. Dashes mean that secondary cataract of the grade did not occur.

Thus, secondary cataract is a common complication after phacoemulsification in rabbits. Its pathogenesis is based on the proliferation of lens epithelial cells with the formation of structurally and functionally defective lens fibers. In addition, an adhesive process occurs, the iris or ciliary body, being involved in inflammation, form adhesions with the capsular bag of the lens. The results of studying the pathogenesis of secondary cataracts allow us to conclude that capsulorhexis of the posterior lens capsule is an effective intraoperative technique for preventing secondary cataracts that can be used in combination with phacoemulsification. This technique causes minimal structural changes in the capsule bag, while the central zone of the capsule located in the projection of the pupil remains optically transparent. This helps restore visual function and allows for a positive clinical effect.

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