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## MOLECULAR-GENETIC, MORPHOMETRIC AND SEXUAL CHARACTERISTICS OF BELUGA (*HUSO HUSO* L., 1758) CULTURED IN THE AQUACULTURE OF THE REPUBLIC OF BELARUS

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### Abstract

The subject of the study was genetic and phenotypic characteristics of Beluga (*Huso huso* (Linnaeus, 1758)) grown in the warm-water aquaculture at the “Selets” fish farm (the Republic of Belarus). Molecular-genetic analysis was performed using STR-markers and species-specific PCR revealed the absence of hybrid individuals in analyzed broodstock of Beluga sturgeon. Affiliation of individuals to the Caspian population was established based on the sequence analysis of the 367 bp region of mitochondrial D-loop. Using six STR loci, the population structure was analysed. The population structure and gene pool of the only broodstock of Beluga sturgeon in Belarus was compared with domesticated Beluga sturgeons from the Russian Federation. The analysis showed a lack of genetic variation and the heterozygote excess in Beluga sturgeons from the Belarusian farm. Analysis of morphometric indicators revealed that the conditions in which the Beluga broodstock is kept at the fish farm “Selets” should be improved. Using ultrasound scanning, the following stages of gonad maturity were established: stages II, III and IV in males; II, II semi-fatty, II fatty, II-III in females. The beluga broodstock at the fish farm “Selets” is mainly represented by females (the females exceeded the males by 2.3 times in terms of their number).

**Keywords:** aquaculture, Beluga sturgeon, molecular genetics, morphometric characteristics, sex determination.

### INTRODUCTION

The Beluga (*Huso huso* (Linnaeus, 1758)), as well as a number of other species of sturgeon (Acipenseridae), are on the IUCN Red List as critically endangered species. They are

also listed in Appendix II of CITES due to a catastrophic decline in the natural population size (Birstein et al., 1997; Raymakers, 2006). The beluga is also included on numerous regional Red Lists: the Red Data Book of Bulgaria, the Red Books of Russia, Ukraine,



etc. (Red Data Book of the Republic of Bulgaria, 2015). As it is considered, a decline in the wild Beluga number is on the score of illegal fishing, the natural habitat destruction and due to the construction of dams on its spawning migration routes. Last time the beluga was registered on the territory of Belarus was in the River Sozh (Gomel Region) in 1907, but now it is hardly possible, since its migration pathways are blocked by the dams of the Dnieper HPP (hydropower plants) cascade. Therefore, the only way to preserve the gene pool of this valuable species of anadromous sturgeon is to create domesticated beluga broodstock in aquaculture, improve artificial reproduction methods and obtain a sufficient amount of high-quality fish stock.

In order to expand the range of fish products, Beluga sturgeon fry with an average weight of 5 grams was imported in Belarus in 2008 from Rostov Region, Russia. Before 2020, the only replacement broodstock of Beluga sturgeon was formed at the fish farm “Selets” (Brest Region, Belarus), but its species purity and population identity remained unknown by the scientific community.

## MATERIALS AND METHODS

The object of research: 256 twelve-year-old Beluga sturgeons grown in concrete basins in warm-water aquaculture. Genetic study was carried out on 122 Beluga broodstock specimens. DNA was extracted by the phenol-chloroform method from the pectoral fin fragments collected in 2019-2020 and fixed by 96% ethanol (tissue/ethanol proportion up to 1:5 by volume). Tissue collecting was followed by individual specimen chipping depending on their sex and reproductive quality with tapes (for immature specimens and part of males),

and/or electronic tags from Hallprint (Australia). The individual identification number was assigned to each sample and the corresponding mark number was registered.

Several molecular tools were used for the aquaculture broodstock management: 1) a species-specific nuclear tool based on a panel of STR loci (An20, AoxD161, AoxD165, AfuG41, Aox23, Spl106) for beluga identification, species purity verification and affinity analysis (Table 1); 2) species purity (hybrid presence analysis) was tested by 3 methods: 4 STR marker analysis (An20, AoxD161, AoxD165, AfuG41), detection of Beluga-specific SNP in the 2nd intron of the nuclear ribosomal protein S6 (RP2S6) and detection of two species-specific SNP positions for the beluga and sterlet used as specific binding sites for diagnostic primers; 3) sequencing of the 367 bp control region of mitochondrial D-loop for population assignment.

STR marker analysis was conducted with the primers listed in Table 1 (Barmintseva, Mugue, 2013.; Fopp-Bayat, et al., 2015; King, 2001). Fragment analysis was conducted using AppliedBiosystems 3500 GeneticAnalyzer. Allele lengths were identified using GeneMarker 5. The specific statistical parameters (Tables 3, 4) were estimated using specialized macro GenAIEx v.6.5.

For *H. huso* X *A. ruthenus* hybrid presence testing, the method published by Havelka et al., 2017 was used. The multiplex HH/AR positive primer mix contained 153\_uni, 247\_uni, 153\_HHp, 247\_ARp primers and the HH/AR negative primer mix contained 153\_uni, 247\_uni, 153\_HHn, 247\_ARn primers (Havelka et al., 2017). Detection of Beluga-specific SNP in the 2nd intron of the nuclear ribosomal protein S6



(RP2S6) was conducted with RP2S6\_huso\_F and RP2S6\_groupA\_R primers published in Boscari et al., 2017. Amplification of the 367 bp region of mitochondrial D-loop was conducted with the primers LproF and DL651(Mugue et al., 2008.). Thus, all sequences obtained were compared with the already known sequences from the internal database of the Russian Federal Research Institute of Fisheries and Oceanography. Sequence alignment and comparison were conducted using the MEGA X program.

Morphometric measurements in Beluga sturgeon (n=47) were carried out during the spring appraisal period by 5 parameters: body

weight (W, kg), body length L (cm), body length l (cm), body coverage (O, cm), body height (H, cm) (Pravdin, 1966).

Non-invasive ultrasound express examination of sex and stages of gonad maturation in Beluga sturgeon was carried out in frontal and transverse planes (Chebanov & Galich, 2013). Ultrasound examination was carried out using the Draminski iScan ultrasound portable scanner by Dr. Barulin N.V. Technical data: image presentation (projection) mode – B mode, probe frequency and type – electronic linear 7.5 MHz (from 4 to 9 MHz), scanning range from 0 to 15 cm.

**Table 1** Characteristics of 6 microsatellite loci investigated in 122 *Huso huso* specimens from the fish farm “Selets”

Annealing temperature (Tm. °C)	STR locus	Fluorescent label	Sequence, 5' > 3'	Repeat motif	Allele sizes, bp
56	An20	Hexachlorofluorescein (HEX)	F:AATAACAATCATTACATGAGGCT R:TGGTCAGTTGTTTTTTTATTGAT	(ATCT)n (TG)m	145 149 161
56	AoxD161	Fluorescein amidite (FAM)	F:GTTTGAAATGATTGAGAAAATGC R:TGAGACAGACACTCTAGTTAAACAGC	(CTAT)n	98 102
56	AoxD165	Carboxytetramethyl-rhodamine (TAMRA)	F:TTTGACAGCTCCTAAGTGATACC R:AAAGCCCTACAACAAATGTCAC	(CTAT)n CTAC (CTAT)m	178
56	AfuG41	Fluorescein amidite (FAM)	F:TGACTCACAGTAGTATTATTTATG R:TGATGTTTGCTGAGGCTTTTC	(GATA)n TA (GATA)m	229 237 261 269
52	Aox23	Carboxytetramethyl-rhodamine	F:TTGTCCAATAGTTTCCAACGC R:TGTGCTCCTGCTTTTACTGTC	(ATT)n (ACT)m	123 126



		(TAMRA)		(AAT)p	129 135 141
56	Spl106	Fluorescein amidite (FAM)	F:CACGTGGATGCGAGAAATAC R:GGGGAGAAAAGTGGGGTAAA	(TAGA)n ((T/G)AAA)m	219 235 243

## RESULTS AND DISCUSSION

### Testing for hybrid presence

Microsatellite loci were used for individual genotyping. Allelic variants were determined for four STR loci (An20, AoxD161, AoxD165, AfuG41) and compared with the data published by Barmintseva, Muge, 2013. The analysis did not reveal any allelic variants not corresponding to the Beluga

(*H. huso*) species. A combination of occurrence with high frequency of Beluga-specific allelic variants (145 An20, 98 AoxD161, 178 AoxD165, 269 AfuG41) and the absence of allelic variants specific for other species indicates with a high probability the species purity of the studied beluga individuals. Sturgeon species-specific allelic variants and the allelic variants identified in our research are listed in the Table 2.

**Table 2.** 4 Sturgeon species-specific microsatellite loci allele variants according the data published by Barmintseva A.E., Muge N.S. (2013)

Species scientific name (identification code)	Allele size, bp			
	An20	AoxD161	AoxD165	AfuG41
Defined allelic variants	145 149 161	98 102	178	229 237 261 269
<i>Huso huso</i> (HUS)	145-153, 161-165, 173-177	98-106	174, 178, 182	225-277
<i>Huso dauricus</i> (DAU)	149, 165-169	118-126, 134-142	174, 186, 190, 194, 198, 202, 206, 210, 216	-
<i>Acipenser schrenckii</i> (SCH)	137, 149-153, 161, 169-173	106-142	164, 172, 176, 180, 184, 188, 192, 196, 200, 204	185-213

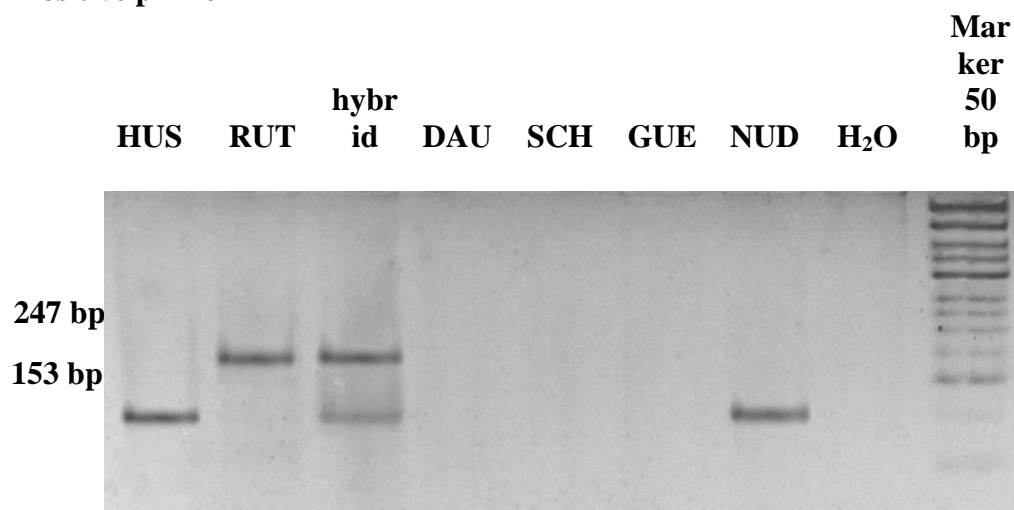


<i>Acipenser-gueldenstaedtii</i> (GUE)	137, 145-181	102-138	174-178, 182-198, 202	173, 193-249
<i>Acipenser medirostris</i> (MED)	161, 169, 181-197	114-126	182, 186-190, 198, 210, 230-254	193-205, 213-217, 225
<i>Acipenser-baerii</i> (BAE)	145-173, 185	102-134, 146	176-180, 184, 188, 192, 196, 200,204, 208	197, 205-249
<i>Acipenser stellatus</i> (STE)	129-145, 161, 169, 177-181	114-142	148, 152, 168-204, 208	193-233
<i>Acipenser ruthenus</i> (RUT)	149-181	102-138	164-168, 172, 176, 180-188, 192, 196	197-253
<i>Acipenser-nudiventris</i> (NUD)	149-153, 161-165	114-142	168, 180, 184, 196, 200, 204, 212	201, 213, 229-253
<i>Acipenser persicus</i> (PER)	161-181	102-142	156, 174-190, 194, 198, 202	173, 193-221, 229-241

Crossing *Huso huso* x *Acipenser ruthenus* is very common in fish farming for obtaining highly productive hybrids. Therefore, the broodstock was examined for cc hybrids with *A. ruthenus* using the method specially

developed for this purpose (the method published by Havelka M. et al, 2017). This method also allows distinguishing *H. huso* and *A. ruthenus* from some other sturgeon species (Fig. 2).

#### Positive primer mix





### Negative primer mix



**Fig. 1.** Electrophoresis of PCR product samples of sturgeon species with positive and negative primer mixes. Abbreviations: HUS – *Huso huso*; RUT – *Acipenser ruthenus*; hybrid – *Huso huso* x *Acipenser ruthenus* hybrid ; DAU – *Huso dauricus*; SCH – *Acipenser schrenckii*; GUE – *Acipenser gueldenstaedtii*; NUD – *Acipenser nudiventris*

When using the positive primer mix, the DNA of non-hybrid *H. huso* individuals is amplified resulting in a fragment of 153 bp, and when using the negative primer mix, in a fragment of 247 bp.. On the contrary, *A. ruthenus* samples give a 247 bp fragment using the positive primer mix and a 153 bp fragment using the negative primer mix. The samples of hybrid (*Huso huso* x *Acipenser ruthenus*) specimens produce two products using both primer mixes. Obtaining two PCR products using the negative primer mix and no products using the positive primer mix is a sign of other sturgeon species (e.g. *H. dauricus*, *A. schrenckii*, *A. gueldenstaedtii*, see Fig. 1). It should be noted that this method does not allow distinguishing *H. huso* and *A. nudiventris* (NUD on Fig. 1).

The third method for the species verification was developed specifically for the Beluga sturgeon by Boscari et al. It is based on the detection of Beluga-specific SNP in the 2nd intron of the nuclear ribosomal protein S6 (RP2S6). PCR was carried out using the RP2S6\_huso\_F and RP2S6\_groupA\_R primer (Boscari et al., 2017).

#### mtDNA analysis

In order to establish the population identity, we sequenced the 367 bp region of mitochondrial D-loop using LproF and DL651 primers (Mugue et al., 2008.). In total, we studied 36 Beluga individuals. The sequences were equal and matched haplotype 3 according to the internal database of the Russian Federal Research Institute of Fisheries and Oceanography. This haplotype corresponds to



the Caspian population of Beluga sturgeon. The obtained sequences also correspond to the sequence (*Huso huso caspicus* voucher nC12 D-loop, partial sequence) published in the GenBank AY846650.1 (Doukakis, 2005).

**Population structure identification**

Six STR loci (An20, AoxD161, AoxD165, AfuG41, Aox23, Spl106) were analyzed from the chipped Beluga breeders (n=122) kept in warm water (Table 3).

Five out of six alleles in the studied Beluga individuals (n = 122) were polymorphic. It should be noted that alleles 229 (AfuG41) and 129 (Aox23) are registered only once in the sample analyzed in the same female specimen. Hardy-Weinberg expectations for genotype frequencies are shown in Table 4.

**Table 3.** Frequencies of 6 microsatellite loci allele variants in the *Huso huso* broodstock of the fish farm “Selets”

Allele size, bp	Allele frequency
An20	
145	0,4918
149	0,2664
161	0,2418

AoxD161	
98	0,74
102	0,26
AoxD165	
178	1
AfuG41	
229	0,0041
237	0,4754
261	0,2049
269	0,3156
Aox23	
123	0,2377
126	0,2787
129	0,0041
135	0,2746
141	0,2049
Spl106	
219	0,2541
235	0,4918
243	0,2541

**Table 4.** Summary of Chi-Square Tests ( $\chi^2$ ) for Hardy-Weinberg Equilibrium

Locus	DF	$\chi^2$	Prob	Significance
An20	3	35,184	0,000	P<0.001
AoxD161	1	15,423	0,000	P<0.001
AoxD165	Monomorphic			
AfuG41	6	36,666	0,000	P<0.001





Aox23	10	116,266	0,000	P<0.001
Spl106	3	30,534	0,000	P<0.001

Abbreviations: *DF*, the degrees of freedom =  $[Na(Na-1)]/2$ ; *Prob*, probability of the observed numbers deviating as far from the expected numbers by chance

It was revealed a significant difference between the observed and expected according to Hardy-Weinberg model number of allele variance. Considering the fact that the main variety of allelic variants in the analyzed group

is limited to 1-4, it is probable that most of individuals are the offsprings of the same broodstock pair. The statistical parameters for population structure analysis shown in the table 5.

**Table 5.** Comparison of population genetic traits of Beluga broodstock from the fish farm “Selets” and the genetic diversity of domesticated Beluga sturgeon

Sample	Beluga sturgeon from the fish farm “Selets”, the Republic of Belarus				Domesticated Beluga sturgeon, the Russian Federation			
	An20	AoxD161	AoxD165	AfuG41	An20	AoxD161	AoxD165	AfuG41
Na	3	2	1	4	12	5	8	14
Ne	2,693	1,631	1,000	2,720	4,036	2,008	2,664	9,640
I	1,045	0,575	0,000	1,065	1,950	1,005	1,303	2,433
Ho	0,746	0,525	0,000	0,795	0,577	0,327	0,442	0,788
He	0,629	0,387	0,000	0,632	0,752	0,502	0,625	0,896
uHe	0,631	0,389	0,000	0,635	0,760	0,507	0,760	0,905
F	-0,186	-0,356	-	-0,257	0,233	0,349	0,292	0,120

Abbreviations: *Na* = No. of Different Alleles; *Ne* = No. of Effective Alleles =  $1 / (\sum pi^2)$ ; *I* = Shannon's Information Index =  $-1 * \sum (pi * \ln(pi))$ ; *Ho* = Observed Heterozygosity = No. of Hets / *N*; *He* = Expected Heterozygosity =  $1 - \sum pi^2$ ; *uHe* = Unbiased Expected Heterozygosity =  $(2N / (2N-1)) * He$ ; *F* = Fixation Index =  $(He - Ho) / He = 1 - (Ho / He)$ .

Beluga sturgeon from the fish farm “Selets” was compared with the domesticated Caspian Beluga sturgeon population from a number of fish farms (the Russian Federation). The conducted analyses showed a lack of the

gene pool diversity of Beluga sturgeon broodstock from the fish farm “Selets”. A number of identified allelic variants (*Na*) at the fish farm “Selets”/domesticated Beluga sturgeon are as follows: An20 3/12, AoxD161

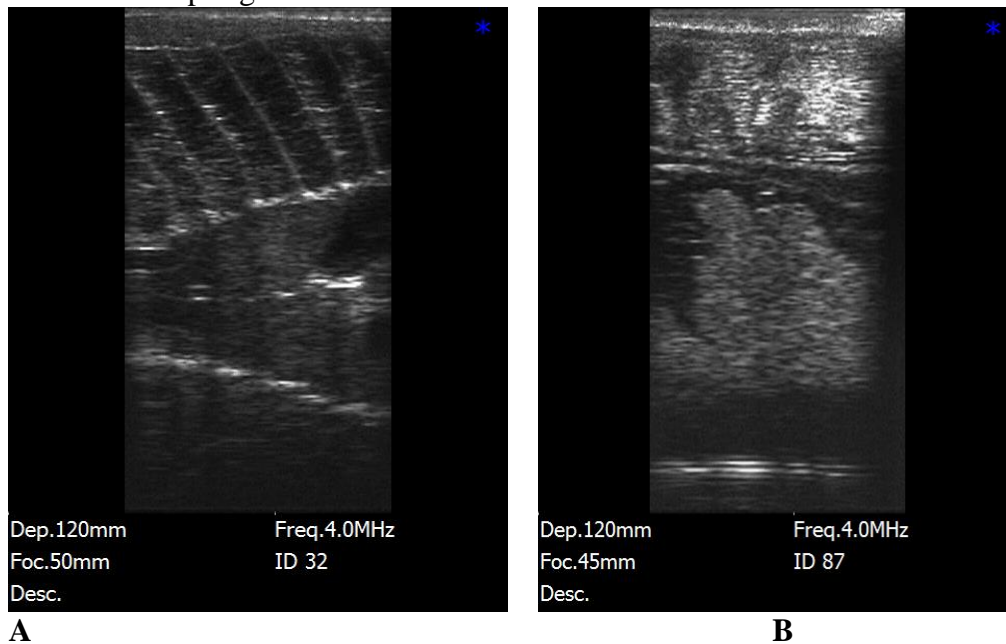


2/5, AoxD165 1/8, AfuG41 4/14. It should be noted that at the fish farm “Selets” Beluga sturgeon showed a negative sign of the fixation index in contrast with a positive index for the domesticated Beluga population. It indicates the genetic balance displacement towards the heterozygote excess in the population of the fish farm “Selets” and shows the unequal allele distribution

in the population, which may be a result of the specimens’ close relation. The intrapopulation breeding of these specimens would lead to a decrease in heterozygosity that increases the probability of recessive genetic abnormalities in their offspring.

### Morphometric analysis and sexing

The morphometric analysis of Beluga sturgeon (n=47) from the broodstock from the fish farm “Selets” revealed their unsatisfactory physiological state after winter and during the feeding period. Comparatively high variability (Cv) of the body mass (18,3%), Fulton’s condition factor (27,2%) and external traits may be a result of unfavorable feeding conditions, since genetic evidence shows a low allelic variation in the Beluga sturgeon broodstock.



**Fig. 2.** Ultrasound images of frontal sections of Beluga sturgeon male (A) and female (B) at the maturity stage II

Using ultrasound scanning, we found that the broodstock of Beluga sturgeon at the fish farm “Selets” was represented by 69.5% of females (178 pcs. ♀) and 30.5% of males (78

pcs. ♂). In our research, we observed the following stages of gonadal maturity: stages II, III and IV for males; stages II, II semi-fatty, II fatty, II-III for females. Starting from the



maturity stage II, testicular tissue was easily visible in frontal and transverse sections (Fig.2). The testicular part was hyperechoic and had distinct margins. The fat part was underdeveloped or poorly developed from the medial side and practically not visible. The margins of the gonad were smoothly curved, while a bright hyperechoic tunic of testis was clearly seen.

At maturity stage III, the echogenicity of testicular tissue increases significantly. The testes appeared on echograms as a homogeneous structure of a light grey (in some cases white) colour with distinct hyperechoic margins. In some cases, two clear hyperechoic lines, gonad margins and peritoneal lining, were well discernable. On the echogram, the testes at stage IV appeared as a bright, hyperechoic, fine-grained homogeneous structure with clear margins and well-defined tunics. Hyperechogenicity of the testis reached its maximum at stage IV. The ripe male maturity status and readiness to spawn were assessed by the brightness of the testis image. On the ultrasound image, the ovarian tissue (stage II) appeared as a grainy “cloud-like” structure of mixed echogenicity with uneven boundaries without a tunic. The fatty portion of the ovary was slight and was visualized in the shape of darker areas as distinct from the lighter ovarian tissue. At maturity stage II semi-fatty, on the ultrasound image, single ovigerous lamellae appeared as areas of higher echogenicity (of a grey or light-grey colour) alternating with hypoechoic (dark) fat regions. Thus ovigerous lamellae “grew” from the lateral to the medial part of the gonad. At maturity stage II fatty, in contrast to previous stages, the proportion of the ovarian and fat tissue visible on echograms was different. The ovarian tissue was surrounded by fat both from

the medial and lateral sides (dark, anechoic regions). The dark anechoic fat layer was well discernable between the muscles and the gonads. At maturity stage II-III, an ovary seen in the ultrasound image showed moderate echogenicity (grey or light grey). Ovigerous lamellae “penetrated” the body of the gonad and appeared as a brachiate vertical structure (“coral-like” or “fringed” in shape) of higher echogenicity, spreading to the dark hypoechoic region (the fat tissue).

## CONCLUSIONS

1. The purity of the species in the Beluga (*Huso huso* L.) broodstock at the fish farm “Selets” (Brest region, the Republic of Belarus) was established.

2. The absence of interspecific hybrids and species purity in the studied Beluga sturgeon group was confirmed by three molecular genetic methods mentioned in Materials and Methods. It is important that the markers we used allow the accurate identification of bester, a hybrid that is most commercially exploited.

3. The sequence of 367 bp in the mitochondrial D-loop region was identified as haplotype 3 that corresponds to the Caspian population of Beluga sturgeon. The population structure analyses showed a lack of the gene pool diversity of Beluga sturgeon broodstock from the fish farm “Selets”.

4. The resulting diversity of microsatellite locus length may indicate a close affinity of the analyzed sturgeon individuals. The comparative analysis of the Beluga sturgeon gene pool of the fish farm “Selets” and the gene pools of domesticated Beluga sturgeons of a number of sturgeon fish farms in the Russian Federation showed that the Beluga



sturgeon gene pool in the Belarusian farm is poorer than in the domesticated Beluga sturgeons from Russian sturgeon fish farms.

5. The coefficients of variation of morphometric parameters and Fulton's condition factor indicate that the conditions in which the Beluga sturgeon broodstock is kept at the fish farm "Selets" should be improved.

6. The following stages of gonad maturity were established using the ultrasound scanning of Beluga sturgeons at the fish farm "Selets": stages II, III and IV in males; II, II semi-fatty, II fatty, II-III in females.

7. The Beluga sturgeon broodstock at the fish farm "Selets" is mainly represented by females exceeding males by 2.3 times in their number.

8. A number of Beluga males (*Huso huso* L.) can be used for hybridization with the Sterlet sturgeon and the Siberian sturgeon.

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