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Physiological and immunological characteristics of red spot disease-resistant hybrid carp named "Surskiy malokostny"

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Abstract: The industrial fish farming is followed by lower immune status of cultured specimens in connection with high stocking density, handling and other technological factors. Specific immune-modulating substances or selection for higher immune status could be used for solving of this problem. The most dangerous multifactorial infection in commercial carp farms is red spot disease (RSD) caused by *Pseudomonas*, *Aeromonas*, and Spring Viremia of Carp Virus *Rhabdovirus carpio*. The authors had obtained a new commercial mirror hybrid carp "Surskiy Malokostny" (with lower number of bones) characterized by high rates of weight growth, survival and immune resistance. The application for selection achievement for this cross had No. 77897. The high immune resistance of new hybrid is connected with characteristics of one of parent lines, Angeline mirror carp exposed to long-term selection after outbreak of RSD more than 60 years ago. The leucogram of new hybrid was characterized by high levels of myeloid segmental cells suggested full development of inborn cell immunity. The lower level of lysosomal cation protein in neutrophils of new hybrid before winter comparing with control full-scaled carp of commercial line is the feature of high immune resistance.

Keywords: hybrid carp, immune resistant, red spot disease

Introduction

The survivability of cultured animals and especially different species of fishes greatly depends on status of immune system. That's the reason why strengthening of immune resistance of cultured fish is an important and relevant task.

Boosting the immune system of animals can be achieved by means of applying immune stimulants or immune modulators and preventive vaccination. A possible alternative to the struggle with outbreaks of diseases is a genetic improvement of natural lines through selection of disease resistant lines (Gjedrem, 2012). The farmers have a certain progress in artificial selection focused on immune resistance to certain diseases. Some disease-resistant lines of cattle, pigs, rabbits, and birds have been produced. A perspective aim is using complementary or antisense genes for inhibiting virus infections (Freeman and Bumstead, 1987).

In contrast to higher vertebrates, fish are free-living organisms from early embryonic stages of life and depend on their innate immune system for survival (Rombout *et al*., 2005). Nonspecific immunity is a fundamental defence mechanism in fish. In addition, it plays a key role in the acquired immune response and homeostasis through a system of receptor proteins. These receptor proteins identify molecular patterns that are typical of pathogenic microorganisms, including polysaccharides, lipopolysaccharide (LPS), peptidoglycan bacterial DNA, viral RNA and other molecules that are not normally on the surface of multicellular organisms. This response is divided into physical barriers and cellular and humoral immune response. These inmunological parameters include growth inhibitors, lytic enzymes, the classic complement pathways, the alternative and lectin pathway, agglutinins and precipitins (opsonins and primary lectins), antibodies, cytokines, chemokines and antibacterial peptides. Various internal and external factors can influence innate immune response parameters. Temperature changes, stress management and density may have suppressive effects on this type of response, while several food additives and immunostimulants can enhance their efficiency (Magnadottir, 2010; Uribe *et al*., 2011).

In fish, the innate response has been considered an essential component in combating pathogens due to limitations of the adaptive immune system, their poikilothermic nature, their limited repertoire of antibodies and the slow proliferation, maturation and memory of their lymphocytes (Whyte, 2007).

Recent research has revealed that neutrophils are

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heterogeneous in phenotype and function and can display outstanding plasticity in both homeostatic and disease states. The great flexibility and elasticity arm neutrophils with important regulatory and controlling functions in various disease states such as autoimmunity and inflammation and other neutrophils possess the capacity of both intra- and extracellular mechanisms to eliminate pathogens (Borregaard *et al*., 2010; Summers *et al*., 2010).

A few works are dedicated to immune resistance of aquatic organisms on the base of selection and genetic programs but fish farmers have some disease-resistant strains of different species. The great progress in development of disease-resistant lines was achieved in salmonid fishes (Yáñez *et al*., 2014; Vallejo at al., 2014).

Red spot disease (RSD) is multifactorial infection caused by *Pseudomonas*, *Aeromonas*, and Spring Viremia of Carp Virus *Rhabdovirus carpio*. From all available lines of common carp *Cyprinus carpio* L, only Angeline full-scaled carp and Angeline mirror carp were exposed to a long-term selection for resistance to RSD after outbreak of infection 60 years ago. 3 generations of fishes were infected and only survived RSD-resistant specimens were selected for reproduction. The advantage of new selected strain comparing with control ones amounted up to 60-70% at bacterial contamination and up to 30-35% at virus contamination. The higher immune resistance of the Angeline lines was proved in field tests at fish farms in Krasnodar Region of Russian Federation (Ilyasov, 2002).

We investigate the physiological and immunological characteristics of juveniles of RSD-resistant Angeline carp. The most significant differences have been noticed in yearlings of this strain: the delay in vernal activation of erythropoiesis, lower percentage of neutrophils and higher percentage of monocytes in leucogram. The juveniles of Angeline strain are characterized by moderately low levels of mean cytochemical coefficient (MCC) of cationic lysosomal neutrophil protein (oxygen-independent mechanisms of phagocytosis) and high level of oxygen-dependent factors detectable in a spontaneous Nitro Blue-Tetrazolium Test (NBT-test). The Angeline strain specimens of different ages are characterized by high activity of aspartate aminotransferase (AST) being an indicator of intensive protein metabolism (Pronina, 2017).

The adult specimens of RSD-resistant strain, as contrasted with the RSD-susceptible fishes, demonstrate higher levels of protein and carbohydrate metabolism, as judged by activity of the enzymes (AST, creatine kinase) and levels of albumin, lactate and urea. The Angeline strain carps have a more intense hematopoiesis. The leucograms of these fishes contain a larger share of neutrophils, especially mature segmental ones (Pronina, 2017).

We have obtained an inter-strain hybrid of productive Anish mirror carps (females) and RSD-resistant Angeline strain mirror carps (males); the cross has been named as the "Surskiy Malokostny" (with lower number of bones). The application for selection achievement for this cross had No. 77897. This hybrid can paternally inherit immune resistance. The embryonal survival of this hybrid is high.

The aim of this research is a comparative assessment of presumably RSD-resistant ("Surskiy Malokostny") and RSD-non-resistant carp hybrids on biological, physiological and immunological parameters.

Materials and Methods

The experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, ETS NO123, Strasbourg, 1986. The experimental protocol was approved by the Ethics Committee of the Federal State-Funded Scientific Institution All-Russian Research Institute of Irrigation Fish-Breeding.

The investigations were conducted in a fish-breeding facility "Kirya", Chuvash Republic, Russian Federation. The objects of research were "Surskiy Malokostny" hybrid and "Pikhtovskiy" carp received as result of cross-breeding full-scaled carp with mirror carp in the "Pikhtovskiy" facility.

Comparative assessment of both lines was performed through valuation of hematological, biochemical and cytochemical indices. 10 specimens in each group were used. The proportions of the different types of white cells were measured in Pappenheim-stained peripheral blood smears. The level of hematopoiesis was defined via calculation of immature forms of erythrocytes and leukocytes.

The biochemical analysis of serum was performed on Chem Well Awareness Technology device with the application of VITAL reagents. The phagocytic activity of neutrophils was defined according to the principle of Astaldi and Verga (1957) by cytochemical method

implying the usage of bromophenol blue, which was adapted for aquatic organisms by Pronina (2017). The content of nonenzymatic cationic protein in the neutrophils' lysosomes of peripheral blood was determined. According to the degree of phagocytic activity, the examined cells were divided into 4 groups:

0 degree – cationic protein granules are absent,
1st degree – few cationic protein granules,
2nd degree – cationic protein granules occupy approx. 1/3 of the cytoplasm,
3rd degree – cationic protein granules occupy 1/2 (and more) of the cytoplasm.

The Mean Cytochemical Coefficient (MCC) was calculated with the formula:
$MCC = (0 \times N_0 + 1 \times N_1 + 2 \times N_2 + 3 \times N_3)/100$

Where N_0, N_1, N_2, N_3 are numbers of neutrophils with 0, 1, 2 and 3 balls activity;
$N_0 + N_1 + N_2 + N_3 = 100$.

The mathematical treatment of data was performed by statistical methods according to Student's test with the application of such software as Excel from the Microsoft Office package. The differences were considered significant at $P < 0.05$.

Results and Discussion

In terms of length and weight indices, the juveniles of the "Surskiy Malokostny" hybrid exceeded the control "Pikhtovskiy" cross. Erythropoiesis of the "Surskiy Malocostny" cross is less intensive in comparison with "Pikhtovskiy" carp. We noted that the lowering of erythropoiesis of former at late autumn is typical for RSD-resistant fishes, according to the low percentages of erythroid blastic forms. The leukopoiesis was more intense in "Surskiy Malokostny" cross validating the earlier data on RSD-resistant fishes during fall period. The share of segmental neutrophils (a macrophage reserve) in "Surskiy Malokosny" cross is statistically higher comparing with "Pikhtovskiy" line due to lymphocytes. The "Surskiy Malokostny" cross demonstrated a higher content of lysosomal cationic protein in neutrophils, which signifies a high potential of phagocytic. At the same time, this index did not exceed the normal values. In the juveniles of "Pikhtovskiy" carp, MCC of the cationic protein was optimal for the fall period (Tab. 1).

Table 1. Comparative hematological and cytochemical indices of juveniles of two carp crosses (SMPC: The sum of mature and polychromatophilic cells; * P<0.05).

Indices	"Surskiy Malokostny"	"Pikhtovskiy"
Dimensional and weight indices		
Body mass (gr)	48.5±1.4	27.9±1.4*
Body length (cm)	12.7±0.2	9.9±0.8*
Erythropoiesis (%)		
Hemocytoblasts, erythroblasts	0.1±0.1	0.8±0.2*
Normoblasts	3.4±0.5	2.5±0.4
Basophilic erythrocytes	12.6±1.6	10.9±2.2
SMPC	83.9±2.1	77.3±2.7
Differential blood count (%)		
Myeloblasts	-	0.1±0.1
Promyelocytes	0.1±0.1	0.2±0.1
Myelocytes	3.3±0.6	2.2±0.2
Metamyelocytes	2.8±0.5	1.7±0.2
Banded neutrophils	1.5±0.5	1.4±0.3
Segmental neutrophils	2.5±0.5	0.9±0.3*
Total neutrophils	4.0±0.4	2.3±0.5*
Eosinophils	0.3±0.1	0.2±0.2
Basophiles	-	0.2±0.2
Monocytes	2.0±0.4	2.6±0.5
Lymphocytes	87.5±1.1	90.5±0.7*
Phagocytic activity		
MCC (units)	1.87±0.04	1.74±0.02*

The second-year fishes also differed in body mass and length; thus the "Surskiy Malokostny" hybrid significantly exceeded the "Pikhtovskiy" cross in these indices. The juveniles and second-year fishes of "Surskiy Malokostny" cross demonstrated a high share of mature neutrophils in the differential blood leucogram, comparing with "Pikhtovskiy" hybrid. MCC of the "Pikhtovskiy" second-year fishes was at almost

highest level of physiological limit being statistically higher than in "Surskiy Malokostny" cross. Earlier, the following peculiarity of RSD-resistant carps was detected: relatively low values of cationic lysosomal neutrophil protein in peripheral blood. This observation suggested that RSD-resistant carps spend cationic protein before wintering as a part of their immune protection (Tab. 2).

Table 2. Mean (SD) of morphometric, hematological and cytochemical indices of second-year carp crosses (SMPC: The sum of mature and polychromatophilic cells; * P<0.05).

Indices	"Surskiy Malokostny"	"Pikhtovskiy"
Dimensional and weight indices		
Body mass, g	686±45	196±26*
Body length, cm	27.7±0.7	19.0±0.9*
Erythropoiesis (%)		
Hemocytoblasts, erythroblasts	0.2±0.1	0.6±0.2
Normoblasts	1.7±0.5	3.2±1.1
Basophilic erythrocytes	5.6±1.4	6.4±1.6
SMPC	92.5±1.7	89.8±2.3
Differential blood count (%)		
Myeloblasts	0.2±0.1	0.6±0.2
Promyelocytes	0.3±0.2	0.4±0.4
Myelocytes	0.5±0.3	0.8±0.4
Metamyelocytes	1.2±0.4	2.2±0.8
Banded neutrophils	0.8±0.3	0.4±0.3
Segmental neutrophils	2.3±0.3	1.2±0.3*
Total neutrophils	3.1±0.5	1.6±0.3*
Eosinophils	-	-
Basophiles	-	-
Monocytes	2.2±0.6	2.6±0.5
Lymphocytes	92.5±1.3	91.8±0.6
Phagocytic activity		
MCC (units)	1.63±0.03	1.93±0.05*

The juveniles of the "Surskiy Malokostny" cross demonstrated statistically higher values of activity of such serum enzymes as AST and pancreatic amylase, in comparison with "Pikhtovskiy" cross, both parameters characterize the intensity of protein and carbohydrate metabolism, respectively (Tab. 3).

The similar tendency related to the values of protein metabolism can be observed in second-year fishes. Thus, the carps from the "Surskiy Malokostny" cross demonstrate statistically higher levels of transaminases and total protein (Tab. 4).

Tab. 3: Mean ±SD of biochemical indices of carp fingerlings (* P<0.05).

Indices	"Surskiy Malokostny"	"Pikhtovskiy"
ALT (u/l)	37.3±7.8	38.8±12.8
AST (u/l)	210±19	154±16*
Glucose (mmol/L)	5.2±0.5	5.6±0.6
CK (u/l)	2489±355	1906±577
Creatinine (mcmol/l)	17.5±4.8	27.4±6.8
LDH (u/l)	1505±238	1587±300
Lactate (mg/dL)	113.2±4.9	119.5±12.2
ALP (u/l)	54±10	74±19
Albumin (g/dl)	11.2±1.0	12.9±0.6
Amylase (u/l)	33.1±13.3	10.6±4.4
Urea (mg/dL)	44.4±15.5	66.7±10.2
Total protein (g/dl)	21.4±3.2	29.8±4.3
Pancreatic amylase (u/l)	27.6±5.8	8.8±4.3*
Triglycerides (mg/dL)	126±27	147±13
Cholesterol (mg/dL)	112±14	153±22

Tab. 4: Mean ±SD of biochemical indices of second-year fishes (* P<0.05).

Indices	"Surskiy little bone" cross	"Pikhtovskiy" carp
ALT (u/l)	28.1±3.1	19.7±1.6*
AST (u/l)	226±18	129±28*
Glucose (mmol/L)	5.2±0.4	5.4±0.8
CK (u/l)	5119±336	6151±141*
LDH (u/l)	1451±204	1313±206
Albumin (g/dl)	11.5±0.6	9.9±0.4
Amylase (u/l)	98.4±25.2	84.0±14.1
Urea (mg/dL)	2.9±1.1	4.0±0.8
Total protein (g/l)	32.8±2.3	25.3±1.8*
Lipase (u/l)	23.4±0.9	23.2±1.2
Triglycerides (mg/dL)	167±7	168±8
Cholesterol (mg/dL)	169±16	189±25
Total bilirubin (mg/l)	0.8±0.2	0.4±0.2
Direct, bilirubin (mg/l)	2.5±0.6	1.2±0.3

The activity of creatine kinase in the "Surskiy Malokostny" cross is lower in comparison with "Pikhtovskiy" one. The "Pikhtovskiy" cross demonstrates high values of this index that may be explained by different factors including cell damage. It is known that the enzyme can catalyze the creation of creatine phosphate from creatinine and ATP (adenosine triphosphoric acid), which is consumed by an organism at increased physical loads.

The new commercial carp hybrid "Surskiy Malokostny" has been introduced at the fish-breeding farm "Kirya" LLC. The cross is characterized by scattered (mirror) scaliness and high rates of weight growth, survivability and immune resistance, which makes it valuable for a commercial breeding and use in selection.

The juveniles of "Surskiy Malokostny" cross demonstrated the high level of leukopoiesis, mature segmental forms of neutrophils and high content of lysosomal cationic protein. The second-year fishes of this cross are characterized by a high share of mature neutrophils in differential blood count. All specimens of this line are characterized by the high level of protein metabolism in terms of transaminase activity and the content of the total protein. The content of lysosomal cationic protein in the microphages of "Surskiy Malokostny" cross is significantly lower than in control RDD-nonresistant "Pikhtovskiy" cross reflecting the boosting of immune defense.

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