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Research paper

Immunoferment, Cytomorphological, Cytochemical Parameters of Fish as Ecological Indicators of Resistance to Helminthes, Passed by Fish-Eating Birds

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Abstract

A search for indicators reflecting resistance of fish to helminth infections passed by fish-eating birds has been carried out. A decrease in the concentration of lysozyme by 13.5 % and an increase in hemagglutinin by 7.7 % for the bighead carp (*Hypophthalmichthys nobilis*), 19.9 % and 7.4 % correspondingly for scaly carp (*Cyprinus carpio*) and 18.6 % and 11.4 %, respectively for white amur (*Ctenopharyngodon idela*) identify the fish at risk for the incidence of helminths of *Trematoda* class (*Posthodiplostomum brevicaudatum* and *Diplostomum spathaceum*) and *Cestoidea* class (*Pseudophyllidea*). The diffuse-gel method revealed that the dynamics of lysozyme marks resistance of fish to invasions. A decrease in lysozyme indices in the kidney of a bighead carp by 30.1 %, that of a scaly carp by 10.9 %, and that of a grass carp by 6.6 % is an indicator of a decrease in the resistance of the fish to helminths. The positive pool of polysaccharide granulation in the cytoplasm of fish lymphocytes detected by periodic acid Schiff reaction attributes them to the risk group for helminth infections passed by fish-eating birds. A dangerous tendency to decrease resistance to invasions is precipitation of large glycogen granules in lymphocytes of silver carp (up to 2.7 %), scaly carp (up to 2.4 %) and white amur (up to 2.9 %), as well as small polysaccharide inclusions up to 24 %, 54 % and 43 % respectively. Among cytomorphologic markers of fish resistance to helminth infections, an increase (2-4 times) in the number of lymphocytes with caudate nuclei, micronuclei, and chromosome aberrations of the dicentric type is benchmark.

Keywords: *resistance to helminthiasis, fish-eating birds, scaly carp, silver carp, white amur*.

1.Introduction

Analyzing the current state and prospects for the development of commercial fish farming [11], scientists actualize the use of indication techniques using aquaculture representatives as test objects [12, 13, 14].

The study of the parasitic fauna of freshwater fish associated with the multifactor contamination of the aquatic environment can be considered as one of the most important research directions that forms the scientific basis for the ecological optimization of nature management [1,2,17]. The significance of the research of the invasion diversity of fish is indicated by N.A. Golovina, N.N. Romanov, P.P. Golovin, A.A. Listopadov, O.V. Sekhin [8, 9, 18, 19], T.A. Platonov, N.V. Kuzmina, A.N. Nyukkanov [17], G.N. Dorovskikh [10], A.I. Novak [16], Soodeh Shokrolahi, Seyed Mehdi Hosseinifard, Mohammad Raza Youssefi, Mina Sadough [22], Mehdipour M, Barzegar M, Jalali [23].

For fish used in aquaculture, a serious problem is helminth infections, passed by fish-eating birds such as herons, gulls and cormorants. The body's immune response, aimed at combating parasites, deprives the fish of physiological resources for growth and development for some time, which adversely affects the economic performance of fish farms. Hence, when breeding with cyprinids, it is necessary to take into account the bio testing indicators for resistance to helminthiasis.

Both individuals of the replacement stock and representatives of the breeding core are exposed to the disease. The basis for the development of a method for regulating the resistance of carp fish to invasive diseases passed by fish-eating birds can be the search for markers for bio testing their resistance. They will allow to judge the level of preparation of the immune system of fish and can be used to assess changes in its general state during natural and fish-breeding effects [20]. Based on the studies, the marker indicator reflecting the susceptibility (resistance) of carp fish to helminthic infections is the enzyme immunoassay status that determines the development of their immunity.

In addition, for bio testing the predisposition to helminthic infections of carp, silver carp and white amur fingerlings, it is effective to use the cytochemical and cytomorphologic parameters of these fish's lymphocytes. Moreover, all these markers, in the event of a disease, in carp, silver carp and white amur have identical dynamics.

The purpose of the research was to justify the use of bioecological research methods in commercial fish farming. The objectives of the research included the search for immune, cytomorphologic and cytochemical indicators of fish's bio testing, reflecting resistance to helminthic infections, which are passed by fish-eating birds.

2.Materials and methods

The studies were carried out in spring and summer of 2015–2018 (March – August) and cytomorphologic analyzes of lymphocytes

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took place during the 2nd – 3rd quarters of the year in the laboratory of veterinary, sanitary and ecological expertise of the department of veterinary medicine and biotechnology at Ryazan State Agrotechnological University.

Silver carp (*Hypophthalmichthys nobilis*), scaly carp (*Cyprinus carpio*) and white amur (*Ctenopharyngodon idella*) were analyzed for resistance to helminthiasis. The method of ichthyoparasitologic examination determined the species composition of parasites [8].

Parameters of hemagglutinin and lysozyme in kidneys of silver carp (group 1), scaly carp (group 2) and white carp (group 3) were taken as markers for bio testing the fingerlings' resistance to helminthiasis. Each group had 50 fish. To obtain control values, analogue groups were created (25 individuals each), which were placed separately from infected fish.

Parameters of enzymatic immune assay for hemagglutinin and lysozyme in kidneys of fingerlings from the control group were taken as the reference values for healthy fish for the studied invasion.

Diffuse-gel enzymatic immune assay and serial dilution method (titration) to determine hemagglutinin and lysozyme were performed according to the methodological guidelines of A.A. Vikhman, L.P. Generalova and O.V. Sitnova [3-7,], MU 3.3.2.1758-03 [21]. The object of the research was the kidneys of fish.

To identify cytochemical (periodic acid Schiff reaction) and cytomorphologic parameters ("caudate nuclei", "bridge nuclei" and "micronuclei") [15] used for bio testing of fish for resistance to invasion, the studies were performed in the same experimental groups as for enzymatic immune assay. Blood smears of experimental fish were fixed no later than a day after preparation, and stained no later than 2-3 weeks. Absolute ethyl alcohol was used for fixation (15-20 min). The blood smears were removed with tweezers and one by one placed in a vertical position on a sheet of filter paper. The fixing liquid was poured into a jar with a ground-in stopper. Fixed smears were dried in a thermostat at 37° C, since during slow drying moisture is attracted, which adversely affects the subsequent color.

Smears for cytomorphologic studies of fish lymphocytes were stained according to Romanovsky-Giems method. The solution was prepared using factory liquid paint. The working solution was prepared at the rate of two drops of paint per 1 ml of distilled water (neutral acidity).

Preparations (previously moistened in distilled water) were stained in special hematological baths. The color time was 20 minutes, then monitored under a small microscope magnification. A smear well washed in distilled water was dried in a vertical position for 3 days. Then it was immersed in 96 % ethanol for several seconds to better differentiate the nucleus.

To determine the frequency of occurrence of nuclei anomalies, lymphocytes with caudate nuclei, bridge nuclei and micronuclei, samples of 500 lymphocytes were examined. To determine the frequency of occurrence of lymphocytes with abnormal nuclei, 3 preparations from each fish were used. Small lymphocytes were not counted, as well as lymphocytes located on the periphery of the smears. These anomalies were recorded in the event that the lymphocyte had no signs of degeneration and damage, and its cytoplasm was evenly rounded.

3.Results

Analyzing resistance of fish fingerlings to helminthiasis, it should be noted that in the experiment scaly carp (2nd group) had high resistance and representatives of the 3rd experimental group (white amur) were in second place for resistance to the analyzed invasions. Representatives of the 1st experimental group (silver carp) showed low resistance to the disease.

When comparing enzymatic immune parameters of fingerlings who got helminthic infections, and their counterparts, which remained resistant to the parasite, the hypothesis was confirmed that hemagglutinin and lysozyme from the kidneys of fish are markers for their bio testing for resistance to the diseases studied.

When using the method of serial dilutions (titration), it was found that a decrease in lysozyme concentration by 13.5 % and an increase in hemagglutinin by 7.7 % compared with the same parameters of fish from the control group attribute the silver carp fingerlings to the risk group for helminthic infections passed by fish-eating birds. The trend is maintained for the remaining experimental groups of fish (Table 1).

Table 1 The concentration of hemagglutinins and lysozyme in kidneys of fish, reflecting resistance to helminthiasis passed by fish-eating birds

Parameters, units	Experimental groups								
	1 (silver carp)			2 (scaly carp)			3 (white amur)		
	resistance to helminthiasis								
	control	liable	resistant	control	liable	resistant	control	liable	resistant
lysozyme									
titre	61.4 ± 0.8	53.1 ± 1.2	61.9 ± 0.9	63.4 ± 1.2	50.8 ± 1.0	65.4 ± 0.7	26.9 ± 0.3	21.9 ± 0.4	27.8 ± 1.3
µg/ml	33.9	23.7	34.3	23.0	20.5	23.6	64.7	60.4	65.3
hemagglutinin									
µg/ml	54.6	58.8	54.9	48.4	52.0	49.3	142.6	158.9	143.9

So, for carps' bio testing for resistance to helminthes, a decrease in lymphocyte by 19.9 % and an increase in hemagglutinin by 7.4 % as well as 18.6 % and 11.4 %, respectively for white amur are a trouble sign for fish farmers dealing with aquaculture.

Diffuse gel method confirmed that lysozyme is a marker of fish resistance to invasions passed by fish-eating birds. The decrease in lysozyme by 30.1 %, detected by this method in the kidney of a silver and scaly carp by 10.9 % and that of white amur by 6.6 % should be considered as bio testing parameters indicating that the fish are less resistant to studied invasions.

To determine the resistance of fish to helminthiases, it is possible to use such indicators for bio testing as periodic acid Schiff reaction of lymphocytes in the form of large granules and blocks and periodic acid Schiff reaction in the form of medium and small granules (Table 2).

Table 2. Evaluation of cytochemical parameters of fish for resistance to helminthic infections passed by fish-eating birds

Groups	The rate of testing fish for resistance to helminthiasis					
	Schiff reaction in the form of large granules, %			Schiff reaction in the form of medium and small granules, %		
	control	liable	resistant	control	liable	resistant
1	0.1-0.2	0.1-0.2	0.3-2.7	10-13	10-14	15-24
2	0.2-0.3	0.2-0.3	0.4-2.4	12-15	12-14	15-54
3	0.4-0.5	0.4-0.5	0.6-2.9	19-23	20-24	25-43

The positive pool of polysaccharide granulation in the cytoplasm of lymphocyte cells of fingerlings of scaly carps, silver carps and white amurs determines the fish with such a reaction to the risk group for invasions passed by fish-eating birds.

It becomes clear that such fish cannot be introduced into the breeding core of the broodstock. A dangerous tendency to lose resistance to helminthosis in the broodstock, it is necessary to consider the fact that precipitation is detected from large glycogen granules in lymphocyte of silver carp (up to 2.7 %), scaly carp (up to 2.4 %) and white amur (up to 2.9 %) as well as small polysaccharide inclusions of up to 24 %, 54 % and 43 % respectively.

Cytomorphologic analysis of lymphocytograms obtained from fish that are resistant and liable to helminthic infections has shown that it is efficient to use such parameters as the number and size of micronuclei for bio-testing.

The cytomorphologic parameters of liable to helminthosis scaly carps, silver carps and white amurs are as follows: they reveal a large number of lymphocytes with chromosomal aberrations of the dicentric type, aberrations such as the caudate nuclei and aberrations of the cytoplasmic type - the micronuclei.

When karyotype assessment of lymphocytes resistant and liable to the analyzed invasions of fish, it turned out that the number of lymphocytes with micronuclei in unstable to the disease silver carps is 3 times higher and that of white amurs is 2 times higher than that of analogues from the control group. It should be noted that it is impossible to use the micronucleus test as a fundamental one for the scaly carp when choosing the promising fish to be used in breeding, since according to this parameter the difference between the two experimental groups (resistant and liable to disease fingerlings) was not found (Table 3).

The species liable to invasions have all micronucleus test parameters higher than the control values. Thus, the number of lymphocytes with micronuclei in the blood of fingerlings predisposed to invasion increases on average 4 times for silver carps and white amurs and 2 times in a case with scaly carps. The 2 times increase of the total number of micronuclei in fish lymphocytes also indicates susceptibility to helminthic infections passed by fish-eating birds.

It should be noted that the size of the micronucleus is not a parameter when carrying out the indication of fish for resistance to helminthiasis, since the statistical analysis has not revealed any difference in this parameter between resistant and liable fingerlings.

Table 3 Dynamics of aberrations of the micronucleus type in lymphocytes of fish depending on resistance to helminthosis

Parameters	Number of lymphocytes with micronuclei	Total number of micronuclei	Micronuclei 0.5-1.0 um, %	Micronuclei 1.1-3.0 um, %	Micronuclei 3.1-5.0 um, %
Group 1 – silver carp					
Control	1.0 ± 0.05	1.5 ± 0.17	13	70	14
Resistant	1.1 ± 0.07	1.7 ± 0.11	14	70	14
Liable	4.3 ± 0.09	3.2 ± 0.31	15	71	16
Group 2 – scaly carp					
Control	3.1 ± 0.12	4.1 ± 1.32	12	40	23
Resistant	3.5 ± 0.12	4.4 ± 1.70	12	41	25
Liable	6.7 ± 0.83	8.4 ± 1.02	14	40	26
Group 3 – white amur					
Control	2.3 ± 0.12	3.8 ±1.8	13	30	7
Resistant	2.6 ± 0.22	4.2 ±1.8	13	32	9
Liable	8.7 ± 0.24	9.4 ± 1.1	13	31	9

When studying the next series of cytomorphologic parameters, it turned out that the number of dicentric type lymphocytes and lymphocytes with nucleus bridges also serves as a test response to the bio testing of fingerlings for resistance to helminthic infections, which are passed by fish-eating birds (Table 4). Such test is least applicable when scaly carp is used in aquaculture.

Table 4 Dynamics of chromosomal aberrations in lymphocytes of fish resistant and liable to invasion

Parameters	Number of dicentric lymphocytes	Number of lymphocytes with nucleus bridges
Group 1 – silver carp		
Control	1.2 ± 0.02	1.4 ± 0.03
Resistant	1.3 ± 0.02	1.5 ± 0.09
Liable	7.8 ± 0.05	7.3 ± 0.11
Group 2 – scaly carp		
Control	1.3 ± 0.02	1.4 ± 0.02
Resistant	1.3 ± 0.02	1.4 ± 0.03
Liable	1.4 ± 0.02	1.6 ± 0.02
Group 3 – white amur		
Control	1.6 ± 0.02	1. 7 ± 0.04
Resistant	1.9 ± 0.03	1.6 ± 0.03
Liable	3.7 ± 0.04	4.4 ± 0.06

The karyotype analysis of the blood of fish from the three experimental groups made it possible to reveal another parameter to indicate resistance of the fingerlings to helminthic infections. This parameter includes the number of lymphocytes with caudate nuclei (Table 5).

Table 5 The dynamics of the parameter "caudate nuclei" in fish lymphocytes, reflecting their resistance to helminthic infections passed by fish-eating birds

Parameters	Number of lymphocytes with caudate nuclei	Number of caudate nuclei in lymphocytes
Group 1 – silver carp		
Control	17.5 ± 0.05	26.3 ± 0.04
Resistant	15.2 ± 0.06	24.5 ± 0.09
Liable	69.3 ± 0.05	116.3 ± 0.06
Group 2 – scaly carp		

Control	15.9 ± 0.03	24.4 ± 0.02
Resistant	13.7 ± 0.02	22.4 ± 0.03
Liable	51.2 ± 0.07	111.6 ± 0.08
Group 3 – white amur		
Control	16.6 ± 0.02	25. 7 ± 0.04
Resistant	16.9 ± 0.03	26.2 ± 0.03
Liable	73.7 ± 0.04	124.1 ± 0.06

Low immunity of fish to invasions passed by fish-eating birds must be considered troublesome when an average increase in the number of lymphocytes with caudate nuclei in their blood is 4 times.

4.Conclusions

The search for fish parameters reflecting resistance to helminthic infections passed by fish-eating birds revealed the need for enzymatic immune, cytomorphologic and cytochemical studies.

Using the serial dilution method (titration) to determine hemagglutinin and lysozyme in kidneys of fish, it has been proved that these parameters are markers of carp fish resistance to helminthic infections.

A decrease in lysozyme concentration by 13.5 % and an increase in hemagglutinin by 7.7 % for silver carp (*Hypophthalmichthys nobilis*), 19. 9% and 7.4 %, respectively, for scaly carp (*Cyprinus carpio*), 18.6 % and 11.4 %, respectively, for white amur (*Ctenopharyngodon idela*) attribute the fish to the risk group of being infected with helminths of *Trematoda* class (*Posthodiplostomum brevicaudatum* and *Diplostomum spathaceum*) and *Cestoidea* class (*Pseudophyllidea*).

The diffuse-gel method revealed that the dynamics of lysozyme reflects the resistance of fish to invasions. A decrease in lysozyme in the kidney of a silver carp by 30.1 %, scaly carp by 10.9 %, and white amur by 6.6 % is an indicator of a decrease in fish resistance to helminths. The positive pool of polysaccharide granulation in the cytoplasm of fish lymphocytes, detected biochemically by periodic acid Schiff reaction, places them at risk for helminthic infections passed by fish-eating birds.

A dangerous tendency to decrease resistance to invasions is the precipitation of large glycogen granules in lymphocytes in the silver carp (to 2.7 %), scaly carp (2.4 %) and white amur (2.9 %), as well as small polysaccharide inclusions up to 24 %, 54 % and 43 %, respectively. Among cytomorphologic markers of fish resistance to helminthic infections, an increase (2-4 times) in the number of lymphocytes with caudate nuclei, micronuclei, and chromosomal aberrations of the dicentric type is indicative.

Thus, it can be concluded that as a result of cytomorphologic studies of the blood of experimental fish groups, it was found that there are various mutations in their lymphocytes, the quantitative indices of which must be used in bio testing susceptibility of fingerlings to helminthic infections passed by fish-eating birds.

Indications for resistance to invasions should be carried out on fish in early spring, in late March - early April, which will allow timely preventive measures to be taken against helminthic infections.

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