Effect of trypanosomiasis on hematologic characteristics of bream (*Abramis brama*)

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Trypanosomes are flagellated protozoa; they parasitize in the blood of a wide range of vertebrates and invertebrates, including fish, for which leeches are carriers. The metabolites released by trypanosomes are toxic to the host, cause disruption of homeostasis, which leads to illness and even death. Parasites in fish living in hot climates are the most common and better studied. Trypanosomes were first detected in common bream (*Abramis brama* L.) from the Uglich Reservoir (Upper Volga) in August 2015. The aim of this work is to study the effect of these parasites on the hematological parameters of the fish. As a control, blood indices of uninfected fish were used. The condition factor of infected fish did not differ from that of healthy fish. There were no significant differences between the two groups of fish in contents of total serum protein and glycemia. This may indicate a low level of bream parasitemia. At the same time, a significant increase in the leukocyte abundance index was detected, which indirectly indicates an increase in the number of these cells in the infected fish compared with the control ones, statistically significant differences were found in the leukogram: the proportion of eosinophils in the diseased fish increased almost 6 times while the relative number of lymphocytes decreased. The pattern of red blood also changed: the proportion of immature erythrocytes increased in the infected fish; a small number of microcytes and amitoses of erythrocytes and differences in the cytometric characteristics of red blood cells were found. The level of hemoglobin significantly decreased. A sharp increase in the content of circulating immune complexes indicates a shift in antigenic homeostasis caused by the presence of parasites. A similarity in the reaction of a number of indicators of the blood system of bream with trypanosomiasis to that of animals of higher systematic groups was revealed. The interpretation of the results obtained during the study of the effect of parasites on the host organism requires consideration of its physiological status and habitat conditions, the stage of the disease and the mechanism of adaptation of the parasite to the host defense system.

Keywords: *Trypanosoma* sp.; common bream; blood cells; hemoglobin; total protein; glucose; circulating immune complexes.

Introduction

Trypanosoma are a large group of flagellate protozoa, which parasitize in the blood of many vertebrate and some invertebrate animals (Goodwin, 1985; Ranzani-Paiva et al., 1997; Haag et al., 1998; Fermino et al., 2015). In fish, *Trypanosoma* have been found both in marine and fresh water species, representatives of Cyprinidae (Grybchuk-Ieremenko et al., 2014), eels (Islam & Woo, 1991a; Zintl et al., 1997), gobies, esocids, Pleuronectidae (Woo, 2001), Gadidae (Khan, 1977), Schilbeidae and Clariidae (Ferreira & Avenant-Oldewage, 2013), Serranidae (Wang et al., 2015), *Tilapia* (de Jesus et al., 2018), Characidae (Rodrigues et al., 2018). Fish living in hot climates are the most affected. Trypanosomes are very small, their length, depending on the species, ranges from 2 to 30 μm. They consume food in the form of dissolved organic and other substances using the entire surface of their body. The products of the metabolism of these parasites can be highly toxic and cause serious, and in many cases lethal diseases to their hosts (Bienek et al., 2002).

The life cycles of most trypanosomes have been insufficiently studied, but it has been established that among the invertebrates, their hosts are leeches, which therefore are the carriers of these protozoa for many fresh water as well as the marine species of fish, (Qadri, 1962; Mork, 2011; Hayes et al., 2014; Lemos et al., 2015). In water bodies with a high number of leeches, fish are often infected by *Trypanosoma*, around 200 species of piscine trypanosomes being currently known (Gupta & Gupta, 2012). During the cultivation of fish in ponds, disease outbreaks are rare due to absence of carriers,

whereas in open water bodies inhabited by leeches, trypanosomiasis of fish can be a serious problem. According to some authors, at the initial stage of infestation, stimulation of humoral and cell immunity occurs (Ribeiro et al., 2010; Oladiran et al., 2011). The extent of the impact of the parasites on the organism of fish is mostly determined by the energetic resources of the host, and also the conditions of the environment (water temperature, feeding, competition, etc.) (Gupta & Gupta, 2012). It has been demonstrated that trypanosomes decrease the vitality and the growth tempi of fish, cause anemia (Dykova & Lom, 1979; Clauss et al., 2008), shifts of physiological and biochemical parameters, disorders in metabolic processes (Lom & Dykova, 1984; Gupta & Gupta, 1987). Histopathological analyses of infected fish, compared to healthy fish, have indicated the following disorders; damaged vessels of spleen, degenerative and infiltrational changes in hepatocytes and epithelium of the kidney tubules, hypertrophy of epithelium cells of secondary lamellae of the gills (Osman et al., 2009).

Despite the fact that trypanosomiasis of fish was determined long ago, data on the parasites` impact on physiological and hematological parameters of infected fish are fragmented, and the problem of the influence of these protozoa on the commercial species which live in the Volga has not been studied. The objective of this paper is the study of the morphophysiological and hematological parameters of fish infected by trypanosomes. The scope of the study included determining the condition factor, hemoglobin concentration, the content of total protein, glucose and circulating immune complexes, leukogram, and also the ratio of different age stages and sizes of the erythrocytes of common bream from the Uglich Reservoir.

Materials and methods

For the study, we used common bream *Abramis brama* L. caught in the Uglich Reservoir in August 2015. For the study, we selected 20 mature fish of 294 ± 25 mm and 547 ± 142 g length and weight respectively. Trypanosomes were found in the blood smear of all 20 individuals. Systematically, they belong to Protozoa kingdom; Euglenozoa type; Kinetoplastida class; Trypanosomatida order; Trypanosomatidae family; *Trypanosoma* genus. No identification of the parasitic species was made. As the control, we used non-infected fish (15 individuals) with similar linear-weight parameters selected during the same period in the Ivankovo Reservoir located upstream of the Volga (Fig. 1).
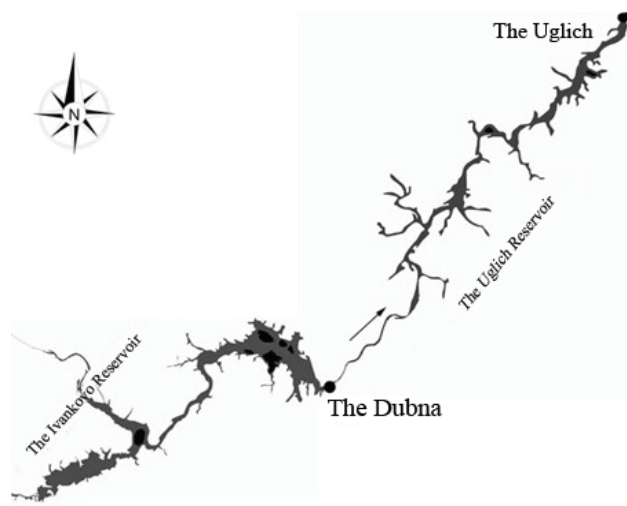

Fig. 1. Scheme of the Reservoirs

The main hydrochemical parameters of the water bodies where the fish was caught (pH, BOD, the content of the main cations and anions, nutrients) have similar values (Debol'skij et al., 2010). According to the results of the final evaluation of the quality of the water in the Uglich Reservoir, the water is considered "dirty" by most of the integral hydrochemical indices (Lazareva, 2016). The water in the Ivankovo Reservoir, in area where the fish were caught, is also considered "dirty" according to the main hydrochemical parameters (Debol'skij et al., 2010). Therefore, no principal difference in the quality of water in the two reservoirs was found.

We measured the body length, weight of the fish, and after the caudectomy, obtained blood. The smears were made, part of the blood was stabilized by heparin and was used for identification of hemoglobin. The remaining blood was used for making blood serum. Then, the fish was anatomized and weighed after the internal organs were removed. The condition factor was calculated according to Clark. The concentration of hemoglobin was analyzed using the HPP-01 [Hemoglobinometer photometric portative] miniHEM+ hemoglobinnometer in accordance with the hemichrome method. The level of circulating immune complexes (CIC) was determined using a standard method of sedimentation with polyethylene glycol. The results are given in conventional units. The content of total protein and glucose in the serum was determined according to the biuret and glucose oxidase methods respectively, using the "Agat-Med" standard sets of reagents for clinical analysis.

The smears were processed according to the standard method, we established the leukocyte formula, and also the relative number of erythroblasts, immature and mature erythrocytes, amitosis. During the calculation of the red blood cells, we took into account no less than 500 cells in the smears, the results were expressed as a % of total number of erythrocytes. The anomalous forms of erythrocytes were considered microcytes and cells without a nucleus. The amitosis were calculated also for 500 erythrocytes and expressed as a %.

In determining the proportion of the leukocytes, we calculated no less than 200 cells in a smear. We identified hematopoietic stem cells, lymphocytes, monocytes, myelocytes, metamyelocytes, stab neutrophils and segmented neutrophils, eosinophils. The results of the calculation were expressed as a %. The abundance index of leukocytes (without thrombocytes) was calculated as the number of the leukocytes in the microscope field of view at $^x1000$ magnification. The number of thrombocytes was calculated in relation to the total number of leukocytes and was expressed as a %.

We measured the small (h) and large (l) diameter of the erythrocytes and their nuclei, calculated the index of form, area of cells and nuclei, nuclear-cytoplasmic ratio. The index of form (If) was calculated as a ratio of the small diameter (h) of the cell to the large (l): If = h/l. The area of the cell and nucleus was calculated using the formula ellipse equation: S = πhl/2, μm². Nuclear-cytoplasm ratio was calculated using the formula: N/C = S_n/(S_c–S_n), where S_n – the area of nucleus, S_c – the area of cell. The research was conducted using a Keyence VHX-1000 microscope using a Z-500 R objective at ×2000–3000 magnification.

The results were analyzed in the Statistica package at the significance level of P < 0.05. The data are presented as mean values and standard errors (x ± SE), for the assessment of the values` reliability, we used ANOVA, taking into account the normal distribution of the parameters.

Results

Trypanosomes were found for the first time in the peripheral blood of common bream from the Uglich Reservoir. The parasites were found in the smears of all analyzed fish to the amount of 1–3 individuals per 10 and more view fields (at $^x1000$ magnification) (Fig. 2).
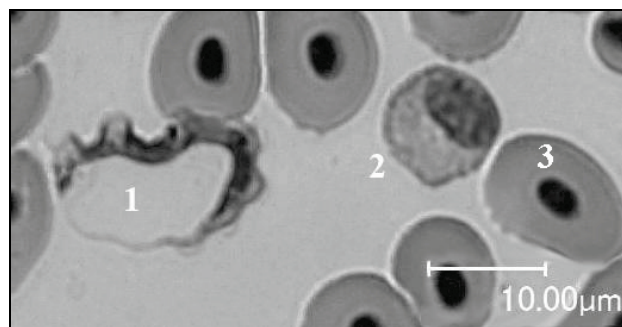

Fig. 2. Peripheral blood of common bream from the Uglich Reservoir: *1* – trypanosome, *2* – neutrophil metamyelocyte, *3* – erythrocyte

Condition factor. The presence of parasites did not affect the fatness coefficient, in both groups of the fish it was the same, equaling 1.8 ± 0.1.

Physiological-biochemical parameters of the blood. The results are presented in Table 1.

Table 1
Physiological-biochemical parameters
of the peripheral blood of bream (x ± SE)

Parameters	Infected fish, n = 20	Control, n = 15
Total protein, g/l	44.9 ± 2.4	40.6 ± 1.2
Glucose, mmol/l	5.0 ± 0.5	5.4 ± 1.0
Hemoglobin, g/l	67.8 ± 3.3*	80.6 ± 1.6
CIC, conventional units	37 ± 4*	18 ± 3

Note: hereinafter, * – symbol in tables indicates the values which are statistically reliably different compared to the control.

As the obtained data demonstrated, the content of the total protein in the serum of the infected fish was slightly higher than in the control, though this difference was not statistically reliable. The level of glycemia the fish of the both groups practically did not differ. The hemoglobin concentration of the infected fish was reliably lower than among the healthy fish, and the CIC content of the fish from the first group was twice as high compared to the control.

Blood cells. Despite the relatively low level of infection, the blood leukogram was sharply shifted towards the granular forms of

the cells (Table 2), mostly due to 6 times increase in the content of eosinophils.

Table 2
The ratio of the forms of leukocytes and erythrocytes
in the peripheral blood of bream (x ± SE)

Parameter	Infected fish, n = 20	Control, n = 15
Erythrocytes		
Erythroblasts	3.51 ± 1.43	1.73 ± 0.81
Immature erythrocytes	4.52 ± 1.24*	2.11 ± 1.51
Mature erythrocytes	93.33 ± 2.01	97.60 ± 1.82
Amitosis	0.53 ± 0.21	0.00
Microcytes	1.23 ± 1.03	0.00
Leukocytes		
Lymphocytes	50.04 ± 0.52*	67.30 ± 2.41
Monocytes	2.92 ± 0.21	0.89 ± 0.89
Blasts	2.38 ± 0.26	1.09 ± 0.78
Myelocytes	5.11 ± 0.67*	14.22 ± 3.24
Metamyelocytes	14.21 ± 1.11	10.38 ± 4.52
Stab neutrophils	5.89 ± 0.09	6.83 ± 2.37
Segmented neutrophils	0.30 ± 0.30	1.03 ± 0.77
Eosinophils	19.26 ± 1.91*	2.83 ± 1.77
Shift of leukocytes	0.77 ± 0.05*	0.53 ± 0.21
Index of leukocyte abundance	20.00 ± 2.00*	8.00 ± 5.00
Thrombocytes	63.52 ± 0.68*	52.08 ± 4.48

We should mention the increase in the index of leukocyte abundance, and also reliable increase in the relative number of thrombocytes in the infected fish compared to the control. The ratio of immature and mature erythrocytes was higher among the infected fish, in their peripheral blood, we also found an insignificant amount of amitosis and microcytes.

The analysis of the cytometric characteristics of erythrocytes indicated that the erythrocytes of the infected fish had a more rounded shape, had a smaller area, but greater nuclear-cytoplasmic ratio (Table 3).

Table 3
Cytometric parameters of the erythrocytes of bream (x ± SE)

Parameter	Infected fish, n = 20	Control, n = 15
Cell		
H, μm	8.85 ± 0.04	9.24 ± 0.03
L, μm	11.81 ± 0.03	12.62 ± 0.06
Sc, μm^2	82.04 ± 0.87*	91.52 ± 0.64
I$_f$	0.75 ± 0.06	0.73 ± 0.04
Nucleus		
H, μm	4.84 ± 0.05	4.64 ± 0.04
L, μm	6.08 ± 0.03	5.76 ± 0.06
Sn, μm^2	23.09 ± 0.22	20.98 ± 0.32
Nuclear-cytoplasmic ratio	0.39 ± 0.01*	0.30 ± 0.02

Discussion

The difference which consisted in the presence or absence of infection of bream in the Uglich and Ivankovo Reservoirs may be caused by the peculiarities of these water bodies. The main fattening places of the fish are shallow-water areas up to 3 m depth. The shallow water area in the Uglich Reservoir is almost 2 times smaller compared to the Ivankovo (90 and 160 km^2, respectively), which is by 12% lower in relation to the total area of the reservoir (48 and 36%). At the same time, ichthyomass of the demersal fish in the Uglich Reservoir since 1985 has been higher than in the Ivankovo Reservoir (Ehdel'shtejn, 1998).

The number of leeches (intermediate hosts of trypanosomes) on fish in the Uglich Reservoir is higher compared to the Ivankovo (Lapkina et al., 2002), therefore the probability of infection of the fish is higher in the Uglich Reservoir than in the Ivankovo Reservoir.

Trypanosomes grow to the full length in 60 days after penetrating the blood of fish (Lom & Dykova, 1984), therefore the occurrence of individuals of more than 80 μm length and 3 to 5 individuals per mm^3 in the bloodstream of the bream indicate that the bream of the Uglich Reservoir were exposed to the chronic stage of the disease. The literature contains very few data on the impact of trypanosomes on the

linear-weight parameters of fish. The are reports that the parasites can cause decrease in growth and weight (Kharat & Kothavade, 2012), at the same time, the analysis of the impact of the extent of infestation by *T. percea* on length and weight of perch revealed differences only in the juvenile groups (Hamnueva, 2001).

Referring to the literature data, Islam & Woo (1991b) provide several reasons for the decrease in food consumption among fish infected by trypanosomes: slowing of food passage through the intestine, damage to the mucous membrane of the intestine, which leads to disorders in metabolism and absorption, disruption in the balance of hormones which regulate the sense of hunger, and development of anorexia. However, in their own studies, the authors did not find any significant decrease in the body weight of the infected fish. They think that the fish with developed anorexia either die or survive a crisis, after which they return to normal feeding. The absence of differences according to the condition factor in our research most likely occurred due to relatively low level of infection.

The literature data on the changes in the level of total serum protein of the infected fish are also contradictory. There are reports about decrease in the parameters of African sharptooth catfish (Osman et al., 2009). According to the authors, the significant decrease in the total protein in the serum and its fractions (albumin and globulin) of the infected fish, which they found, could be caused by hemodilution. The representatives of 7 species of ornamental loricariids, infected by trypanosomes were observed to have both decrease and insignificant increase, as well as absence of the changes in the parameter compared to the control (Fudjimoto et al., 2013).

On the whole, literature data and our own study allow us to draw the conclusion that the level of total serum protein in fish with trypanosomiasis depends on many factors, including the extent of invasion, species and physiological condition of fish, etc.

Disorders in the carbohydrate metabolism of the host infected with protozoa were mentioned by von Brand (1973). He made the assumption that trypanosomes consume such an amount of sugar in the blood that the carbohydrate reserves of the host become depleted, causing a heavy load on the liver and disorders in its functioning. Such phenomenon occurs both among amniotes and fish. At the same time, the extent of depletion of the carbohydrate reserves of the host depends on the species of parasite and extent of infestation (Gupta & Gupta, 1987). According to the results of our studies, the average level of glucose in the blood of infested fish practically did not differ from that of the control fish, which was most likely caused by the low extent of invasion.

We found no literature data on the impact of protozoa on the level of CIC of fish. However, taking into account that the formation of immune complexes is an important and indispensable component of the mechanism of supporting the antigene homeostasis, and they are constantly present in blood stream, one should expect a reaction of the indicator to the parasites` penetration to organism. The CIC concentration is determined by the conditions of the fish` habitat. They are removed as with the amniotes – by cells of the mononuclear phagocyte system (MPS) (Levinsky, 1981).

It has been demonstrated that birds suffer the development of pathological process when subject to helminthiases with accompanying formation of CIC which take an important part in pathogenesis of the disease, and the period of their circulation in the host`s organism correlates with the time and severity of the pathological process (Kuklina & Kuklin, 2011). Increase in the content of CIC by 59% was also determined among horses suffering from strongyloidiasis (Tkachenko, 2009).

Because trypanosomes, as mentioned above, produce toxic metabolic products in the blood of the host, their neutralization requires an additional number of non-specific antibodies, which can also cause increase in the CIC level. Increased production of antibodies, including specific antibodies, by the host organism was noted by a number of authors during introduction of hemoflagellates (Evans & Gratzek, 1989; Plouffe & Belosevic, 2006; Joerink et al., 2007; Sitia-Bobadilla, 2008; Wiegertjes & Forlenza, 2010). Intensity of production of antibodies depends on the extent of the invasion and the temperature of the habitat (Woo, 1981; Sypek & Burreson, 1983). Therefore, literature

data and our study suggest that the immune complexes take an active part in an organism`s reaction to parasites.

Most researchers mention a decrease in the level of total number of erythrocytes among infected fish (Steinhagen et al., 1990; Hamnueva, 2001; Klauss et al., 2008; Shahi et al., 2013). It is considered that this parameter is the most vulnerable hematological parameter during trypanosomiasis. At the same time, apart from decrease in the total number of erythrocytes, a decrease in the content of hemoglobin has been mentioned (Shahi et al., 2013), and anemia in a number of cases (Gupta & Gupta, 2012). Anemia is considered the most typical symptom of trypanosomiasis, the fish are often slow and apathetic (Shahi et al., 2013).

On the basis of their own and literature data, Islam & Woo (1991a) came to the conclusion that decrease in the total number of erythrocytes in cases of trypanosomiasis is caused by at least two factors: first of all, hemolysin produced by the parasite, which causes direct lysis of erythrocytes; secondly, by hemodilution (increase in the blood volume), a process which correlates with the protozoa number. It was experimentally demonstrated that the hemolysis of erythrocytes is also caused by metalloproteinases produced by *Criptobia salmonocida* (Zuo & Woo, 2000).

The decrease in the proportion of mature and, correspondingly, increase in the immature erythrocytes that we observed has also been mentioned by other authors (Maqbool & Ahmed, 2016). Most likely, this phenomenon is related to stimulation of erythropoiesis caused by the accelerated death of mature cells.

The literature contains very few data on the change in morphometric characteristics of erythrocytes of fish with trypanosomiasis. There is information about decrease in hematocrit and increase in the cell volume of the infected fish (Shahi et al., 2013; Maqbool & Ahmed, 2016). On contrary, during direct counting of cell sizes, we determined a decrease in their area, increase in the area of the nucleus, and therefore increase in nuclear-cytoplasmic ratio. Perhaps, this decrease in the cell sizes is related to reduction of the time of their presence in the bloodstream.

We can draw a conclusion that cytometric parameters are affected by a number of factors, including the extent of the invasion, stage of disease, etc. The differences between the data we obtained and the literature data, is probably connected with the different methods of counting the volume (area) of cells. With recounting from hematocrit volume and number of erythrocytes to ml of blood, one will obtain average cell volume, without consideration of the stage of development, whereas during direct measurement of cells, one considers only the sizes of mature erythrocytes.

On the whole, measuring the morphometric parameters explains the rather steep decrease in the hemoglobin level: decrease in the share of mature erythrocytes, decrease in their area, including the area of cytoplasm, where hemoglobin is concentrated.

The leukocytes` reaction to parasites is similar to that occurring in guinea pigs and fresh water fish in cases of both acute and chronic processes. Usually, there is a steep increase in the total number of leukocytes and shift of the leukogram towards granular forms of cells (Shahi et al., 2013; Correa et al., 2016; Maqbool & Ahmed, 2016), which was also found in our study. Some authors mention that the reaction of phagocytes to trypanosome infection depends on the intensity of the invasion, its stage, and also the species of parasite and fish (Forlenza et al., 2008). We determined that the factors produced by monocytes (macrophages) in peripheral blood can stimulate growth and development of trypanosomes in hosts (Bienek & Belosevic, 1999). In its turn, transferrins which penetrate the blood during lysis of erythrocytes significantly activate the killer ability of macrophages (Stafford & Belosevic, 2003).

The significant increase which we found in the share of eosinophils among the infected fish coincides with the assumption that eosinophils are cells "responsible for the organism`s reaction to parasites" (Clauss et al., 2008). Eosinophilia was also observed in cases of infestation of fish by flat worms and nematodes (Kutyrev et al., 2011).

The reaction of thrombocytes to the invasion of parasites is not usually analysed, and data in the literature on the reaction of these cells in cases of parasitemia appear rarely and are contradictory. Therefore, protozoa caused no change in the share of thrombocytes in loricariid *Hypostomus* sp. (Correa et al., 2016), but carp with severe tryponosomiasis were observed to have a decrease in population of these cells in the spleen and blood and their apoptosis caused by increase in the level of nitrogen oxide in the blood (Fink et al., 2015), during our study, the bream showed a reliable increase in the share of these cells. Contradictions in the abovementioned data could most likely indicate insufficient study of this parameter in the response reaction of the host organism to parasitemia.

On the whole, the data we obtained indicate that the trypanosomes which have penetrated the organism of fish produce compounds that destroy erythrocytes, cause decrease in the level of hemoglobin, increase in the share of immature erythrocytes and stimulate production of eosinophils, and also cause increase in the level of CIC.

It is worth noting the similarity between the determined shifts of the hematological parameters in fish with trypanosomiasis and those caused by different species of parasites in different systematic groups of vertebrates: inhibition of erythropoiesis, eosinophilia, lymphopenia, increase in the CIC level among horses with strongloidiasis (Tkachenko, 2009), according to a number of parameters, the pattern of changes was similar to that of birds suffering from cestode invasion (Kuklina & Kuklin, 2011).

The ambivalence of the reaction of some parameters in different species of fish to trypanosome` invasion is caused by the fact that "parasite – host" is a complicated system of interdependence, which is determined by many factors, of which the most important are age, immune-physiological status and conditions of the fish habitat on the one hand and mechanisms of interaction of parasite and immune system of host on the other hand (Khan, 2012).

Conclusion

The absence of deviations in fatness, level of total serum protein and blood sugar level of the infected fish prove the conclusion drawn on the basis of analysis of blood smears that the level of parasitemia was low. The presence of a relatively low number of parasites and their sizes indicate the chronic stage of disease. The clear reaction of the CIC level in these conditions allows one to presume that using this parameter is promising for diagnosing parasitic infections, including those of protozoa. We determined changes in the condition of red blood, which allows one to assume a decrease in the lifespan of the erythrocytes, and also a reliable decrease in the concentration of hemoglobin in the blood. At higher levels of infestation, such shifts cause anemia. Change in the composition and ratio of the leukocytes accompanied increase in their total number. Shift of the leukocytes was caused mostly by growth of the share of eosinophils. On the whole, it could be stated that the reaction of the hematological parameters of bream to trypanosome infection is in general similar to the reaction of animals of higher systematic groups to presence of parasites.

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