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ORIGINAL PAPERS

The Effects of Anthropogenic Pollution on the Immunological and Biochemical Indices of the Round Goby *Neogobius melanostomus* (Pallas, 1814) (Perciformes: Gobiidae), Inhabiting Coastal Waters of the Black Sea

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Abstract—This study investigated several immunophysiological indices of the round goby *Neogobius melanostomus* (Pallas, 1814), which inhabits Caucasian coastal waters of the Black Sea with different levels of anthropogenic load. Fish specimens caught in areas of a high level of pollution differed significantly from fish from pure water areas in low indices of antimicrobial properties of the blood serum, antioxidant activity, and phospholipid contents, and, in contrast, by high levels of immune complexes, total lipids, triacylglycerols, and malonic dialdehyde.

Keywords: round goby *Neogobius melanostomus*, humoral immunity, lipids, lipid peroxidation, antioxidant activity

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INTRODUCTION

Coastal water areas of the Black Sea in the vicinity of large industrial centers, port complexes, oil terminals, agricultural enterprises, and large river estuaries are subject to the constant influence of pollutants. Pollution has led to a deterioration of conditions in fish habitats, to reduction in the diversity of fish species and, consequently, to losses for commercial fisheries in the areas [8, 18, 26]. Fish of the coastal ichthyofauna that lead a sedentary lifestyle in particular, the round goby *Neogobius melanostomus* (Pallas, 1814) (Perciformes: Gobiidae) suffer most severely. In the 1950s to 1970s, the catch of the round goby was approximately 900 centners per year; it had decreased by more than 40 times by the end of the 1990s. Negative phenomena in the coastal fish fauna of contaminated areas of the Black Sea indicate that under unstable environmental conditions toxicants induce disturbances in the function of the immunological and biochemical mechanisms of fish homeostasis that provide the optimal growth, development, reproduction, resistance to parasitic factors, and survival during ontogenesis [5, 13, 14, 16, 22, 26, 27, 29]. Changes in species diversity and the age structure of fish populations, as well as a decrease in the abundance and the longevity of fish under conditions of technogenic pollution of the water environment are preceded by profound pathophysiological and immunopathological processes that lead to a decrease in adaptive capacity and resistance of fish and to changes in the pattern of their development.

The purpose of this work was to assess the immunological and biochemical indicators of the round goby from areas of the Black Sea that differ in pollution level in order to identify the causes for the decrease in the adaptive potential of fish and to develop indicators of their health.

MATERIALS AND METHODS

We used 50 sexually mature round gobies as material for the study (ages of 3+, 13.9–18.6 cm in length and 150–185 g in weight). The fish were caught during feeding in the Caucasian coastal waters of the Black Sea, in the central part of the water area near the port of Tuapse (Station 1; 23 individuals) and near the settlement Tsandripsh, Abkhazia (Station 2, 27 individuals), 5 km from the border of Russia. The water area at the cargo and passenger port of Tuapse is one of the most polluted areas of the Caucasian coast. These coastal waters are polluted, especially with oil products, their concentration is more than 14000 times higher than the maximum permissible standards. It was also established that the maximum permissible concentration limits for the content of urea, phosphates, nitrates, synthetic surfactants, phenols, and hydrogen sulphide were significantly exceeded. Part of the water area near the settlement Tsandripsh is considered as relatively clean, since there are no industrial enterprises here; only domestic sewage from small resort settlements discharge into the sea.

We analyzed the immunophysiological state of the fish via the bacteriostatic activity of the blood serum (BAS), the portion of immunodeficient (IMD) specimens, the contents of immune complexes (IC), total lipids (TL) and the individual lipid fractions, products of lipid peroxidation (LPO), and the level of antioxidant protection (AP) in liver tissues. To study the content of ICs, LPOs, and the level of AP in the liver, we prepared a homogenate, grinding the tissues with 0.65% physiological saline at a ratio of 1 : 5.

The BAS was assessed by the nephelometric method in the modification of Mikryakov [11]. Blood for serum was taken from the tail vein. On the 5th day from the moment of blood collection, this parameter was determined based on the effect of 5-fold diluted serum on growth and the development of 50 million *Aeromonas hydrophila* test microbes. The choice of *A. hidrophila* is explained by the fact that this species belongs to opportunistic microorganisms, is widespread in nature, and belongs to the etiological factors of rubella, that is, aeromonas infection of freshwater and marine bony fish species [30]. The test culture was obtained from the Laboratory of Ichthyopathology of the All-Russian Scientific Research Institute of Freshwater Fisheries. The growth rate of bacteria in the presence of the blood serum (experiment) and without it (control) was determined after a 5–6 h incubation at 26°C. The rate of growth of test microbes was assessed from the change in the optical density before and after the incubation of bacteria in the experiment in comparison to the control. Depending on the level of BAS, we detected the proportion of IMD specimens whose blood serum did not inhibit the development of test microbes.

The IC content was determined spectrophotometrically at 450 nm by the method of selective precipitation with 4% polyethylene glycol (MW 6000), as recommended earlier by Grinevich and Alferov [4].

Lipids from liver tissues were extracted and identified gravimetrically by the standard Folch method [28]. The samples were fixed with a mixture of chloroform: methanol at a ratio of 2 : 1 by volume. Separation of total lipids was carried out by ascending thin-layer chromatography on "Silufol" plates in the petroleum ether solvent system, that is, sulfuric ether : glacial acetic acid (90 : 10: 1, by volume). The fractional composition of lipids was determined in a sealed chamber in iodine vapor [6]. We determined the relative content of structural phospholipids (PL), cholesterol (Ch), non-esterified fatty acids (NEFAs), reserve triacylglycerides (TAGs), sterol esters (SEs), and hydrocarbons (Hs).

The intensity of LPO products was determined from the accumulation of malondialdehyde (MDA), one of the final lipid peroxidation products. The MDA concentration was determined by the amount of LPO products that react with thiobarbituric acid with the formation a colored complex. The intensity of staining was evaluated spectrophotometrically from the change in the absorption maximum at 532 nm [1]. We calculated the MDA content taking the molar extinction coefficient of 1.56×10^5/(M cm) into account and expressed it as nanomoles per 1 gram tissue.

The general antioxidant activity was judged by the kinetics of the oxidation of the substrate of the reduced form of 2,6-dichlorophenolindophenol with air oxygen according to the generally accepted method [19]. The essence of the method lies in the fact that the higher the rate of oxidation of the substrate in the presence of a biological material is, the lower the content of antioxidants in tissues is. The inhibition constant of substrate oxidation (ICSO), which is a parameter of the tissue antioxidant activity, was determined with respect to the control according to the formula: $K_i = K_{\text{control}} - K_{\text{exp}}/C$, where K_{control} and K_{exp} are the rate constants for substrate oxidation rates in the control and in the experiment, respectively, and C is the concentration of the biological material in the cuvette.

Statistical processing of the results of the study was carried out using standard algorithms implemented in the Statistica v. 6.0 software package, using the t-test. Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

A comparative analysis showed that fish caught in an area with a high level of contamination differed significantly from fish from the conventionally clean area according to the studied immunological and biochemical characteristics. The value of BAS in fish caught at Station 1 was lower and the proportion of IMD individuals and IC content exceeded those in fish from Station 2 (Fig. 1). The observed differences between fish samples indicate a damaging effect of pollutants on the functional state of the immunological mechanisms of homeostasis. It is known that BAS is an integral characteristic of the functional state of congenital factors of humoral immunity: complement systems, lysozyme, properdin, immunoglobulins, antimicrobial peptides, lectins, precipitins of B-lysine, etc. [7, 11, 17, 31, 32 and others], and the degree of their manifestation depends on the functional state of the body, and the nature of the influence on fish of unfavorable factors, including toxic effects [13, 14]. Previous studies of the patterns of BAS changes in different fish species showed the relationship between the antimicrobial function of blood serum and the physiological and biochemical state of the fish, the intensity of parasite infestation and the death rate of the organism at different stages of ontogenesis [10, 15, 20, and others]. As a result of the study of the biochemical composition of the blood serum of the IMD individuals we revealed a

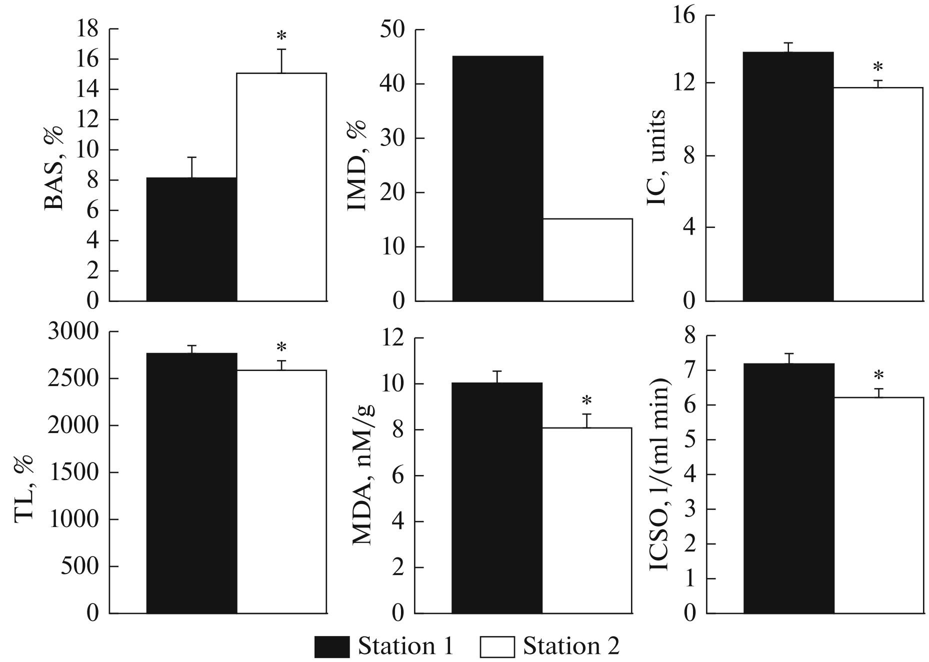

Fig. 1. Indicators of the immunophysiological state of the round goby *Neogobius melanostomus* from different regions of the Black Sea. Here and in Fig. 2: Station 1 in the water area of the port Tuapse; Station 2 in the water area near the Tsandripsh settlement (Abkhazia). Vertical lines give the standard error; *, significant difference between the areas at $p \leq 0.05$.

link with the lipid and protein status. We found the dependence of BAS on the quantitative ratio of biochemical components, in particular, proteins and lipids, albumins, alpha, beta and gamma globulins, as well as on the contents of phospholipids, triacylglycerides, cholesterol, unesterified fatty acids, hydrocarbons, and free amino acids [12, 23]. It was suggested that the functional state of the humoral immune system is determined by the balanced relationship between the biochemical components of blood serum [23]. We found that modifications caused by stress factors, including pollutants, are accompanied by disregulation of both metabolic processes and the immunobiochemical status, and also lead to fish with secondary immunodeficiencies within fish populations [14].

It is known that the antigen–antibody complexes ICs are associated with complement components formed as a result of interaction with low-molecular-weight foreign compounds (haptens, soluble antigens, and autoantigens) [7]. ICs play an important role in regulation of immune reactions, elimination of xenobiotics from the body, and in the maintenance of immunological and biochemical homeostasis. When the body is saturated with foreign components, an excess level of ICs is caused by suppression of the clearing function of the cell phagocytic system [9]. The high IC content in fish liver extracts from Station 1 in comparison with that of fish from Station 2 indicates a violation of the mechanisms of elimination of these complexes; this elimination is associated with circulating and tissue phagocytes, including those in the liver. An increased IC content is one of the factors that cause suppression of the mechanisms of humoral immunity and uncontrolled pathological processes (allergization and autoimmunization); as a consequence, it is the cause of decreased immunoreactivity, depletion, and death of fish [11, 14]. Apparently, the increased IC content in fish from Station 1 is one of the causes for a decrease in BAS level and IMD individuals with a low adaptive potential and viability in the round goby populations. A low level of BAS, a high proportion of IMD fish and a high IC content of the round goby caught in the area of Tuapse, compared to those for fish from the coastal region of Abkhazia, are caused by the immunotoxic effect of pollutants that damage the mechanisms of innate immunity.

The fish were characterized not only according to immunological indices, but also according to the content of total lipids and the relative contents of individual lipid fractions (Figs. 1 and 2). Lipids, with their heterogeneity and variety of functions in the body, are important informative indicators of adaptive processes and reflect their role as either favorable or negative factors in their effects on fish [25]. They serve as sources of metabolic energy in the body, as biological effectors and mediators involved in the regulation of the vital functions of body systems to provide optimal growth, development, and health at all stages of ontogenesis and adaptation to adverse environmental factors [3, 12, 20, 24]. The revealed modifications in the lipid exchange of the round goby from different habi-

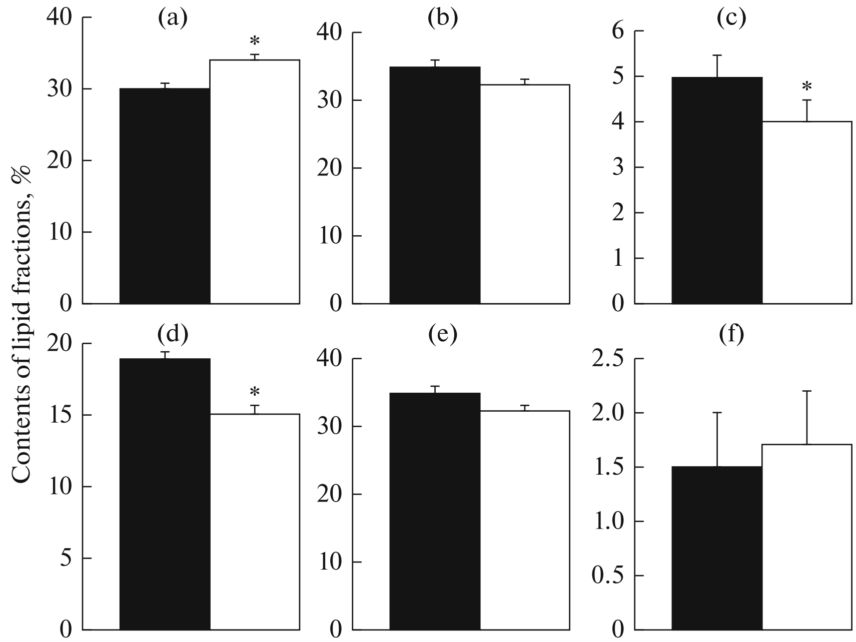

Fig. 2. The contents in the liver tissues of the round goby *Neogobius melanostomus* from different regions of the Black Sea of phospholipids (a), cholesterol (b), non-esterified fatty acids (c), triacylglycerides (d), sterol esters (e), and hydrocarbons (f).

tats reflect the influence of pollutants on metabolism. An increased concentration of total lipids can reflect the conditions of feeding and intensity of fish nutrition and also indicates a toxicant-induced dystrophy of lipid metabolism.

The fish caught in the coastal waters near Tuapse were characterized by a significantly high TAG content, but a low concentration of structural phospholipids. The reduced content of Ph in the liver of fish from Station 1 may be due to the suppression of the formation or entry of lipotropic substances (choline, methionine, etc.) into this organ. It was shown that at their low concentration, the synthesis of PL from neutral fat (glycerol and fatty acids) is significantly reduced [3, 25]. A high TAG content can be explained both by a less intensive use of reserve lipids for energy needs and by a more intense anthropogenic load on the physiological state of the fish population in the vicinity of Station 1. The change in the lipid status of the fish in the contaminated zone may be due to the toxicant-induced disruption of lipid metabolism. Similar changes in the studied parameters were also noted earlier in other fish species from anthropogenically transformed ecosystems and technogenic-transformed water bodies [2, 12, 16, 21].

An analysis of the prooxidant-oxidant status of the fish liver showed that the MDA content of fish from Station 1 was 39% higher than that for fish from Station 2, which may be caused by activation of lipid-peroxidation processes (Fig. 1). The value of the inhibition constant of substrate oxidation (ICSO) in Abkhazian waters was minimal, which reflects a higher content of antioxidant structures in fish from these waters. The high MDA content in the liver tissues, as well as a high rate of the kinetics of oxidation (ICSO) in fish from contaminated water areas, indicate a shift of the equilibrium of the prooxidant–oxidant system towards the intensification of oxidative stress processes [5, 14, 21, 26, and others).

Activation of LP processes induced by free-radical oxygen forms is one of the non-specific mechanisms of the negative toxic effect of xenobiotics on the body. Toxic products of lipid peroxidation can also cause secondary damage to cell membranes, reduced antioxidant contents, suppressed immunological and regenerative functions, premature aging, reduced rates of growth and development, and lower vitality of fish.

Thus, as a result of the study, it has been established that the round goby individuals from the anthropogenically polluted coastal water area differed from the individuals from the relatively clean area according to the structural–functional state of the homeostasis mechanisms.

Earlier, Silkina et al. [22] found similar differences in the level of BAS, in the portion of IMD individuals, the content of AL, MDA, and the AP index for the dogfish *Squalus acanthias* caught in the same model waters, i.e., in the port of Tuapse and from the vicinity of the Tsandripsh settlement. The established modifications in the immunobiochemical status of fish from the contaminated area are a pathogenetic cause of the reduction of their adaptive potential and vitality.

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