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Article

Health parameters and antioxidant response in Black Sea whiting *Merlangius merlangus euxinus* (Nordmann, 1840) parasitized by nematode *Hysterothylacium aduncum* (Rud., 1802)

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Abstract

Biometrical and biochemical parameters in healthy and parasitized by nematode *Hysterothylacium aduncum* Black Sea whiting *Merlangius merlangus euxinus* were studied. The increase of hepatosomatic index and decrease of spleen index in infected fish were found, while condition factor was similar in both groups. Antioxidant enzyme activities (superoxide dismutase (SOD), catalase (CAT), peroxidase (PER), glutathione reductase (GR) and glutathione-S-transferase (GST)) were higher in red blood cells in the parasitized fish as compared with the healthy animals. In the liver, the antioxidant response was not uniform: activities of SOD and GST decreased and activities of PER, GR and CAT increased in the infected individuals. The obtained results reflect host defense against oxidative stress caused parasitic infection. The tissue specific fluctuations of antioxidant response in parasitized and healthy fish are discussed. Data in this study can be used for understanding the defense mechanisms of fish against parasites.

Keywords: antioxidant enzymes, fish, parasite invasion, red blood cells, liver

Introduction

Throughout their life wild fish populations areexposedtonaturalandanthropogenicstressors. Many fish, including important commercially and aquaculture species infect by parasites, which may have considerable impact on physiological and biochemical processes in the host organisms and harmful for their health. Parasites are widely distributed in water bodies, which are the main recipients of wastewaters, containing chemicals and pathogens, and the deleterious effects of them are well known (Pasquaud et al., 2013; Cazenave et al., 2014). The interactions between parasites, pathogens and chemicals possess a high potential for accumulation or synergetic effects. The roles, functions, and life-styles of parasites help to characterize an ecosystem (Iwanowicz, 2011; Costa et al., 2012). Therefore, parasites are good indicators of both marine and freshwater ecosystems health and may serve an alternative tool for monitoring studies of aquatic environment. Increase of parasite infection in wild fish populations indicates the unfavorable living conditions, led the decrease of fish resistance, modulate population structure, change its composition and loss biodiversity. Although understanding the mechanisms of fish defense against parasite infection is very important, however, the information remains scarce and not uniform.

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Hysterothylacium aduncum is highly distributed parasite belonging to *Anisakidae* family. These parasites are very harmful for people and domestic animals, because adult helminthes are localized in the stomach, intestine and in the abdominal cavity. *H. aduncum* is a good indicator of both marine and freshwater ecosystems health and may serve an alternative tool for monitoring studies. Many fish, including important commercially and aquaculture species infect with parasite. Additionally, parasitic infection causes bacterial diseases in cultivated fish species leading to economic loses. Additionally, *Anisakidae* parasites cause the allergic response and they are capable of infecting humans (Gaevskaya, 2005).

One of the natural definite hosts of *H. aduncum* in the Black Sea is whiting *Merlangius merlangus euxinus,* which is an important commercially species. Whiting plays a role in the transfer of the parasites via food chains to predators. Parasite is found in intestine, stomach and abdominal cavity in the whiting and may cause oxidative stress and inflammation in these tissues (Zav'yalov & Kuzminova, 2011).

The aim of the present study was to compare the physiological and biochemical response of Black Sea whiting *Merlangius merlangus euxinus* (Nordmann, 1840) (Gadidae) infected by parasite nematode *H. aduncum*. For this purpose we use the analysis of physiological indices of the liver, spleen and condition factor and the antioxidant enzyme activities in red blood cells and in the liver as biomarkers of healthy and infected fish.

Materials and Methods

Materials

Whiting *Merlangius merlangus euxinus* was caught in winter period 2010 in the coastal waters in the Sevastopol region (Black Sea, Crimea). Animals were immediately placed in the aerated tanks containing original marine water and transported to the laboratory. The fish were processed according the protocol approved by the Scientific Commission of IMBR RAS.

Parasitological determinations

Complete parasitological dissection for Anisacide parasites was immediately performed using the light microscope. Parasitic infection of fish was determined according the standard parasitological methods of parasites estimation Invasion characteristics such as prevalence (percentage of infected host individuals in each sample), intensity of infection (number of parasites per infected host, min-max), and abundance (mean number of parasites per host individual in each sample) were determined (Gaevskaya, 2005).

Biometric determinations

Fish were individually measured, weighed and captured. The total, standard lengths, weight of fish and soma weight, weight of liver and spleen were measured. Liver and spleen were weighted for somatic indices calculations as following (Anisimova & Lavrovsky, 1983):

Hepatosomatic indices (HSI) were calculated according the formula:

$$HSI‰ = \frac{Wl}{Ws} * 1000 \qquad (1)$$

Spleen somatic indices (SSI) were calculated according the formula

$$SSI\% = \frac{Wsp}{Ws} * 100 \qquad (2)$$

Condition factors (CF) were calculated using value of soma weight from the equation:

$$CF\% = \frac{Ws}{Ls^3} * 100, \qquad (3)$$

where Wsp, Wl, Ws – spleen weight, liver weight and soma weight in grams of fish; Ls - standard length in centimeters.

Preparation of blood and liver samples

Blood was collected from the ventral artery using needle syringe or Pasteur pipette. Whole blood was collected and serum was separated within 24 hrs of collection in refrigerator at +4^{0}C. After blood collection fish were dissected and the liver was quickly removed and stored on ice. The organ was washed in the cold 0.85% NaCl solution several times, then homogenized in the cold physiological solution (1:5 w/v) using glass homogenizer. The resulting homogenate was centrifuged at 8000 g for 20 min.

Blood samples used for measuring antioxidant activity immediately after liver preparation. Specifically, the sediments of red

blood cells (RBC) were washed three times with cold 0.85% NaCl solution and then lysed by addition of 5 vol of distilled water and stored for 24 hrs at +4^{0}C as we described previously (Rudneva et al., 2014). The enzyme activity was then determined in the RBC lysates immediately after preparation. Spectrophotometer Specol-211 (Carl Zeiss, Germany) was used for all biochemical determinations.

Enzymatic activities assays

Antioxidant activity (SOD, CAT, PER, GR): Antioxidant activities in the liver extracts and red blood cells from the 3 species in this study were determined according to methods described previously (Rudneva et al., 2012; 2014), with a few minor modifications. The number of enzyme activity determinations in healthy and infected fish RBC and liver was shown in Table 1.

Specifically, superoxide dismutase (SOD) was assayed on the basis of inhibition of the reduction of nitroblue tetrasolium (NBT) with NADH-mediated by phenazine methosulfate (PMS) under basic conditions (Nishikimi et al., 1972). All measurements were performed in 0.017 M sodium pyrophosphate buffer pH 8.3 at 25^{0}C. The reaction mixtures contained 5 μM NBT, 78 μM NADH, 3.1 μM PMS, and a 0.1 mL sample; the final volume was 1.5 mL. The reactivity was measured using OD at 560 nm.

Catalase (CAT) was measured using a previously described method that involves a hydroperoxide reduction.

Peroxidase (PER) activity was detected by spectrophotometry at 600 nm using benzidine reagent (Litvin, 1981). Specifically, the reaction mixture contained 1 ml acetate buffer pH 5,4, 0.4 ml 0.09% benzidine, 0.2 ml 0.03% H_2O_2, and 0.2 ml sample.

Glutathione reductase (GR) activity was assayed spectrophotometrically using a method modified after Goldberg and Sparner (Goldberg & Sparner, 1987). The reaction mixture contained 0.1 mL mM NADPH, 0.5 mL 7.5 mM oxidized glutathione, 0.2 mL mM EDTA, and 2 mL 0.05 M phosphate buffer pH 8.0. After incubation for 10 min at room temperature, the OD of the mixture was determined at 340 nm.

Glutathione-S-transferase (GST) activity was determined by the method of Habig et al. (1974) by following the increase in absorbance at 340 nm due to the formation of the conjugate 1-chloro-2,4-dinitrobenzene (CDNB) using as substrate at the presence of reduced glutathione (GSH).

Statistical analysis

Biochemical measurements were detected in duplicate for each sample. The number of tested individuals ranged from 7 to 21 animals (Table 1). Simple, descriptive statistics were performed using an ANOVA to determine means (+/-SEM) (Halafyan, 2008). P value of 0.05 was used for determination of statistical significance in all cases. The graphs were made using the computer program EXELL.

Table 1. Number of analyses of examined biochemical characteristics of whiting caught in Sevastopol coastal waters (Black Sea, Crimea)

Enzyme activities	Blood		Liver	
	healthy	infected	healthy	infected
SOD	7	13	10	20
CAT	7	14	7	11
PER	7	13	7	11
GR	7	12	9	21
GST	7	13	9	12

Results

Parasitological characteristics of fish

The examination of stomach, intestinal and abdominal cavity contents revealed that approximately 70% of tested fish were infected with nematode. The main parasitological characteristics are present in Table 2.

The values of intensity of infection were approximately similar in infected fish organs, the highest abundance was shown in stomach and prevalence was greater in intestine than in other tested organs.

Table 2 Parasite abundance, intensity of infection and prevalence of whiting infected by *H. aduncum*

Localization	Intensity of infection, (min-max)	Abundance	Prevalence , %
Intestine	1-4	0.85	43
Stomach	1-6	0.71	62
Abdominal cavity	1-9	0.57	14

Biometric characteristics of infected and healthy fish

Biometric values of healthy and infected animals are present in Figure 1.

The HSI value was significantly higher in infected fish than in healthy ones, while SSI demonstrated the opposite trend. CF in parasitized fish was not significantly differed from those in healthy animals.

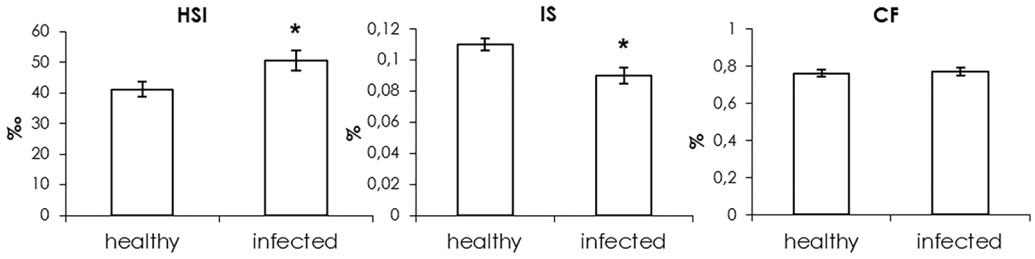

Figure 1. Biometric characteristics of whiting healthy (n= 15) and infected (n = 48) by parasites (HSI – hepatosomatic index, SSI –spleen somatic index, CF - condition factor, Mean ±SEM, * - significant differences, p<0.05)

Antioxidant enzyme activities in fish RBC and in the liver

SOD, CAT and GST activities were significantly higher in RBC in infected fish than in healthy ones. GR activity tended to grow in parasitized whiting, while no significant difference in PER activity was observed in RBC between infected and non-infected fish (Figure 2).

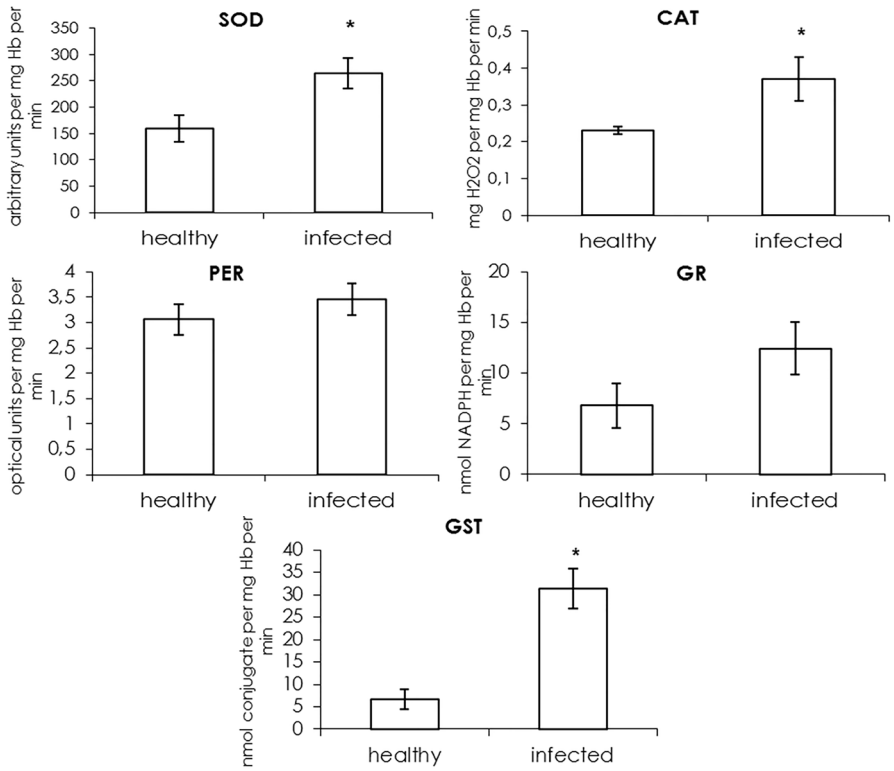

Figure 2. RBC antioxidant enzyme activities in whiting healthy and infected by parasites (SOD – superoxide dismutase, CAT – catalase, PER – peroxidase, GR – glutathione reductase, GST – glutathione-S-transferase, Mean ±SEM, * - significant differences, p<0.05).

CAT and PER activities in the liver of parasitized fish was significantly higher as compared with the values of healthy animals; whereas GST activity was significantly lower. GR activity tended to increase in infected fish, while SOD activity tended to decrease in infected fish. However, the differences were not significant (Figure 3).

Therefore, the antioxidant enzyme response was not uniform in infected fish in RBC and liver and demonstrated tissue specific peculiarities.

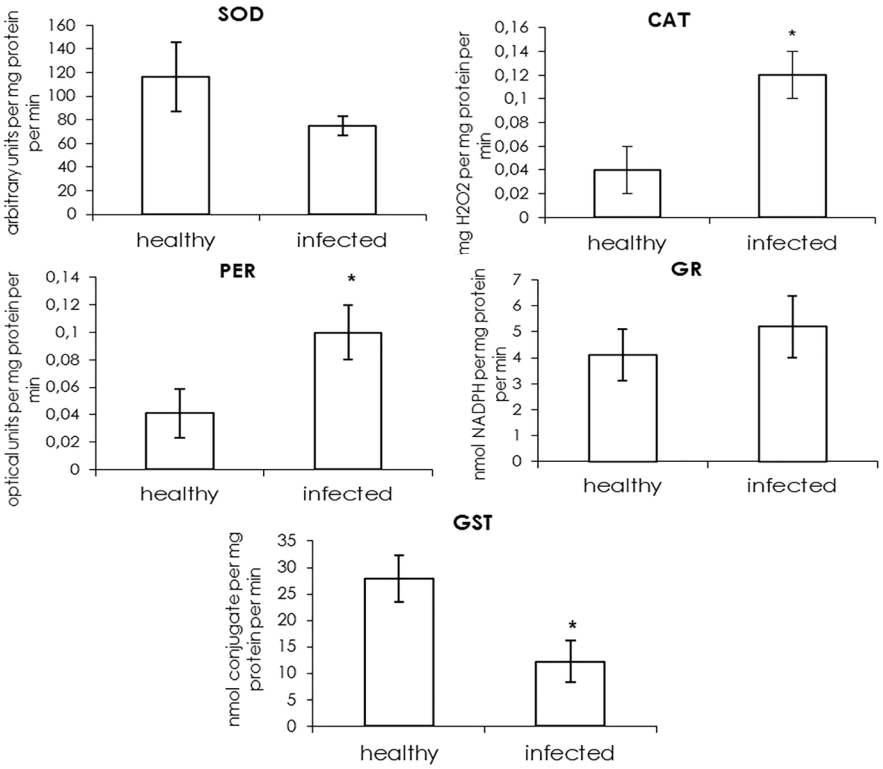

Figure 3. Hepatic antioxidant enzyme activities in whiting healthy and infected by parasites (SOD – superoxide dismutase, CAT – catalase, PER – peroxidase, GR – glutathione reductase, GST – glutathione-S-transferase, Mean ±SEM, * - significant differences, $p<0.05$).

Discussion

The evaluation of animal's health, especially wild fish populations has required the development of biological markers of their status in different levels of their biological organization. Sensitivity or resistance of the wild life to various stressors depends on many exogenous and endogenous factors and for the first time, on the status of defense mechanisms, which protect an organism against diseases and environmental pollution caused anthropogenic impact. Generally, various biochemical and biometrical parameters are used for the evaluation of fish health. They often reflect physiological status of the animal and the influence of certain biotic (pathogens, parasites, disease state, etc) and abiotic factors (anthropogenic pollution, habitat degradation, etc) on it (Martinez-Alvarez et al., 2005; van der Oost et al., 2003; Fonseca et al., 2011). Among them biometrical parameters of the liver, spleen and condition factor, blood and hepatic biochemical characteristics are used widely as early warning indicators of changes health status (Haman et al., 2012).

The main tissues and organs which respond to unfavorable living conditions are blood, spleen and liver. They play an important role in fish defense against contamination, diseases and parasite infection. HSI and SSI are simple and informative parameters characterizing fish response to stressors insult, including parasites, because parasites act as an important factor modulating the levels of fish physiology and metabolic activity.

Liver plays an important role in the uptake, biotransformation and detoxification of pollutants and pathogens and HSI reflects the fish

health status. In healthy carp with normal liver HSI were significantly lower as compared with the histopathological livers (Kaptaher et al., 2014). Enlargement in liver size in the juvenile Arctic charr *Salvelinus alpinus* infected by *Diplostomum spp.* compared to healthy fish was reported by Seppänen et al. (2009). Chronic parasite infection resulted in a decrease in standard metabolic rate. The authors propose, that higher liver mass in infected *S. alpinus* may have been related to disorders in energetic function, which could have had major effects on biochemical regulation by the liver, especially with a possible reduction in insulin sensitivity in tissues, which resulted in ineffective glucose consumption and damage lipid metabolism. Besides that, elevation of metabolic activity in the liver caused the increase of the energetic costs for detoxification, may also stimulate the growth of the liver mass and HIS value correspondingly. To the other hand, no changes in HSI were observed in the fish from reference and polluted marine sites (Cazenave et al., 2014). In agreement with the aforementioned studies, our findings show that HSI is significantly higher in infected fish related to healthy individuals, that may be explained the increase of toxic impact on the liver and its damage.

Immune system is the main defense system, the basic function of which is to protect an organism against infection in order to minimize the fitness costs of being infected (Rohlenová, et al., 2011). The main immune organ in fish is well-developed spleen, its size and spleen somatic index are widely used as a simple measurable immune parameter with a potential role in response against parasite infection (Taskinen & Kortet, 2002; Kortet & Taskinen, 2004). For instance, in carp significant relationship was observed between the abundance of digeneans and SSI. Positive relationship was found between larval digeneans of *Diplostomum* species and relative spleen size. A weak correlation was shown between spleen size and abundance of trematodes, while significant relationship between condition factor and spleen size did not note (Rohlenová et al., 2011). In our present study SSI in parasitized fish was significantly lower than in healthy ones, which may by explained with the toxic impact on the organ by parasite metabolites. Contradictory, enlargement in spleen mass in infected Arctic charr *Salvelinus alpinus* was documented by Seppänen et al. (2009). Authors explained this phenomenon by increased leucocyte synthesis. The links between spleen size and parasites infection have been tested in many intraspecific studies (Taskinen & Kortet, 2002; Kortet & Taskinen, 2004). However, in several studies any associations between spleen size and parasite counts did not been found. The authors suggest that determination of relationships between immune variables and parasite infection needs further investigations (Vainikka et al., 2009).

CF values did not present significant variations among healthy and parasitized fish. Thus we could propose that fish adapted to parasites invasion and the changes of their physiological indices are the response to infection. We suggested also that *H. aduncum* infection in whiting might not impose direct energetic costs, but it may weaken the efficiency of energy metabolism, which agree with the studies of Seppänen et al. (2009). Thus, CF was not sensitive parameter for parasitized invasion. Several researchers also documented that this parameter was not sensitive for pollution level in the environment (Sadauskas-Henrique et al., 2011; Cazenave et al., 2014).

Many endogenous and exogenous factors cause oxidative stress in living organisms. To protect against oxidative stress caused many kinds of biotic and abiotic agents, including parasite infection, organisms have developed different mechanisms such as the induction of antioxidant enzymes SOD, catalase CAT, peroxidase PER and glutathione related enzymes (GR, glutathione peroxidase (GP) and GST) (Livingstone, 2001). Parasite infections have been reported to change biotransformation system (Armstrong et al., 1993), immune response (Mikraykov & Silkina, 2006) and antioxidant activities (Dautremepuits et al., 2003). Antioxidant and detoxifying enzymes have been widely used as biomarkers of oxidative stress in fish and invertebrates that were exposed to xenobiotics in experimental conditions or inhabited polluted environment (Rudneva et al., 2012;

Kaptaher et al., 2014). Parasite invasion is also stressed organism and may stimulate reactive oxygen species (ROS) production. Our previous studies also demonstrated the modification of antioxidant enzyme activities in the liver and muscle of Black Sea turbo *Psetta maxima maeotica* infected by cestode *Botriocephalus gregarious* (Rudneva et al., 2004). Antioxidants are very useful in the evaluation of health status, however, the response of antioxidant system on parasite infection in various fish species is not uniform, and it depends on fish species and parasites specificity.

Comparison of the obtained data revealed the significant influence of parasite invasion on antioxidant status of whiting tissues. Our data agree with the results of several authors demonstrating the modulations in antioxidant enzymatic activities in fish by parasites invasion. However, the response of antioxidant system in parasitized fish is not uniform and it depends on parasite species, tested tissues and specificity of enzyme. In additional, environmental pollution may modulate the antioxidant response of parasitized fish (Dautremepuits et al., 2002; Hursky & Pietrock, 2015). Blood and hepatic biochemical characteristics are used widely as early warning indicators of changes physiological and health status (Haman et al., 2012).

Parasitic invasion is often associated with the bacterial infection, inflammation and oxidative stress in hosts (Forlenza et al., 2008; Bello et al., 2000; Pinlaor et al., 2008; de Faria et al., 2014). The response in host is characterized by enhancement of oxygen consumption in leykocytes ("respiration burst"). Superoxide anion-radical involves on detoxification reactions and simultaneously it is the source of H_2O_2, OH^-, HOCl radicals, involving into biotransformation process. However, in our study the response of antioxidant system in host was not uniform in the red blood cells and in the liver.

Increase of antioxidant enzyme activities of CAT, PER and GR were shown both in the liver and in the RBC in infected fish. Our results agree with the data of Dautremepuits et al. (2002; 2003), who documented the elevation of enzymatic activity in the liver and head kidney of carp parasitized by cestode *Ptychobothrium* sp., compared with healthy fish. Our previous results also demonstrated the induction of antioxidant enzymatic activities in the liver and muscle of Black Sea *Psetta maxima maeotica* infected by cestode *Botriochephalus gregarious* (Rudneva et al., 2004).Contradictory, decrease of the total antioxidant activity and increase of lipid peroxidation were shown in the blood and liver in *Abramis brama* infected by *Ligula intenstinalis* L. (Cestoda) (Mikraykov & Silkina, 2006) and in the muscle of Black Sea sprat infected by *H. aduncum* (Skuratovskaya & Zav'yalov, 2006). A significant increase of the level of TBA-reactive substances and decrease of the concentration of total thiol groups in the gills of the *Carassius auratus* parasitized by *Dactylogyrus spp* were observed (Mozhdeganloo & Heidarpour, 2014). The investigators also demonstrated the inhibition of chemiluminescence of host's phagocytes (*Paralichthys olivaceus*) with tissue extracts of parasite *Uronema marinum* (*Ciliophora, Scuticociliatidae*) (Kwon et al., 2003). At the other hand, no differences of antioxidant enzyme activities CAT and SOD were observed in the muscle of *Rhamdia queken* infected by *Clinostomum detruncatum* (Bello et al., 2000).

In our study we found the significant increase of SOD and GST activity in RBC of parasitized fish, while the enzymatic activity in the liver tended to decrease as compared with healthy animals. SOD is a key antioxidant enzyme which inactivates superoxide anion via the reaction of dismutation, GST plays a protective role against oxidative stress, because its ability to detoxify some of the secondary ROS intermediates. SOD and GST activities were strongly correlated with increased resistance to oxidative stress. High antioxidant enzyme activities in RBC together with the high activity of GST demonstrated the protective effect of antioxidant system of the host against parasitic infection. In addition, we could proposed, that increase of antioxidants in RBC may strengthen by the additional bacterial infection associated with parasites (Adeyemi, 2014). High SOD activity in RBC could be explained by the increase of the ROS generation caused inflammation or parasites metabolites presence.

Thus, in RBC we showed the uniform

response of antioxidant defense system, characterized the increase of the activity of tested enzymes. This response could be attributed with the involving of parasites metabolites and, perhaps, together with parasites bacterial infection in the blood and the inhibition of their toxic effects by biotransformation system. Contradictory, the hepatic GST activity was significantly lower in the infected fish as compared with the healthy animals, which could be explained with the high intoxication or with inflammation, caused damage of the hepatocytes in the host liver.

Therefore, we could suggest that the response of antioxidant system on parasitic invasion depended on fish species, parasites specificity, tested tissues, fish resistance and peculiarities of host – parasite interaction, and, perhaps on the intensity of infection and parasites abundance. Our findings show the tissue-specific response of the antioxidant system in Black Sea whiting infected by nematode *H. aduncum*.

Conclusions

The findings of this study show the changes of physiological and biochemical status of Black Sea whiting infected by nematode *H. aduncum*. The response of the host tissues on parasitic infection was not uniform. The antioxidant response on oxidative stress caused parasitic infection was tissue specific. It was more pronounced and uniform in RBC which was attributed with the involving parasite intermediates in the blood stream and (or) the associated with the parasites bacterial infection, caused inflammation. In the liver the trends of antioxidant enzyme activities were different which could depend on intensity of infection and abundance of the parasites. Because parasites are often used to assess environmental change due to stressors or anthropogenic disturbance the further investigations of the influence of the degree of parasite infection on fish are required.

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