Arch. Biol. Sci., Belgrade, 62 (1), 97-105, 2010 DOI:10.2298/ABS1001097R

SUPEROXIDE DISMUTASE AND CATALASE ACTIVITIES IN THE LIVER AND MUSCLE OF BARBEL (*BARBUS BARBUS*) AND ITS INTESTINAL PARASITE (*POMPHORYINCHUS LAEVIS*) FROM THE DANUBE RIVER, SERBIA

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Abstract – The activities of total superoxide dismutase (Tot-SOD), manganese-containing superoxide dismutase (Mn-SOD), copper zinc containing superoxide dismutase (Cu/Zn-SOD) and catalase (CAT) protein concentration, as well as protein and SOD electrophoretic profiles in the liver and muscle of barbel (*Barbus barbus*) and its intestinal parasite *Pomphoryinchus laevis* from the river Danube, within the suburban area of Belgrade, Serbia (the stretch between Višnjica and Grocka) in spring and summer were investigated. Specific activities of Tot-SOD, Mn-SOD, Cu/Zn-SOD were higher in spring, while specific CAT activity was higher in summer in all investigated samples. Temperature influence on the antioxidant defense enzymes in barbel tissue and in its intestinal parasite *Pomphoryinchus laevis,* as well as seasonal patterns, are evident. Our work represents the first study of SOD and CAT activities in the barbel and its intestinal parasites and shows that barbel and acanthocephalans are very useful for biomonitoring studies in aquatic ecosystems.

Key words: Barbel, parasite, liver, muscle, biomarkers, oxidative stress, antioxidant enzymes, season.

UDC 597.551.2(282.243.7):576.89

INTRODUCTION

The antioxidative defense system (AOS) is necessary for the maintenance of redox homeostasis in organisms. Oxygen free radicals and other reactive oxygen species (ROS) can react with the main cellular components, thus damaging tissues and causing oxidative stress, which includes oxidation of proteins, DNA, as well as peroxidation of unsaturated lipids in cell membranes. AOS includes enzymatic and nonenzymatic components that neutralize ROS that are continuously produced during aerobic metabolism. Enzymatic components are superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and biotransformation phase II enzyme glutathione-S-transferase (GST). These enzymes have been proposed as biomarkers of contaminant or seasonally mediated oxidative stress in a variety of marine and freshwater organisms and their induction reflects a specific response to pollutants (Borković et al., 2005).

Of these enzymes, SOD is an oxido-reductase which catalyzes the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide (Fridovich, 1989). This enzyme exists as several isotypes, characterized by their redox-active metals at the catalytic sites. CAT detoxifies hydrogen peroxide and has no electron donor requirement. Although CAT is a well-known antioxidative enzyme and has been implicated in protection against hydrogen peroxide, its localization is limited to the peroxisome (Kono and Fridovich, 1982).

As thermoconformer organisms, most fish must overcome environmental temperature changes and consequently the changes in their metabolic rate. The relatively high antioxidant defense (AD) levels

that typify fish, even when compared to endotherms such as birds and mammals, protect against the consequences of temperature oscillations.

In this study, barbel (Barbus barbus L.) was chosen as a suitable test-organism in biomonitoring studies owing to its wide geographical distribution and benthic way of life (Kennedy, 1997). Barbel has an intestinal parasite Pomphoryinchus laevis (Acanthocephala), (Nachev and Sures, 2009). It has not been widely investigated in the study of AOS, but literature data show that it is very useful for biomonitoring studies in aquatic ecosystems (Sures, 2003). Barbel represents the final host of the adult worms that live inside its intestine. Fish become infected when feeding on crustaceans that contain infective larvae in their hemocoels (Taraschewski, 2000).

The liver was chosen for the present study due to its responsibility for the regulation of overall body metabolism and thereby intense involvement in the detoxification of xenobiotics. The muscle is important because of its consumable value. The parasite was chosen because some literature data show that it is very useful as a bioindicator in heavy metal pollution (Sures, 2003). Thus, the evaluation of antioxidant responses in these tissues and parasite is highly relevant since toxic chemicals, causing temporary or permanent disturbance of homeostasis, can disrupt their functions (Miller, 2002).

The aim of this study was to investigate and compare the activities of total superoxide dismutase (Tot-SOD, EC 1.15.1.1), manganese-containing superoxide dismutase (Mn-SOD), copper/zinc-containing superoxide dismutase (Cu/Zn-SOD) and catalase (CAT, EC 1.11.1.6), the total protein concentration, as well as protein and SOD electrophoretic profiles in the liver and muscle of barbel (*Barbus barbus,* L.) and in its intestinal parasite *Pomphoryinchus laevis* from the Danube river. The metabolic activities of fish are influenced by changes in abiotic and biotic factors that depend on season, thus the purpose of this study was to compare the activity of AOS components in barbel in spring and summer.

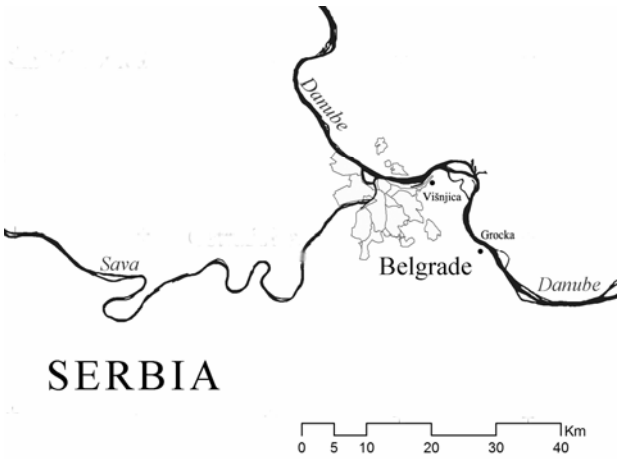

Figure 1. The geographical position of specimen collection from the Danube river.

MATERIAL AND METHODS

Site description, sample collection and preparation

Barbels (*Barbus barbus* L.) naturally infected with acanthocephalans *Pomphoryinchus laevis* were caught by a local fisherman in the Danube river in the suburban area of Belgrade – the stretch between Grocka (44° 42, 256' N, 20° 42, 314' E - 44° 40, 824' N, 20° 43, 922' E, RKM 1135) and Višnjica (44° 50, 836' N, 20° 34, 296' E - 44° 49, 716' N, 20° 91, 398' E, RKM 1158) in summer (August) and spring (April), (Fig. 1).

At Grocka, 10 specimens were collected with an average length of 36.20 ± 0.92 cm and average mass of 473.70 ± 33.08 g. At Višnjica, 14 specimens were collected with an the average length of 41.07 ± 1.55 cm and average mass of 835.0 ± 118.40 g. All collected specimens were males. The fish were brought alive to the laboratory and then killed by a blow to the head while kept on ice, where the tissue samples (liver and muscle) were isolated. Intestinal tissue adjacent to the acanthocephalans was removed, avoiding the remains of worms. Tissue samples and parasites were frozen at -80°C until

further processing. Sigma Chemicals (St. Louis, MO, U.S.A.) were used for the analyses.

The barbel tissues and parasites were minced and homogenized in 5 volumes (Lionetto et al., 2003) of 25 mmol/L sucrose containing 10 mmol/L Tris-HCl, pH 7.5 at 4°C with an Ultra-Turrax homogenizer (Janke and Kunkel, IKA-Werk, Staufen, Germany), (Rossi et al., 1983). The homogenates were sonicated for 30 s at 10 kHz on ice to release enzymes. The sonicates were centrifuged in a Beckman ultracentrifuge at 4°C at 100 000 x g for 90 min (Takada et al., 1982), and the resulting supernatants were used for further biochemical analyses.

All enzyme activities were measured simultaneously in triplicate for each sample using a Shimadzu UV-160 spectrophotometer and a temperature controlled cuvette holder. The activity of Tot-SOD was assayed by the epinephrine method (Misra and Fridovich, 1972) based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline at 26°C. The activity of Mn-SOD was obtained after the inhibition of Cu/Zn-SOD with KCN. Cu/Zn-SOD activity was calculated as a difference between Tot-SOD and Mn-SOD activities. Total protein concentration was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a reference and expressed in mg/mL protein. Protein electrophoretic profiles were examined by the standard method of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), (Laemmli, 1970). SOD electrophoretic profiles were examined using nitroblue tetrasolium (NBT) by the method of Mavelli et al. (1984).

CAT activity was evaluated by the method of Claiborne (1984), which is based of H_2O_2 degradation by the action of CAT contained in the examined samples. All enzyme activities (SOD and CAT) were expressed as specific activities (U/mg protein).

Table 1. Protein concentration (mg/mL protein) in the liver and muscle of the barbell *Barbus barbus* and in its intestinale parasite *Pomphoryinchus laevis* from the Danube River in spring and summer season.

Protein concentration (mg/mL protein)			
	Liver	Muscle	Parasite
Spring	14.03 ± 0.29	9.22 ± 0.17	10.89 ± 0.49
Summer	13.19 ± 0.33	11.73 ± 0.26 *	10.40 ± 0.44

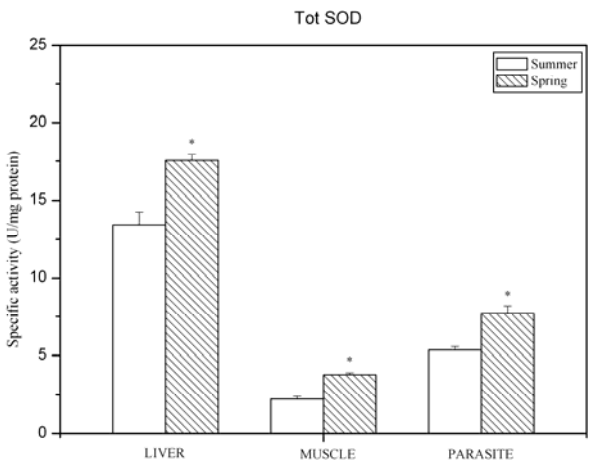

Figure 2. The specific activity (U/mg protein) of total superoxide dismutase (Tot-SOD) in the liver and muscle of barbel *Barbus barbus* and in its intestinal parasite *Pomphoryinchus laevis* from the river Danube in summer and spring. The data are expressed as the mean ± S.E. The non-parametric Mann-Whitney *U*-test was used to establish significant differences between the means. A minimum significance level of $p<0.05$ was accepted.

Statistical analyses

The data are expressed as mean ± Standard Error (S.E.). The non-parametric Mann-Whitney U-test was used to search for significant differences between means. A minimum significance level of $p<0.05$ was accepted. Analytical protocols described by Darlington et al., 1973 and Dinneen and Blakesley (1973) were followed.

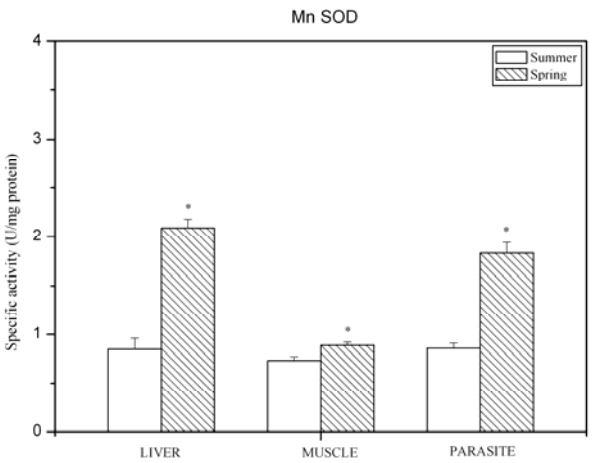

Figure 3. The specific activity (U/mg protein) of manganese containing superoxide dismutase (Mn-SOD) in the liver and muscle of barbel *Barbus barbus* and in its intestinal parasite *Pomphoryinchus laevis* from the Danube in two seasons: summer and spring. The data are expressed as the mean ± S.E. The non-parametric Mann-Whitney *U*-test was used to establish significant differences between the means. A minimum significance level of $p<0.05$ was accepted.

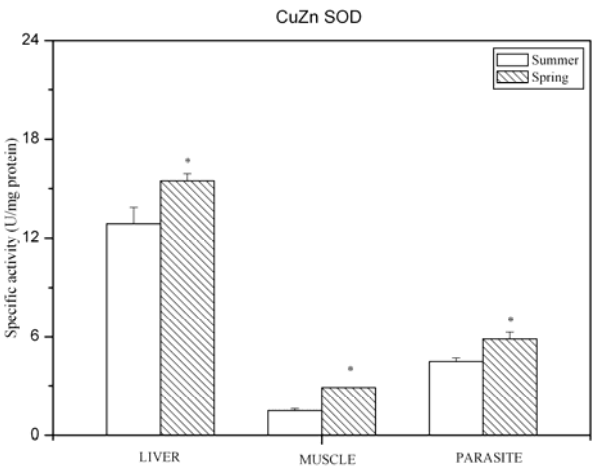

Figure 4. The specific activity (U/mg protein) of copper zinc containing superoxide dismutase (Cu/Zn-SOD) in the liver and muscle of barbel *Barbus barbus* and in its intestinal parasite *Pomphoryinchus laevis* from the Danube river in two seasons: summer and spring. The data are expressed as the mean ± S.E. The non-parametric Mann-Whitney *U*-test was used to establish significant differences between the means. A minimum significance level of $p<0.05$ was accepted.

RESULTS

Protein concentration in the liver, muscle and parasite of the barbel *Barbus barbus* is shown in Table 1. The obtained results in the muscle demonstrate significantly higher protein concentration in the summer season in respect to spring ($p<0.05$). There are no considerable differences in specific protein concentrations between the seasons in the liver and parasites.

The results of our investigations show that the specific activities of Tot-SOD (Fig. 2), Mn-SOD (Fig. 3) and Cu/Zn-SOD (Fig. 4) were considerably higher in the liver, muscle and parasite of barbel in the spring ($p<0.05$) compared to the summer.

Contrary to these results, specific CAT activity was higher in the liver, muscle and parasite ($p<0.05$) in the summer than in the spring (Fig. 5).

Electrophoretic analysis of proteins shows characteristic protein profiles in the barbel liver, muscle and the barbel parasite *Pomphoryinchus laevis* in summer (Fig. 6) and spring (Fig. 7).

SOD electrophoretic profiles show typical distribution patterns of SOD isoform activities in all investigated barbel tissues and in the barbel parasite in both seasons, revealed by the NBT method (Fig. 8, Fig. 9).

DISCUSSION

The rates of physiological and biochemical processes can considerably fluctuate over the year depending on temperature, food availability, growth rates of organisms and the stages of reproductive cycle (Aleshko and Lukyanova, 2008). Fish, being poikilotherms, are strongly influenced by water temperature; therefore they continuously adjust to environmental conditions. They are widely used in biomonitoring studies. Near-bottom species are preferred because they are in the contact with bottom substrates, where pollutants are accumulated (Aleshko and Lukyanova, 2008).

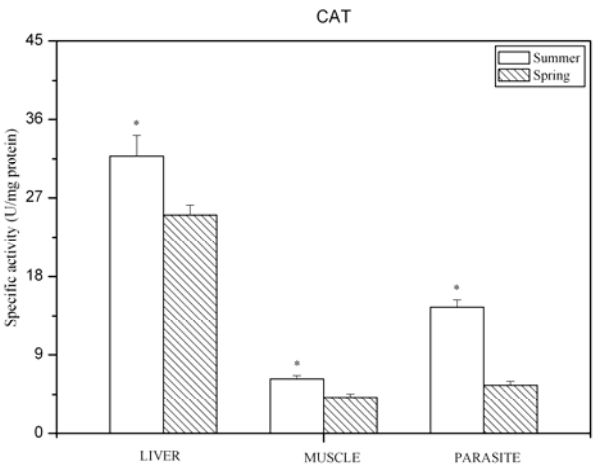

Figure 5. The specific activity (U/mg protein) of catalase (CAT) in the liver and muscle of barbel *Barbus barbus* and in its intestinal parasite *Pomphoryinchus laevis* from the Danube in two seasons: summer and spring. The data are expressed as the mean ± S.E. The non-parametric Mann-Whitney *U*-test was used to establish significant differences between the means. A minimum significance level of $p<0.05$ was accepted.

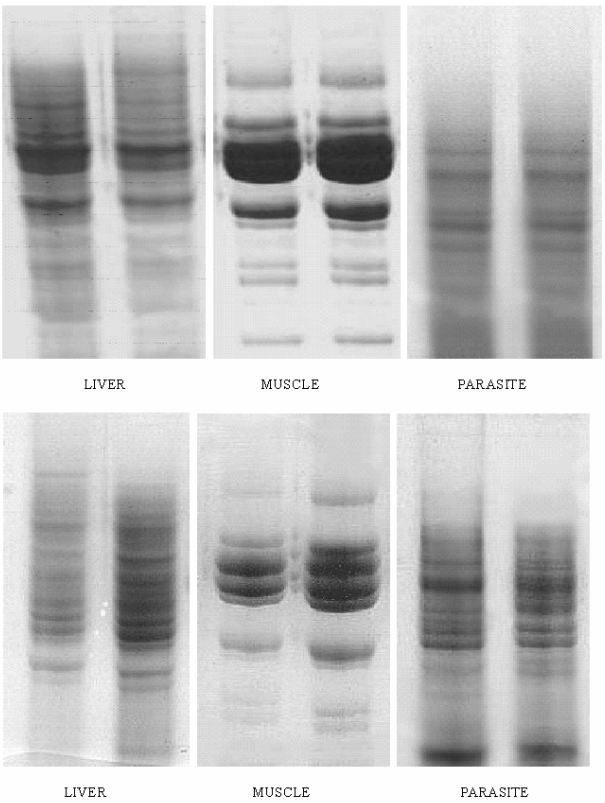

Figure 6. SDS-PAGE analysis of proteins in summer (A) and spring (B) in the liver and muscle of barbel *Barbus barbus* and in its intestinal parasite *Pomphoryinchus laevis* from the Danube.

The body composition of fish is well known to change in response to seasonal, reproductive and environmental conditions (Dygert, 1990). Temperature changes either from low to higher or from high to lower result in alterations of enzyme activities as has been shown for fish (Pavlovic et al., 2004) and mussel (Borković et al., 2005). It is known that a temperature rise induces a higher metabolic rate, oxygen consumption, ROS formation and oxidative stress, and theoretically, a low temperature could reduce the metabolic activities and thereby lower enzymatic activities in general. Yet, a temperature decrease from 28°C to 18°C in zebrafish also caused oxidative stress (Malek et al., 2004).

Data on seasonal variations of fish antioxidative defense are quite rare (Meyer et al., 2003). This study reveals seasonal-specific alterations in SOD and CAT activities. We show that in April the specific activities of the Tot-SOD, Cu/Zn-SOD and Mn-SOD of barbel were higher than in August in both the tissues and in the parasite. The solubility of oxygen increases in cold temperatures and there is a direct relationship between ROS production and the partial pressure or concentration of oxygen (Jamieson et al., 1986), which contributes to the enhanced antioxidant enzyme activities compared to August. Solé et al. (1995) reported the seasonal dynamics of antioxidant enzyme activities of the mussel Mytilus galloprovincialis. They observed minimal activities in June and maximal activities in April in a six-month study of SOD and CAT and concluded that the observed seasonal variations were related to the metabolic status of the animal itself depending on such factors as food availability, gonadal maturation and the hydrological cycle, which regulated productivity in the area. Lau et al. (2004) measured antioxidative and biotransformation enzyme activities in Perna viridis and determined higher enzyme activities in winter samples,

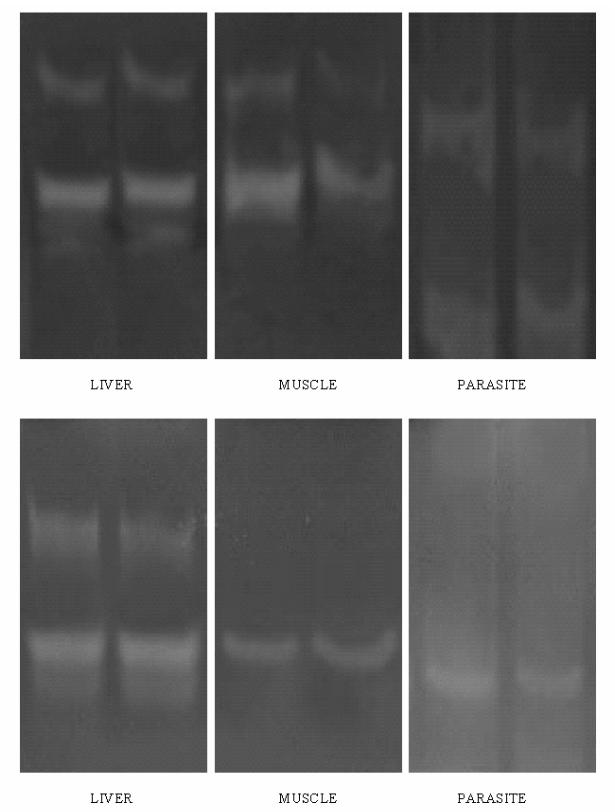

Figure 7. Superoxide dismutase (SOD) electrophoresis in summer (A) and spring (B) in the liver and muscle of barbel *Barbus barbus* and in its intestinal parasite *Pomphoryinchus laevis* from the Danube.

which was explained by the concentration effect as a result of the generally decreased protein level. Another cause might be the stress induced by chemicals from the water.

CAT also shows seasonal-specific alterations in activities but, unlike SOD, the specific activities were lower in April than in August in both tissues and parasites. Kopecka and Pempkowiak (2008) obtained the same results. In their study, the activity of catalase in the liver of the flounder Platichthys flesus was higher in August than in April. Bocchetti et al. (2008) obtained the opposite results. They measured CAT activities in the digestive gland of the clam, Tapes philippinarum, and mussels, Mytilus galloprovincialis, from the Adriatic Sea and the enzyme activity increased in spring in comparison to summer.

The enzyme system SOD-CAT represents the first line of defense against free radicals. SOD catalyzes the dismutation of the superoxide anion radical. As a result, H2O2 is produced and decomposed by the CAT. It is usual to expect a simultaneous induction of SOD and CAT, but not in our case. In the present study, the SOD level was low in summer and the CAT level was high whereas the SOD level was high in spring and the CAT level was low. The low levels of CAT could be attributed to an increased production of superoxide anion radical which has been reported to inhibit CAT activity in case of excessive production (Kono and Fridovich, 1982).

It should be emphasized that barbel adults spawn once a year in mid- or late spring (April-June, exceptionally July) (Hancock et al., 1976), therefore these changes in enzyme activities could also implicate the intense reproductive activity that occurs in that period. In support of our results, Tripathi and Verma (2004) showed seasonal fluctuations in the levels of some metabolic enzymes during the annual reproductive cycles of fish.

In addition to seasonal variations, reproductive status and pollution, other factors also have a great impact on the physiological levels of these biomarkers and could also explain some of the variations observed for AOS components and protein concentration. These factors are food availability (Pascual et al., 2003), dissolved oxygen concentration (Cooper et al., 2002), age (Sanz et al., 2001), light intensity (Fitzgerald, 1992) and swimming activity (Filho et al., 1993).

Antioxidant enzymes are rarely investigated in parasites. These enzymes are essential for parasites to defend themselves against the ROS generated by the macrophages, neutrophils and eosinophils of the host, in addition to their normal functions in aerobic organisms (Sies, 1993). These enzymes may be particularly important for long-lived parasites. The SOD and CAT have been quite extensively

characterized in parasitic nematodes (Selkirk et al., 1998).

In our study, we found seasonal changes in SOD and CAT activities in the parasite *Pomphoryinchus laevis*. Both these enzymes had the same trend in activity as the enzymes in barbel tissues. We assume that these changes in the enzyme activities in a parasite could be a consequence of seasonal changes in its host. The investigation of this enzyme system represents a major advance towards the understanding of how parasitic acanthocephalas deal with both internal and environmental oxidative stress.

The analysis of protein and SOD electrophoretic profiles shows strong seasonal characteristics in the liver and muscle of the barbel *Barbus barbus* and the parasite *Pomphoryinchus laevis.*

In conclusion, to our knowledge, this is the first report on SOD and CAT activities in the liver and muscle of barbel and in its intestinal parasite *Pomphoryinchus laevis* on the Serbian bank of the Danube. From the presented results it can be concluded that the seasonal pattern of AD found in *Barbus barbus* seems to be closely correlated with the seasonal variations of temperature and the reproductive cycle. Seasonal influences on AD and biotransformation enzymes need to be better understood in order to make further deductions regarding thermoconformers.

Acknowledgements – This study was supported by the Ministry of Science and Technological Development of the Republic of Serbia, Grants Nos. 143035B and 143023B. The authors are thankful to Radmila Paunović Štajn, MSc, for proofreading the manuscript.

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АКТИВНОСТ СУПЕРОКСИД-ДИСМУТАЗЕ И КАТАЛАЗЕ У ЈЕТРИ И МИШИЋУ МРЕНЕ (*BARBUS BARBUS*) И У ЊЕНОМ ЦРЕВНОМ ПАРАЗИТУ (*POMPHORYINCHUS LAEVIS*) ИЗ ДУНАВА, СРБИЈА

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Одређивали смо сезонску динамику активности ензима антиоксидационе заштите: укупне супероксид-дисмутазе (Tot-SOD), манган садржавајуће супероксид-дисмутазе (Mn-SOD), бакар цинк садржавајуће супероксид-дисмутазе (Cu/Zn-SOD) и каталазе (CAT), специфичну концентрацију протеина, као и електрофоретски профил протеина и SOD у јетри, мишићу речне мрене (*Barbus barbus*) и њеном цревном паразиту *Pomphoryinchus laevis* из Дунава, у пролећној (Вишњица) и летњој (Гроцка) сезони. Добијени подаци показују да су специфичне активности Tot-SOD, Mn-SOD, Cu/Zn-SOD и CAT биле повећане у пролеће у јетри, мишићу и паразиту. Код речне мрене утицај температуре на систем заштите од оксидационих оштећења је очигледан и сезонски образац евидентан. Наш рад представља прву студију SOD и CAT активности у мрени и њеном цревном паразиту и показује да су ови испитивани објекти веома погодни у биомониторинг студијама акватичних екосистема.