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ACTIVITY OF OXIDATIVE STRESS BIOMARKERS IN THE WHITE MUSCLE OF RED MULLET (*MULLUS BARBATUS* L.) FROM THE ADRIATIC SEA

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Abstract — The aim of this study was to investigate the activity of oxidative stress biomarkers (total superoxide dismutase – Tot SOD; and copper and zinc-containing superoxide dismutase – CuZn SOD; manganese-containing superoxide dismutase – Mn SOD; catalase – CAT; glutathione peroxidase – GSH-Px; and glutathione reductase – GR), as well as the biotransformation phase II enzyme glutathione-S-transferase (GST), in the white muscle of red mullet (*Mullus barbatus* L.) at Platamuni (PL) and Valdanos (VAL) in the Adriatic Sea during the winter and spring seasons. The obtained results show both site and seasonal influences on the investigated parameters, with lower enzyme activities at VAL than at PL and in spring than in winter.

Key words: Antioxidant enzymes, white muscle, red mullet, Montenegro, Adriatic Sea

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INTRODUCTION

Marine and freshwater environments are home to a vast diversity of organisms. Unfortunately, these environments also act as sinks for a great variety of anthropogenic pollutants, many of which are toxic (Carney Almroth et al., 2008). When entering the environment, xenobiotics often exert their effects through their ability to influence the redox cycle. Negative side effects include the formation of reactive oxygen species (ROS), which are continuously produced during aerobic metabolism, and their toxicity to the main biological components (proteins, lipids, and DNA) is counteracted by the activities of many cell defense mechanisms (Stohs et al., 2000). The removal of xenobiotics and even some endogenous substances from the cell is catalyzed by a number of different enzymes, so called oxidative stress biomarkers. According to the assumptions behind environmental risk assessment (ERA), oxidative stress biomarkers include both enzymatic [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and the biotransformation phase II enzyme glutathione-S-transferase (GST)] (Van der Oost et al., 2003) and nonenzymatic (Cadenas, 1989) components. Phase I enzymes are involved in xenobiotic biotransformation via the introduction of a polar moiety that renders a lipophilic contaminant more hydrophilic. The cytochrome P450 (CYP) family is the best studied phase I biotransformation enzyme in fish, especially CYP1A. Examples of phase II biotransformation enzymes that are commonly used in biomonitoring programs involving fish include glutathione S-transferase (GST) and UDP-glucuronyl transferase (UDPGT) (Halliwell and Gutteridge, 1999). Generation of ROS in fish may be influenced by variations in the concentration of dissolved oxygen of the aquatic environment, water salinity, developmental stage, nutritional challenges, and the presence of physical stress or xenobiotics, as deduced from changes observed in the activity of antioxidant enzymes (Trenzado et al., 2009).

Variations in the activity of oxidative stress biomarkers have often been proposed as biomarkers of pollutant-mediated oxidative stress (Regoli and Principato, 1995). Since oxidative stress responses

are directly related to cellular function, they may provide a clear indication of the local pollution status (Jena et al., 2009). In our study, we set out to investigate some of white muscle biomarkers proposed by international governmental bodies (ICES) and the scientific community (Van der Oost et al., 2003).

In this study, the benthic fish red mullet (*Mullus barbatus* L.) was chosen as the bioindicator species because it is a territorial fish of commercial interest that has been used in several studies of coastal pollution monitoring. Due to its close association with sediments and wide geographic distribution, the red mullet can be considered a key indicator species for the Adriatic Sea (Porte et al., 2002; Regoli et al., 2002).

The activities of oxidative stress biomarkers – total superoxide dismutase (Tot SOD), copper and zinc-containing superoxide dismutase (CuZn SOD), manganese-containing superoxide dismutase (Mn SOD) (EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSH-Px, EC 1.11.1.9), and glutathione reductase (GR, EC 1.6.4.2) – were measured in the white muscle of red mullet (*M. barbatus*) at Platamuni and Valdanos, Montenegro (Adriatic Sea), in two seasons: winter and spring. The same samples were also used to determine activity of the biotransformation phase II enzyme glutathione-S-transferase (GST, EC 2.5.1.18). The aim of this study was to compare the activity of oxidative stress biomarkers at the two investigated localities, as well as during two different seasons.

MATERIAL AND METHODS

Specimens of red mullet (*Mullus barbatus* L.) were caught by trawling in winter (February) and late spring (May) at two localities, Platamuni (PL) and Valdanos (VAL), whose geographic co-ordinates are given in Table 1. The two localities were chosen in order to compare the activity of oxidative stress biomarkers at unpolluted (Platamuni) and polluted (Valdanos) sites. In addition, the two localities were chosen in order to compare the activity of oxidative stress biomarkers during periods of lower (winter) and higher (spring) metabolic activity. Ten specimens were collected at Platamuni (five in the winter and five in the spring) and 10 at Valdanos (five in the winter and five in the spring). The specimens were collected and immediately transferred to seawater tanks, where they were identified. Fish were killed on board by severing the spinal cord and dissected within 3 min on ice. The white muscle was rapidly dissected, washed in ice-cold 0.65% NaCl, and frozen in liquid nitrogen (-196°C) before storage at -80°C. White muscle was isolated from each sample and then ground and homogenized in 5 vol of 25 mmol/L sucrose containing 10 mmol/L Tris-HCl, pH 7.5 (Lionetto et al., 2003) using a Janke & Kunkel (Staufen, Germany) IKA-Werk Ultra-Turrax homogenizer at 4°C (Rossi et al., 1983). The homogenates were sonicated for 30 s at 10kHz on ice to release enzymes (Takada et al., 1982); the sonicates were then centrifuged at 4°C at 100000g for 90 min. The resulting supernatants were used for biochemical analyses.

Total protein concentration in the supernatant was determined according to the method of Lowry et al. (1951) and expressed in mg/g of wet mass. The activity of antioxidant defense enzymes was measured simultaneously in triplicate for each sample using a Shimadzu UV-160 spectrophotometer and a temperature controlled cuvette holder. The total activity of SOD was assayed using the epinephrine

Table 1. Geographic co-ordinates of the examined locations in winter and spring.

		Start		End			
Season	*Location*	latitude	longitude	latitude	longitude	trawling duration (h)	depth (m)
Winter	Platamuni	42°19'56"	18°35'05"	42°17'70"	18°42'56"	1.33	80
	Valdanos	41°58'**62"**	19°07'**80"**	42°00'**39"**	19°06'**36"**	1.00	30
Spring	Platamuni	42°16'56**"**	18°41'66"	42°17'74"	18°35'54"	1.92	110
	Valdanos	42°00'**20"**	19°06'**10"**	42°01'**70"**	19°04'**50"**	1.08	35

method (Misra and Fridovich, 1972) and expressed as specific activity (U/mg of protein). For determination of Mn SOD activity, the assay was performed after preincubation with 8 mmol/L KCN. Activity of CuZn SOD was calculated as the difference between total SOD and Mn SOD activities. Catalase activity was evaluated from the rate of hydrogen peroxide (H_2O_2) decomposition and expressed as μmol H_2O_2/min/mg of protein (Claiborne, 1984). Activity of GSH-Px was determined following oxidation of nicotine amide adenine dinucleotide phosphate (NADPH) as a substrate with t-butyl hydroperoxide (Tamura et al., 1982) and expressed in nmol NADPH/min/mg of protein. The activity of GR was measured as described by Glatzle et al. (1974) and expressed as nmol NADPH/min/mg of protein. As for the biotransformation phase II enzyme GST, its activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined using the method of Habig et al. (1974) and expressed as nmol GSH/min/mg of protein. All chemicals were from Sigma (St. Louis, MO, USA).

The data are expressed as means ± S.E. (standard error). The non-parametric Mann-Whitney U-test was used to seek significant differences between means. A minimum significance level of $p<0.05$ was accepted. In addition, principal component analysis (PCA) was employed to detect variables that significantly contributed to differences in activities of the investigated enzymes at the examined sites in winter and spring. The analytical protocols described by Darlington et al. (1973) and Dinneen and Blakesley (1973) were followed.

RESULTS

The geographic position of the investigated localities is presented in Fig. 1. Table 1 gives the geographic coordinates, trawling duration, and sea depth at each site.

Total protein concentration in the white muscle of *M. barbatus* at both sites in winter and spring is presented in Table 2. The presented results show that

Fig. 1. Geographic position of the localities of Platamuni (PL) and Valdanos (VAL) in the Southern Adriatic Sea.

Table 1. Total protein concentration (mg/g wet mass) in the white muscle of red mullet (*Mullus barbatus* L.) at Platamuni (PL) and Valdanos (VAL) (Adriatic Sea) in two seasons: winter and spring. The data are expressed as means ± S.E. The non-parametric Mann-Whitney U-test was used to seek significant differences between means. A minimum significance level of $P<0.05$ was accepted. Effects of site: APL winter vs. VAL winter. Effects of season: *PL winter vs. PL spring.

Locality	*Season*	Total protein concentration (mg/g wet mass)
PL	Winter	109.00 ± 4.53
	Spring	169.90 ± 16.20*
VAL	Winter	179.70 ± 13.81^A
	Spring	206.06 ± 1.10

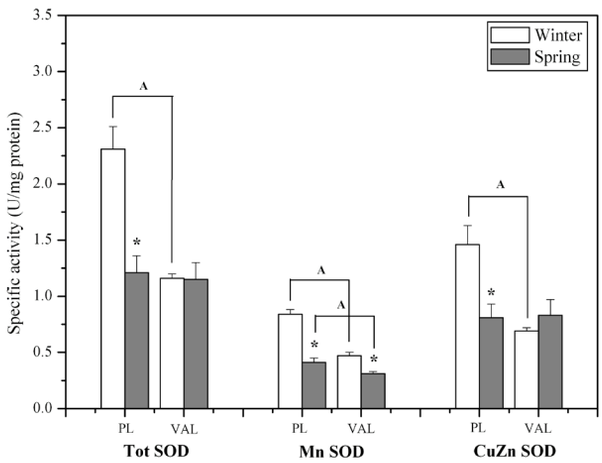

Fig. 2. Specific activity (U/mg protein) of total superoxide dismutase (Tot SOD), copper and zinc-containing superoxide dismutase (CuZn SOD), and manganese containing superoxide dismutase (Mn SOD) in the white muscle of red mullet (*M. barbatus*) at Platamuni (PL) and Valdanos (VAL) (Adriatic Sea) in two seasons: winter and spring. The data are expressed as means ± S.E. The non-parametric Mann-Whitney U-test was used to seek significant differences between means. *p<0.05 represents the minimum significant level for effects of season. AP<0.05 represents the minimum significant level for effects of site.

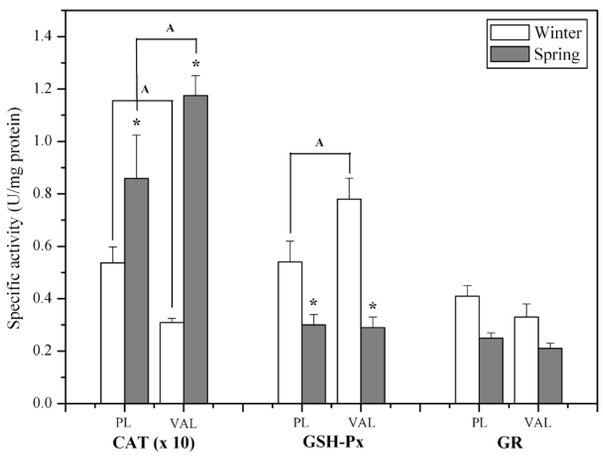

Fig. 3. Specific activity (U/mg protein) of catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) in the white muscle of red mullet (*M. barbatus*) at Platamuni (PL) and Valdanos (VAL) (Adriatic Sea) in two seasons: winter and spring. The data are expressed as means ± S.E. The non-parametric Mann-Whitney U-test was used to seek significant differences between means. *p<0.05 represents the minimum significant level for effects of season. AP<0.05 represents the minimum significant level for effects of site.

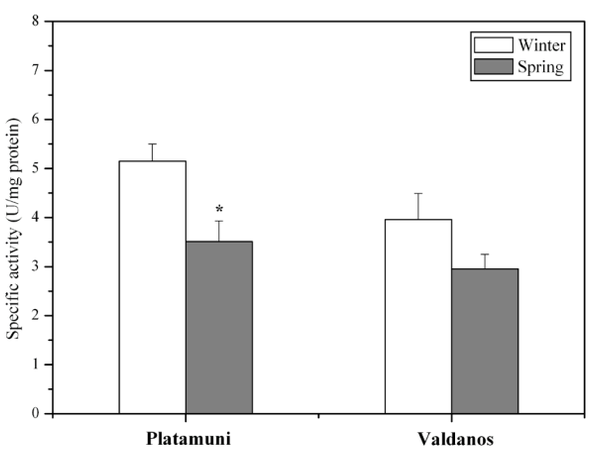

Fig. 4. Specific activity (U/mg protein) of the biotransformation phase II enzyme glutathione-S-transferase (GST) in the white muscle of red mullet (*M. barbatus*) at Platamuni (PL) and Valdanos (VAL) (Adriatic Sea) in two seasons: winter and spring. The data are expressed as means ± S.E. The non-parametric Mann-Whitney U-test was used to seek significant differences between means. *p<0.05 represents the minimum significant level for effects of season.

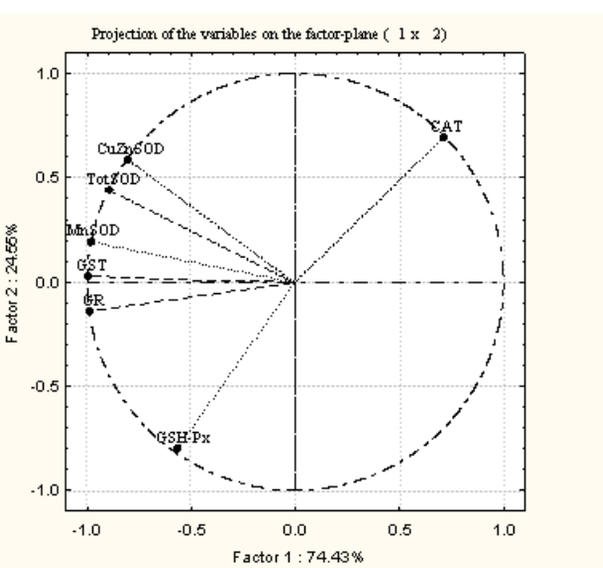

Fig. 5. Principal component analysis (PCA) based on correlations; projection of specific antioxidant enzyme activities on the factor plane.

protein concentration was significantly higher in the white muscle of fish from PL in spring compared to winter (p<0.05). In addition, total protein concentration was significantly higher at VAL than at PL in winter (p<0.05). These data suggest different metabolic activity of the investigated tissue depending on

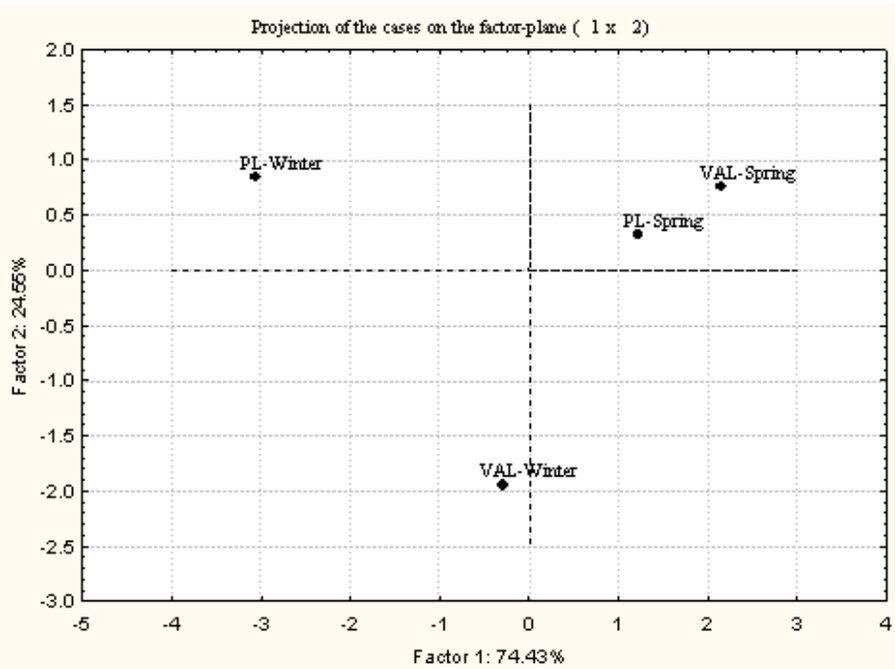

Fig. 6. Principal component analysis (PCA) of specific antioxidant enzyme activities at each site and in both seasons on the factor plane.

the season at PL, as well as between PL and VAL in the same season, and probably reflect the availability of food and feeding behavior.

Our results show that the muscle specific activities of Tot SOD (Fig. 2), Mn SOD (Fig. 2), CuZn SOD (Fig. 2), and CAT (Fig. 3) in winter were significantly lower at VAL in comparison with PL ($p<0.05$). In contrast to that, specific activity of GSH-Px (Fig. 3) was markedly higher at VAL than at PL in winter ($p<0.05$). In spring, specific activity of Mn SOD was significantly lower ($p<0.05$), while that of CAT was significantly higher ($p<0.05$) at VAL than at PL.

Seasonal patterns of oxidative stress biomarkers were obtained by comparing results obtained at the same localities in different seasons. Our results show that at PL, specific activities of Tot SOD (Fig. 2), Mn SOD (Fig. 2), CuZn SOD (Fig. 2), GSH-Px (Fig. 3), and GST (Fig. 4) were significantly lower in spring than in winter ($p<0.05$). Only the activity of muscle CAT at PL was significantly higher in spring than in winter ($p<0.05$). At VAL, specific activities of Mn SOD and GSH-Px were significantly lower, while specific CAT activity was significantly higher in spring than in winter ($p<0.05$).

Which component contributes to the overall antioxidative defense system and to what degree was further analyzed using principal component analysis (PCA), which separates groups according to complete organization of individual components. The results of PCA of the investigated oxidative stress biomarkers in the white muscle of red mullet are shown in Figs. 5 and 6. The treatment of overall data by PCA indicated a clear separation of data for different sampling sites. In addition to differences between sampling sites, PCA clearly shows differences between seasons as well.

DISCUSSION

Oxidative stress is a general response to toxicity induced by many contaminants and is often used as a biomarker of the effects of exposure to environmental pollution in aquatic environments. The biomarker approach, as opposed to chemical

characterization, is also environmentally relevant, since it integrates the action of complex mixtures of chemicals in living organisms (Solé et al., 2009). Its assessment is therefore included in biomonitoring programs as a nonspecific biochemical marker (Winston and Di Giulio, 1991; Livingstone, 2001). The main reason for studying oxidative stress in aquatic organisms is not only to understand whether animals are detrimentally affected by exposure, but also to comprehend the mode of action of the toxicants. Several studies have shown that oxidative stress biomarkers affected by ROS show adaptive responses to xenobiotics that produce oxyradicals (Di Giulio et al., 1995) and are potential biomarkers of oxidative stress in fish (Van der Oost et al., 2003). In field studies, oxidative destruction and activation of antioxidant defense enzymes are usually observed under conditions of moderate pollution (Pandey et al., 2003; Ferreira et al., 2005) or seasonal influences (Pavlović et al., 2004, 2008). In addition, aquatic animals that are widely explored in biomonitoring exhibit high seasonal dependence of these markers. They are therefore considered to be ambiguous for monitoring purposes (Leiniö and Lehtonen, 2005). Studies of aquatic animals demonstrated that seasonal variations in different biomarkers were attributable to environmental and biological factors, mainly temperature and metabolic status of the animals, rather than to the site (Niyogi et al., 2001; Leiniö and Lehtonen, 2005).

In many biomonitoring studies, the liver is the main target organ for investigation because of its fast answer to environmental influences, high metabolic activity, and essential function in the organism. Although white muscle has a lower metabolic rate, its importance for investigation is of great significance to humans because of its nutritional importance, especially in the case of commercially important fish species such as red mullet.

The overall trend obtained in our study revealed decreased activities of the investigated enzymes in spring compared to winter. Proteins constitute a target for oxidative damage, with subsequent alteration of their functions. Studies by other authors reported that flounders living in waters contaminated with xenobiotics showed increased levels of oxidized proteins (Fessard and Livingstone, 1998). Our results show that protein concentration was significantly higher in the white muscle of fish from Platamuni in spring compared to winter. In addition, total protein concentration was significantly higher at Valdanos than at Platamuni in winter. These data suggest different metabolic activity of the investigated tissue depending on the season, as well as between the investigated localities in the same season, and probably reflect the availability of food and feeding behavior. Our results show that specific activities of most of the investigated enzymes in winter were lower at VAL than at PL. The exception is the specific activity of GSH-Px, which was higher at VAL than at PL. In spring, specific activity of Mn SOD was significantly lower, while CAT activity was significantly higher at VAL than at PL. Most of the investigated enzymes show decreased activities in spring compared to winter at both localities. The opposite trend was obtained for specific CAT activity, with higher activities in spring season at both localities. Generally, the investigated enzyme activities were significantly lower at Valdanos than at Platamuni and in spring than in winter. Oxidative stress biomarkers function in detoxification processes and represent suitable parameters in environmental risk assessment (ERA). The major oxidative stress biomarkers in marine fish are SOD, CAT, and GSH-Px, as well as the biotransformation phase II enzyme GST (Van der Oost et al., 2003). The two investigated sites were chosen in order to compare the activity of oxidative stress biomarkers at an unpolluted locality (Platamuni) and a polluted one with significant anthropogenic impact (Valdanos). Our previous investigations at the same localities (Šaponjić et al., 2006) showed no significant differences in concentrations of polychlorinated biphenyls (PCBs) in either season. At the same time, the data on polycyclic aromatic hydrocarbons (PAHs) showed that the concentration of phenantrene was higher at Platamuni in spring, while levels of fluorene and anthracene were higher at Valdanos in spring. It is difficult to predict the direct influence of these compounds on oxidative stress biomarker activities in our study because the situation is complicated by seasonal influences. Similar results were obtained in the liver of red mullet at the same

localities (Pavlović et al., 2008). The major difference vis-à-vis the liver is in muscle-specific CAT activity, which was significantly higher in spring at both investigated localities. These results suggest different hydrogen peroxide metabolism in these two tissues depending on the season. Since CAT is localized in the peroxisomes of most cells and is involved in fatty acid metabolism, the changes in activities may often be difficult to interpret (Van der Oost et al., 2003). However, the differences between CAT activity in liver (Pavlović et al., 2008) and white muscle probably represent different tissue reactions to seasonal changes, rather than a reaction to pollution in the environment. Many enzymes (such as the biotransformation phase II enzyme GST) have reduced activities at lower environmental temperature (Ronisz et al., 1999). Some investigations show that the biotransformation phase II enzyme GST is influenced by levels of organic substrates, and both enhancement and inhibition of these enzymatic activities have been reported in field studies (Regoli et al., 2002). Our results show that GST activity was less sensitive to environmental changes in the white muscle than in the liver of red mullet (Pavlović et al., 2008).

Principal component analysis (PCA) was applied in order to statistically define the differences of oxidative stress biomarkers at the investigated localities in winter and spring. The treatment of overall data by PCA indicated a clear separation of different sampling periods at both localities. A more evident effect of seasonality was observed. The obtained results show a similar trend of positive and negative correlations in specific activity of the investigated oxidative stress biomarkers in winter and spring. Projection of oxidative stress biomarkers activities is based on the factor plane (Fig. 5). At the same time, examination of the seasonal pattern of oxidative stress biomarkers revealed clear differences of specific (factor 1: 74.43%, factor 2: 24.55%) oxidative stress biomarker activities between Platamuni and Valdanos in both seasons, as well as between winter and spring at both localities (Fig. 6). A projection was made for oxidative stress biomarker activities at each site and in both seasons based on the factor plane.

CONCLUSIONS

In can be concluded from the presented results that differences in the activities of oxidative stress biomarkers exist in the white muscle of red mullet (*Mullus barbatus*). These differences are pronounced at the investigated localities, with generally lower activities at Valdanos, as well in different seasons, with lower activities in spring. There is a marked difference of CAT activity in the white muscle, which (contrary to the other investigated enzymes) is significantly higher in spring at both investigated localities. Our work shows that climatic stress is a predominant factor, one that induced changes in activity and organization of the antioxidative defense system.

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АКТИВНОСТ БИОМАРКЕРА ОКСИДАЦИОНОГ СТРЕСА У БЕЛОМ МИШИЋУ ТРЉЕ (*MULLUS BARBATUS* L.) ИЗ ЈАДРАНСКОГ МОРА

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Циљ овог рада био је испитивање активности биомаркера оксидационог стреса (укупне супероксид-дисмутазе-Тот SOD, бакар цинк садржавајуће супероксид-дисмутазе-CuZn SOD, манган садржавајуће супероксид-дисмутазе-Mn SOD, каталазе-CAT, глутатион-пероксидазе-GSH-Px и глутатион-редуктазе-GR) као и ензима фазе II биотрансформације глутатион-С-трансферазе (GST) у белом мишићу трље (*Mullus barbatus* L.) са локалитета Платамуни и Валданос у Јадранском мору (Црна Гора) у зимској и пролећној сезони. Добијени резултати показују разлике испитиваних параметара између различитих локалитета као и испитиваних сезона са нижим активностима ензима у VAL него у PL и у пролеће у односу на зиму.