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Integrated response of antioxidant biomarkers in the liver and white muscle of European hake (*Merluccius merluccius* L.) females from the Adriatic Sea with respect to environmental influences

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Abstract: We investigated the integrated response of antioxidant defense enzymes (total superoxide dismutase (TotSOD), manganese-containing superoxide dismutase (MnSOD), copper-zinc-containing superoxide dismutase (CuZnSOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and phase II biotransformation enzyme, glutathione-S-transferase (GST)) in the liver and white muscle of females of European hake (*Merluccius merluccius* L.) from the Adriatic Sea (Montenegro) in winter and spring. The activity of GSH-Px in the liver was significantly increased, while GST activity was decreased in spring compared to the winter. In white muscle, the activities of TotSOD and CuZnSOD were increased, while the activities of MnSOD, CAT, GSH-Px, GR and GST were decreased in spring when compared to the matching values in winter. The activities of TotSOD and CuZnSOD in winter were markedly lower in the muscle than in the liver, while the activity of MnSOD in the muscle was higher when compared to the liver. Principal component analysis (PCA) revealed clear separation of the investigated antioxidant biomarkers between tissues and seasons, while the integrated biomarker response (IBR) showed that the most intensive antioxidant biomarker response was in the liver in spring. Star plots of IBR showed a dominant contribution of glutathione-dependent biomarkers (GSH-Px, GR and GST) and CAT in both tissues and seasons with respect to SOD isoenzymes. All enzyme activities (except MnSOD) were greater in the liver in comparison to the white muscle. Our results show that the liver possesses a greater capacity to establish and maintain homeostasis under changing environmental conditions in winter and spring. At the same time, seasonal effects are more pronounced in muscle tissue.

Key words: antioxidant enzymes; marine fish; oxidative stress; seasonal; tissues

INTRODUCTION

All aerobic organisms produce reactive oxygen species (ROS) during normal physiological processes, such as energy production or metabolism. Overproduction of ROS can damage DNA, proteins, and lipids, leading to oxidative stress [1]. Many environmental factors may also initiate signaling pathways that are activated in response to oxidative stress, which is associated with the etiology and pathology of many diseases [2]. ROS can play beneficial roles in cells as they contribute to pathways of intracellular signaling and redox regulation [3]. The cellular antioxidant defense system (AOS) represents an important biochemical strategy that provides protection to cells against the deleterious effects of endogenous ROS by maintaining their levels relatively low [4]. To maintain homeostasis, the cells possess a range of antioxidant defense enzymes (SOD, CAT, GSH-Px and GR), as well as phase II biotransformation components, such as GST and the reduced/oxidized glutathione system [5]. Antioxidant defense biomarkers are also related to changes in environmental factors such as temperature, salinity, food availability and concentrations of dissolved oxygen, as well as to intrinsic biological factors, such as the reproductive cycle [6].

Often there are difficulties to summarize and analyze data obtained from a battery of biomarkers. Therefore, some authors have developed integrative tools to summarize the response of a set of biomarkers in a single value or a graph. One of the most

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frequently used tools in field and laboratory studies is the IBR that was developed by [7]. In this method, visual integration of data is achieved using star plots, a simple multivariate graphic method.

Fish are often used in ecophysiological studies because they play a number of roles in the food chain or as biomonitoring organisms for the presence of various toxic substances [8]. Fish are poikilothermic organisms and their ROS levels are directly dependent on environmental temperature, but this situation may be complicated by the influence of other environmental factors. Studies on the antioxidant defense enzymes in poikilothermic organisms revealed that a lower metabolic rates are accompanied by lower antioxidant defenses. Fish are usually on the top of the aquatic food chain and they strongly respond to stressful conditions [9]. Fish liver plays a major role in various processes, such as the uptake, biotransformation and excretion of pollutants. In many biomonitoring studies, the liver is the main target organ for investigation because of its rapid response to environmental influences, high metabolic activity and essential function in the organism. White muscle has a lower metabolic rate and its investigation is important because of its great importance in human nutrition, especially in the case of commercially important fish species such as European hake [10].

Many studies that have reported on the effects of environmental influences and chemicals have focused on bioindicator species, such as the red mullet, while many species that are important to fisheries have been neglected [11]. The gadiform fish, *Merluccius merluccius* (*Merluciidae, Gadiformes*) was selected for this study because it is both commercially and ecologically one of the most important demersal marine fish species in the area [12]. It is also one of the most heavily exploited fish, as well as one of the main commercial species of the demersal fishery in Adriatic coastal countries [13]. The European hake is found usually between 70 and 370 m depth. Adults live close to the bottom during the day, but move away from the bottom at night. Adults feed mainly on fish (small hakes, anchovies, pilchard, herrings, cod, sardines and gadoid species) and squids. The young feed on crustaceans, especially Euphausiids and Amphipods [14]. In the Adriatic Sea, the European hake spawns throughout the year, but with different intensities. The spawning peaks are in the summer and winter [15].

There is a limited amount of information regarding the sexual and developmental differences of antioxidant defense enzymes in European hake, but it is well known that gender affects the influence of various pollutants [5].

There are no studies reporting on the integrated response of the complete antioxidant defensive enzyme activities in the liver and white muscle of European hake in the context of seasonal influences. Thus, the aim of our study was to provide baseline data that could support their future inclusion in a wide variety of applications, such as monitoring studies, implications in nutrition and use in fishery studies. We investigated the activities of antioxidant defense enzymes: TotSOD, MnSOD, CuZnSOD (EC 1.15.1.1), CAT (EC 1.11.1.6), GSH-Px, (EC 1.11.1.9) and GR (EC 1.6.4.2) in the liver and the white muscle of the European hake (*Merluccius merluccius*) at the Platamuni locality (Montenegrin coastline, Adriatic Sea) in two seasons: winter and spring. In the same samples, we also determined the activity of GST (EC 2.5.1.18). The principal aim of this study was to determine the integrated biomarker response of antioxidant biomarkers, and to compare the activities of the antioxidant defense enzymes between a period of low metabolic activity (winter) and a period with higher metabolic activity (late spring), as well as between two tissues with different rates of oxidative metabolism.

MATERIALS AND METHODS

Study area and sampling

Fish were caught by trawling in winter (February 2013) and late spring (May 2013) at the locality Platamuni (Montenegrin coastline) in the Adriatic Sea (geographical coordinates in winter: longitude-42°19'56", latitude-18°35'05"; in spring: longitude-42°16'56", latitude-18°41'66"; Fig. S1). The localities were chosen based on our earlier investigations [16,17], in order to determine natural variations in activities of antioxidant defense enzymes in the liver and white muscle of European hake, between winter and spring [18]. The bottom of the biotope at the investigated locality is covered with a thick layer of fine terrigenous sludge containing particles of detritus. Ten specimens of female European hake were collected in winter, and 10 in spring. All ani-

mal procedures complied with the European Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

Measurements of environmental parameters

Environmental parameters (depth, salinity, temperature, oxygen concentration and oxygen saturation) were measured with the WTW (Wissenschaftlich-Technische Werkstatten, Weilheim, Germany) Multilab System (spot measurements at the time of sampling).

Tissue preparation

After collection, samples of European hake were immediately separated by gender, weighed and transferred to sea water tanks. In order to avoid differences attributed to biological parameters (such as size and age, which are factors that affect the biomarker response in some fish species), females of the same size class weighing 100-120 g were selected to ensure uniformity of samples. In this research, we used females as they were more numerous. Fish were killed on board with a sharp blow to the head and dissected within 3 min on ice. In each group, the liver and white muscle were rapidly dissected, washed in ice-cold 0.6% NaCl and frozen in liquid nitrogen (-196°C) before storing at -80°C. The liver tissue was ground and homogenized in 5 volumes of 25 mmol/L sucrose containing 10 mmol/L Tris-HCl, pH 7.5 at 1500 rpm [19], using a Janke & Kunkel (Staufen, Germany) IKA-Werk Ultra-Turrax homogenizer at 4°C. The homogenates were sonicated for 30 s at 10 kHz on ice to release enzymes [20]; the sonicated fraction was then centrifuged at 100,000 × g for 90 min at 4°C. The resulting supernatants were used for further biochemical analyses. All chemicals used in this study were obtained from Sigma (Germany).

Antioxidant enzyme activities

Protein concentrations in the supernatants were determined according to the method of Lowry *et al.* (1951) [21], using bovine serum albumin as a standard, and were expressed in mg/g 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.

SOD activity determination

TotSOD activity was measured by the epinephrine method [22]. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline at 26°C and was expressed as specific activity (U/mg protein). For the determination of MnSOD activity the assay was performed after the pre-incubation with 8 mmol/L KCN. CuZnSOD activity was calculated from the difference between TotSOD and MnSOD activities.

CAT activity determination

The activity of CAT was evaluated by the rate of hydrogen peroxide (H_2O_2) decomposition [23]. The method is based of H_2O_2 degradation by the action of CAT contained in the examined samples. In this procedure, 30 mmol H_2O_2 as substrate was used. CAT activity was expressed as μmol H_2O_2/min/mg protein.

GSH-Px activity determination

GSH-Px activity was assayed following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate with t-butyl hydroperoxide [24]. This reaction is comprised of the action of GSH-Px contained in the samples in the presence of *t*-butyl hydroperoxide (3 mmol) as substrate in 0.5 M phosphate buffer, pH 7.0, at 37°C. The activity of GSH-Px was expressed as nmol NADPH/min/mg protein.

GR activity determination

The activity of GR was measured as described by Glatzle *et al.* (1974) [25]. The method is based on the capability of GR to catalyze the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as substrate in the phosphate buffer (pH 7.4). GR activity was expressed as nmol NADPH/min/mg protein.

Table 1. Physico-chemical parameters of sea water (depth, salinity, temperature, O_2 concentration, O_2 saturation, nitrite (NO_2^-) and nitrate (NO_3^-) concentrations at the examined location (Platamuni) in the Southern Adriatic Sea in winter and spring.

	Depth (m)	Salinity (‰)	Temperature (°C)	O_2 conc. (mg/L)	O_2 sat. (%)	NO_2^- (mg/L)	NO_3^- (mg/L)
Winter	80	33.07	12.8	8.1	91	0.724	2.880
Spring	80	38.00	17.2	7.6	98	0.175	1.223

GST activity determination

GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined by the method of Habig *et al.* (1974) [26]. The method is based on the reaction of CDNB with the sulfhydryl (-SH) group of GSH catalyzed by GST contained in the samples. The reaction proceeded in the presence of 1 mmol GSH in phosphate buffer (pH 6.5) at 37 °C. GST activity was expressed as nmol GSH/min/mg protein.

Calculation of the IBR

Antioxidant biomarkers were combined into a stress index referred to as IBR, described by Beliaeff and Burgeot (2002) [7] and modified by Guerlet *et al.* (2010) [27]. This index was calculated for each tissue in different seasons as follows:

individual areas *Ai* connecting the *i-th* and the *(i+1)-th* radius coordinates of the star plot were obtained, according to the formula:

$$Ai = \frac{1}{2} \sin\left(\frac{2\pi}{\pi}\right) S_i \cdot S_{i+1}$$

where S_i and S_{i+1} represent the individual biomarker scores (calculated from standardized data) and their successive star plot radius coordinates, and n represents the number of radii corresponding to the biomarkers used in the survey. Antioxidant biomarkers used for IBR index calculation were ranged clockwise according to their hierarchy in ROS detoxification as follows: TotSOD, MnSOD, CuZnSOD, CAT, GSH-Px, GR, as well as biotransformation phase II enzyme GST. IBR was calculated according to the formula:

$$IBR_{tissue/season} = \sum_{i=1}^{n} Ai$$

Statistical analysis

The data are expressed as the means±S.E. (standard error). Before testing, all data were checked for normality and homogeneity using Kolmogorov-Smirnov and Levene statistics to meet statistical demands [28]. The factorial analysis of variance (ANOVA) was performed to determine all interactive effects between localities and seasons. Since interactive effects were observed, the Fischer`s LSD *post hoc* test was used to seek significant differences between the means [29]. A minimum significance level of $P<0.05$ was accepted for all cases. PCA was employed to detect variables that significantly contributed to differences in activities of the investigated antioxidant enzymes [30].

RESULTS

The physicochemical parameters of the sea water are presented in Table 1. They show increased water temperature and consequently lower oxygen concentration in the water in spring. The concentrations of nitrites and nitrates were lower in spring than in winter. Table 2 shows the total protein concentrations in the liver and white muscle of the European hake. Generally, the protein concentrations were higher in the liver in comparison to the white muscle in both seasons. In white muscle, the protein concentration was higher in spring than in winter ($p<0.05$), while sea-

Table 2. Concentration of total proteins (mg/mL) in the liver and white muscle of European hake (*Merluccius merluccius*) from the Montenegrin coastline.

	Winter	Spring
Liver	358.54±31.25	357.79±10.37
White muscle	60.80±4.32 #	127.10±10.02*#

The data are expressed as the means±S.E. ANOVA was performed to determine all effects between localities and seasons. The Fischer`s LSD *post hoc* test was used to obtain significant differences between the means. A value of $^*p<0.05$ was considered to indicate differences of same tissue between seasons; a value of $^\#p<0.05$ was considered to indicate differences between the respective tissues in the same season.

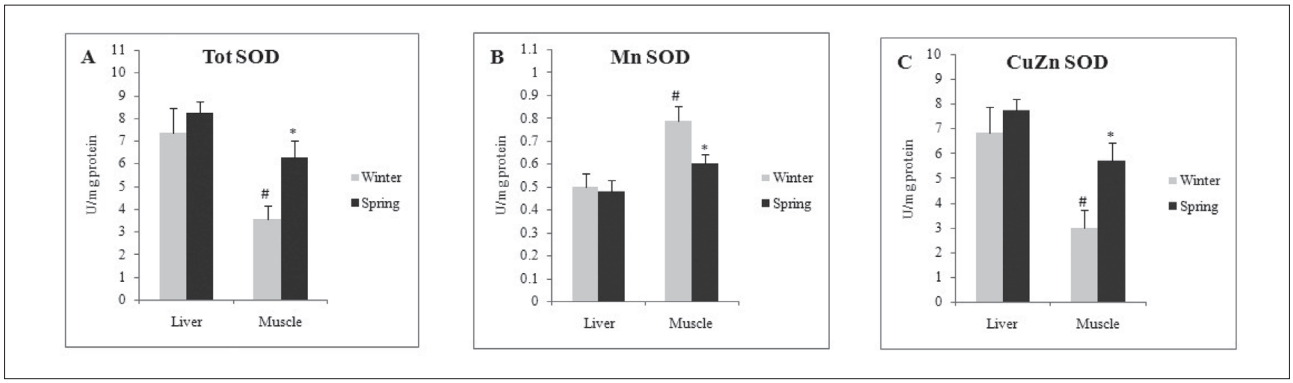

Fig. 1. TotSOD (**A**), MnSOD (**B**) and CuZnSOD (**C**) activities (U/mg protein) in the liver and white muscle of European hake (*Merluccius merluccius*) in winter and spring. The data are expressed as means±S.E. ANOVA was performed to determine all effects between seasons and tissues. Fischer's LSD *post hoc* test was used to seek significant differences between means. A value of $^{*}p<0.05$ was considered to indicate differences of the same tissue between seasons; a value of $^{\#}p<0.05$ was considered to indicate differences between respective tissues in same season.

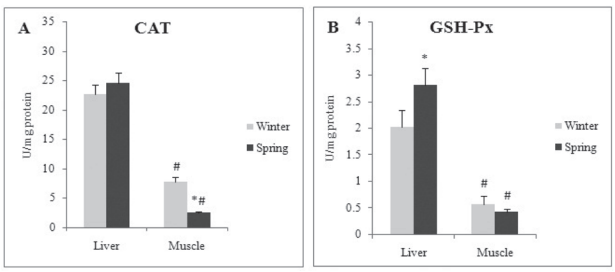

Fig. 2. CAT (**A**) and GSH-Px (**B**) activities (U/mg protein) in the liver and white muscle of European hake (*Merluccius merluccius*) in winter and spring. The data are expressed as the means±S.E. ANOVA was performed to determine all effects between seasons and tissues. Fischer's LSD *post hoc* test was used to seek significant differences between means. A value of $^{*}p<0.05$ was considered to indicate differences of same tissue between seasons; a value of $^{\#}p<0.05$ was considered to indicate differences between respective tissues in same season.

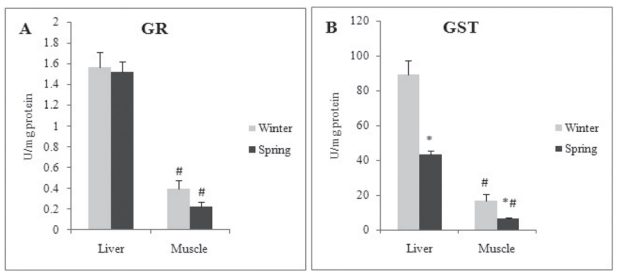

Fig. 3. GR and biotransformation phase II enzyme GST activities (U/mg protein) in the liver and white muscle of European hake (*Merluccius merluccius*) in winter and spring. The data are expressed as means±S.E. ANOVA was performed to determine all effects between seasons and tissues. Fischer's LSD *post hoc* test was used to seek significant differences between means. A value of $^{*}p<0.05$ was considered to indicate differences of same tissue between seasons; a value of $^{\#}p<0.05$ was considered to indicate differences between respective tissues in same season.

sonal variations in liver protein concentrations were not observed. The activities of TotSOD, MnSOD, Cu ZnSOD (Fig. 1) and CAT (Fig. 2A) in the liver were not markedly changed with respect to the season. The activity of liver GSH-Px was significantly increased (Fig. 2B), while the activity of GST was decreased in spring in comparison to winter (Fig. 3B) ($p<0.05$). In white muscle, increased activities of TotSOD (Fig. 1A) and CuZnSOD (Fig. 1C) were observed, while the activities of MnSOD (Fig. 1B), CAT (Fig. 2A), GSH-Px (Fig. 2B), GR (Fig. 3A) and GST (Fig. 3B) were decreased in spring ($p<0.05$). The activities of TotSOD and CuZnSOD were markedly lower in muscle than in the liver, while the activity of muscle MnSOD was higher when compared to the liver in winter (Fig. 1) ($p<0.05$). The activities of CAT, GSH-Px (Fig. 2), GR and GST (Fig. 3) were significantly lower in white muscle in respect to the liver in both seasons ($p<0.05$).

The PCA of all investigated antioxidant defense enzymes is presented in Fig. 4A. The PCA referred to the relative contribution of every antioxidant enzyme in the liver showed that principal component 1 and principal component 2 can explain about 70% of the

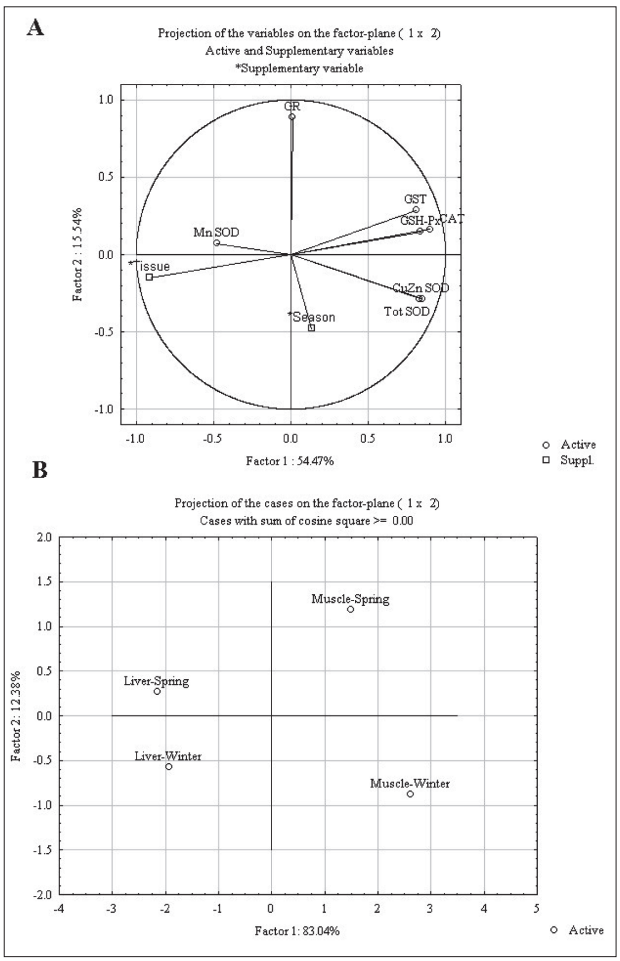

Fig. 4. PCA based on correlations. **A** – projection of all investigated antioxidant defense enzyme activities on the factor plane. **B** – projection of each tissue and season on the factor plane.

total variance in the data matrix. Principal component 1 explains 54.47% of the total variance and is mainly characterized by negative loading of the MnSOD, and by positive loading of all other variables. Principal component 2 explains 15.54% of the total variance and is mainly characterized by positive loading of MnSOD, CAT, GSH-Px, GR and GST.

Fig. 4B presents summarized results of PCA for the investigated sampling locality in winter and spring. It shows that principal component 1 and principal component 2 explain over 95% of the total variance. With regard to the position of the sites, principal component 1 (83.04%) discriminates between the investigated tissues (e.g. the liver in winter and spring on one side, and white muscle in winter and spring on the other). Principal component 2 (12.38%)

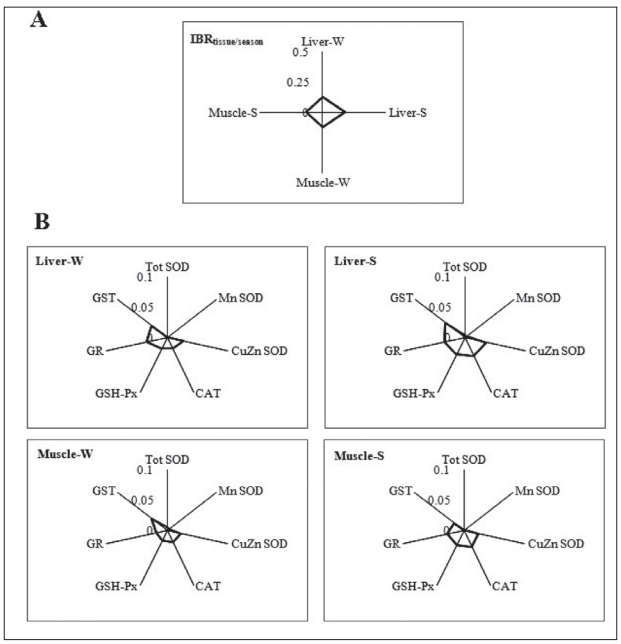

Fig. 5. A – Integrated response of antioxidant biomarkers in the liver and white muscle of European hake in winter (W) and spring (S). **B** – Tissue star plots for each antioxidant biomarker and season.

discriminates between the investigated seasons, showing a clear separation between the liver and muscle in winter on one side, and the liver and muscle in spring on the other.

The IBR index show that the most intensive antioxidant biomarker response was in the liver in spring (IBR=0.1821). The IBR index values for the liver in winter (IBR=0.1320), muscle in winter (IBR=0.1221) and muscle in spring (IBR=0.1289) were similar (Fig. 5A). Star plots of the biological response for each individual antioxidant biomarker revealed a dominant contribution of glutathione-dependent biomarkers, GSH-Px, GR and GST, and of CAT in both tissues and seasons as compared to SOD isoenzymes (Fig. 5B).

DISCUSSION

Compared to other environmental factors, the main difference between winter and spring was in the ambient temperature of the sea water. Lower temperature was detected in winter and consequently a higher oxygen concentration was dissolved in sea water, because oxygen solubility increases in cold water. Aquatic hypoxia triggers a complex set of physiological and

biochemical alterations in fish, including decreased metabolic rate, increased ventilation rate and increased anaerobic respiration [31]. Fish are ectothermic animals and their rates of biological activity are deeply influenced by the thermal environment [32]. At low temperatures, intracellular lipids are elevated and the risk of lipid peroxidation is also increased. In the cold environment, phospholipids are also more vulnerable to oxidation. Increased risk of oxidative damage at low temperatures is enhanced because ectotherms maintain oxidative metabolism of skeletal muscle by increasing the density of mitochondria, which are the main sources of ROS production [33]. Depending to their functions and location in the body, different tissues of the same organism react differently to the temperature change, and are more or less exposed to temperature changes. Therefore, they can have different cellular response to stress caused by temperature changes [34].

In winter, water salinity was lower and the concentrations of nitrites (4 times) and nitrates (2 times) were elevated when compared to spring. In marine aquaculture, salinity is one of the most important abiotic factors influencing fish growth and survival, as its variation may cause a variety of physiological stress responses, which were associated with enhanced ROS generation [35]. Some investigations also showed that the activities of SOD, CAT, GST, GSH-Px and GR in the liver and white muscle of *Nile tilapia* were significantly increased after exposure to nitrogen compounds, such as ammonia [36].

The Montenegrin coastline of the Adriatic Sea is exposed to very intensive demographic activity and receives different levels of industrial, urban and agricultural discharges. The present study is the part of a larger investigation and our previous results from the same locality show that the concentrations of polychlorinated biphenyls (PCBs) were below 10 ng/L water in both winter and spring (PCB-28, PCB-52, PCB-101, PCB-138, PCB-153 and PCB-180 were measured). We have also investigated a set of 13 polycyclic aromatic hydrocarbons (PAHs): dibenzo(A)ahthracene, fluorene, phenantrene, anthracene, pyrene, benz(A)anthracene, chrysene, benzo(B)fluoranthene, benzo(K)fluoranhene, benzo(A)pyrene, benzoperylene, indeno(1.2.3.cd)pyrene, acenaphthylene, and observed that only the concentration of phenantrene in the water was higher in spring (373 ng/L). The concentrations of all other classes of PAHs were below 50 ng/L. We also measured five heavy metals (Mn, Zn, Cu, Co, Ni) and no significant seasonal differences were observed [16,31]. It can be concluded that the investigated pollutants had a similar distribution during both seasons (except phenantrene), and that it is difficult to predict the direct influence of these compounds on the antioxidant defense. Thus, all detected changes obtained in our study are the result of seasonal factors.

Most teleost fish species experience periods of starvation during their normal life cycle due to lack of available prey, changes in behavior due to reproductive and seasonal changes [37]. Experiments on cod (*Gadus morhua*) show that periods of starvation lead to mobilization of body reserves in a specific order: first hepatic lipids, followed by glycogen from the liver and white muscle [33]. In response to lowered food availability, fish can decrease their locomotor and metabolic activities, modify tissue metabolic capacities and degrade muscle proteins. Lowered food availability in winter is the cause of markedly reduced total protein concentration in white muscle of European hake in our investigation.

Antioxidants provide cells with a comprehensive defense from ROS-induced damage. These defenses include antioxidant enzymes and low molecular weight compounds (glutathione and ascorbate). Toxic and organ-specific ROS responses are related to anatomical localization, the exposure route and its defense capacity [38]. These data point to different metabolic activities of the investigated tissues with respect to the season and the dependence of food availability and feeding behavior [39]. Earlier investigations of some authors show that among marine teleosts, *M. merluccius* possesses a very homogeneous response of some enzymes, such as CAT, GR and GST to different ecological variables [40].

SOD and CAT have a remarkable importance for aquatic organisms because these enzymes protect them from free radicals that cause oxidative stress. CAT was also found to be the most sensitive antioxidant enzyme when compared to the others. The SOD-CAT system, the first line of defense against oxidants, varied accord-

ing to the response of the fish antioxidant system to counteract toxicity [41]. Our results show, that the activities of MnSOD and CAT were significantly higher in white muscle during winter, while in the liver they were unchanged. These changes suggest that the liver establishes oxidative homeostasis more efficiently than muscle tissue. Increases in MnSOD and CAT activities in white muscle in winter show elevated ROS production in mitochondria and peroxisomes and, as a result, compensatory increases of these enzymes. At low temperatures, the increase in polyunsaturated moieties of the mitochondrial membranes in fish should raise the rates of mitochondrial respiration, which would in turn enhance ROS formation ROS [31].

Hake liver is essentially a hepatopancreas and it accumulates large amounts of lipids, which is a typical characteristic in gadiform fish, such as cod (*Gadus morhua*) or haddock (*Melanogrammus aeglefinus*). It has been suggested that lipid accumulation might lead to increased production of oxyradicals and further to hepatic lesions [42]. Biological membranes are uniquely susceptible to oxidative damage because lipids damaged by ROS can induce lipid peroxidation (LPO), a self-propagating chain reaction that results in the generation of highly-reactive, complex lipid radicals within the membrane core. In response to increased organic hydroperoxides, GSH-Px increases its activity in order to decrease overproduction of lipid radicals. At warm physiological temperatures, elevated activities of the glutathione-dependent antioxidant enzymes have been reported [3]. This is in accordance with the observed increase in GSH-Px activity in the liver in spring. Glutathione peroxidases are a family of seleno-protein isozymes that have similar catalytic functions. These enzymes reduce soluble hydroperoxides, including hydrogen peroxide and hydroperoxy fatty acids, at the expense of glutathione [1].

GST is a member of an enzyme family that protects against an array of hydrophobic and electrophilic compounds, including peroxidized lipids and xenobiotics. GSTs catalyze the nucleophilic conjugation of endogenous GSH with reactive electrophiles, allowing for their excretion after metabolism [43]. Additionally, GST can also provide protection against endogenous toxic compounds, such as secondary metabolites of lipid peroxidation [44]. As a general rule, GST activity is higher in the liver than in white muscle. It is interesting to note, that GST activity was higher in winter in both liver and white muscle. Some researchers showed that the biotransformation phase II enzyme GST is influenced by the levels of organic substrates, and both increased and inhibited enzyme activities have been reported in field studies. A similar increase in GST activity in winter was obtained by us in the liver of both thinlip gray mullet (*Liza ramada*) and longfin gurnard (*Chelidonichthys obscurus*) [18,31].

The results obtained using PCA show clear separation between the investigated tissues and seasons. Principal component 1 discriminates between investigated tissues (liver in winter and spring on one side, and white muscle in winter and spring on the other side). Principal component 2 discriminates between the investigated seasons (liver and muscle in winter on one side, and liver and muscle in spring on the other side). This analysis revealed clear separation of antioxidant defense enzymes between the liver and muscle. Also, more pronounced seasonal differences were obtained for white muscle than for the liver. In studies including multiple biomarkers, IBR was obviously a tool that allowed for a better understanding of the complex sets of results. In this way, it is more relevant than analysis of each single biomarker response [7]. Using IBR index analysis, comparisons were made between two different seasons with different environmental influences and two metabolically different tissues by two visual criteria: the size and geometric form of the areas of the polygons. Star plots showed a higher biomarker response in the liver in spring, as well as a higher response of glutathione dependent enzymes (GSH-Px, GR and GST) and CAT in both tissues.

As we already mentioned, there is a limited amount of information regarding sexual and developmental differences of the antioxidant defense enzymes in the European hake, but it is known that in some other species, such as rainbow trout and black bullhead, the activities of the endogenous antioxidant systems (CAT, GSH-Px, GST) are influenced by age and maturation [45]. Also, in experiments with hepatocytes of male and female flounder it was demonstrated that many responses to oxidative stress were sex-related [46].

Our work represents a study of the complete antioxidant defense enzymes and phase II biotransformation enzyme GST in the liver and white muscle of

M. merluccius from the Montenegrin coastline and incorporation of the obtained data by applying an IBR approach. From the presented results it can be concluded that the antioxidant defense enzymes behave differently in the liver of the European hake when compared to white muscle. All enzyme activities (except MnSOD) were higher in the liver in comparison to white muscle. The liver possesses a greater capacity to establish and maintain homeostasis in different environmental conditions in winter and spring. At the same time, seasonal effects are more pronounced in muscle tissue. Seasonal changes of environmental factors mainly include temperature and light irradiance, while biological intrinsic variables include the phase of the reproductive cycle. The observed changes probably represent normal physiological adaptation to varying environmental factors in the Adriatic Sea and also suggest that natural variations of analyzed responses are associated with seasonality of both environmental and biological factors.

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Author contributions: All of the listed authors have contributed sufficiently to the work to be included as authors. Slađan Z. Pavlović conceived and designed an experiment. Slađan Z. Pavlović, as well as Jelena P. Gavrić, Marko Prokić, Svetlana G. Despotović, Branka R. Gavrilović, Tijana B. Radovanović, Slavica S. Borković-Mitić and Zorica S. Saičić participated in analysis and interpretation of the data and critical revision. Slađan Z. Pavlović wrote the paper and is responsible for its final content. All of the authors have approved the final manuscript.

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