INFLUENCE OF SEASON ON ANTIOXIDANT DEFENSE SYSTEMS OF *Silurus glanis* Linnaeus (Siluridae) AND *Barbus capito capito* Güldenstädt (Cyprinidae)

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ABSTRACT

Seasonal fluctuations in antioxidant (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; glutathione reductase, GR; glucose-6-phosphate dehydrogenase, G6PD) and biotransformation enzyme activities (glutathione *S*-transferase, GST) and lipid peroxidation (LPO) levels of livers and gills in European catfish (*Silurus glanis*) and Bulatmai barbel (*Barbus capito capito*) were determined. Superoxide dismutase, CAT, GPx, GR, G6PD and GST activities were higher in the liver compared to gills for both species. The exception was being in *B. capito capito* where GR was higher in the gills compared to the than liver during autumn and winter. However, LPO levels were higher in the gills compared to the liver. At the end of present study, it was concluded that antioxidant defense system for both species were affected by many endogenous and exogenous factors. However, it is difficult to determine what is the most critical factor affecting ADS in these species because of complex effects of these factors on the antioxidant defense system.

KEYWORDS: Bulatmai barbel, european catfish, oxidative stress, reactive oxygen species, seasonal changes

1. INTRODUCTION

Oxidative metabolism of cells is a continuous source of reactive oxygen species (ROS). Reactive oxygen species, including free radicals that have an unpaired electron in their outer orbital, are highly reactive molecules. At low concentrations, they may be beneficial or even indispensable in processes such as defenses against microorganisms, contributing to phagocytic bactericidal activity. In contrast, their high doses and/or inadequate removal result in oxidative stress. The main damage induced by oxidative stress is in alterations of cellular macromolecules such as membrane lipids (lipid peroxidation), DNA and proteins [1]. The key reactive species are superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radical ($OH^{\cdot}$) [2]. Under a normal physiological state, harmful effects of ROS are effectively neutralized by antioxidant defense system (ADS) of organisms, which in general comprises both enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.16), glutathione peroxidase (GPx, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), and low molecular weight antioxidants, such as vitamins E and C [3].

Lipid peroxidation (LPO) has been extensively used as a marker of oxidative stress. Tissue lipid and polyunsaturated fatty acid (PUFA) contents and compositions are critical factors in the LPO [4]. Fish tissues contain large quantities of PUFAs essential for membrane function [5] and PUFAs imply an elevated risk of oxidative stress, as PUFAs are major targets for ROS [6].

Antioxidant defense system of fish can be influenced by many factors such as systematic position, age, feeding behavior, food consumption, diet type, stress, toxins presents in the water, seasonal and daily changes in dissolved oxygen and temperature [5, 7-11]. Most of the researches on ADS in fish have focused on toxicological aspects [12-13] and there are limited investigations showing relationship between season and oxidative cycle [11, 14-16]. Although little is known about ADS of *Silurus glanis* Linneaus, 1775 (Siluridae) [17], nothing is known on ADS of *Barbus capito capito* Güldenstädt, 1773 (Cyprinidae).

European catfish (*S. glanis*) is the largest freshwater fish species of Europe and naturally distributed in the large rivers and lakes from central and eastern Europe to southern part of central Asia [18] and also occurs in Turkey's lakes (i.e. Manyas, İznik, Çıldır, Big Menderes) and rivers (Sakarya, Fırat, Ceyhan, Seyhan, Kura and Aras). While main distribution areas of Bulatmai barbel (*B. capito capito*), commonly called "caner" in Turkey, are Caspian

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Sea and Aral Lake Basins, it also lives in Çoruh and Aras Basins in East Anatolian Region of Turkey [19]. They are economically valuable fish species because of their tasty fleshes and high growing up ratios [19-20]. However, both species are endangered by illegal catching methods, human pressures, degradation of spawning habitats and pollution. Consequently, there is need for good management practices for the conservation of stocks in Turkey. To fulfill this task, it is necessary to achieve a sound knowledge of the biology of these species. Therefore, the purpose of the present study was to investigate the seasonal changes in the antioxidant and biotransformation enzyme activities and lipid peroxidation levels of the liver and gills in the European catfish and Bulatmai barbel and to provide reference values throughout one year.

2. MATERIALS AND METHODS

2.1. Fish sampling

Immature European catfish (769.90±85.95 g) and Bulatmai barbel (345.20±63.44 g) were caught quarterly between autumn 2006 and summer 2007 from Aras River, Karakurt, Kars, Turkey (040° 09' 36''N, 042° 35' 32.4''E). The fish were caught using monofilament gill nets. Five fish per season were killed with a sharp blow on the head; liver and gills were excised and frozen immediately in liquid nitrogen. Samples were transferred to laboratory and stored at -84 °C until analyses. Water temperature and dissolved oxygen values were measured with a digital analyzer (WTW 360i, Germany).

2.2. Determination of enzyme activities and lipid peroxidation levels

Liver and gills were washed in 0.9 % NaCl, dried with a filter paper and about 1 g samples were weighted. Samples were homogenized in 10 vol (w/v) 1.15 % KCl for 5 min. The homogenates were centrifuged at 10000 rpm for 30 min at +4 °C and the resulting supernatants were used for analysis.

The following enzyme activities were measured by using a double beam spectrophotometer (Thermo, Evolation 100, England).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by inhibition of xantine/xantine oxidase generated O_2^- reduction of nitroblue tetrazolium (NBT) [21]. NBT was reduced to blue formazon by O_2^-which has a strong absorbance at 560 nm. One unit was defined as the amount of protein producing 50% inhibition of reaction. The reaction mixture of 1mL contained 1 M Tris-HCl, 5 mM EDTA buffer (pH 8.0), 150 µM NBT, 400 mM Na_2CO_3, 1 g L^{-1} bovine serum albumine, 3 mM L^{-1} Xantine, 0.833 U mL^{-1} Xantine oxidase, 0.8 mM $CuCl_2$ and tissue homogenate (30 µL).

Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined by following the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm by the coupled reaction with glutathione reductase (GR) [22-23]. The assay system contained 1 M Tris-HCl, 5 mM EDTA buffer (pH 8.0), 0.1 M GSH, 10 U mL^{-1} GR, 2 mM NADPH, 7 mM t-buthylhydroperoxide, distillated water and tissue homogenate (10 µL).

Catalase (CAT, EC 1.11.1.16) activity was measured according to the method of Beutler [23] after the decomposition of H_2O_2 at 230 nm (ε= 0.071 mM^{-1} cm^{-1}). The assay mixture consisted of 0.05 M phosphate buffer (pH 7.0), 0.019 M H_2O_2 and tissue homogenate (10 µL).

Glutathione reductase (GR, EC 1.6.4.2) activity was determined as described by Beutler [23] by measuring the oxidation of NADPH at 340 nm using the extinction coefficient 6.22 mM^{-1} cm^{-1}. The reaction mixture of 1 mL contained 1 M Tris-HCl, 5 mM EDTA buffer (pH 8.0), 0.033 M GSSG, 2 mM NADPH, distillated water and tissue homogenate (10 µL).

Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) activity was measured as described by the method of Beutler [23]. The activity assay was done by monitoring the increase in absorption at 340 nm (ε= 6.22 mM^{-1} cm^{-1}) due to the reduction of 2 mM $NADP^+$. The assay system contained 1 M Tris-HCl, 5 mM EDTA buffer (pH 8.0), 0.1 M $MgCl_2$, 2 mM $NADP^+$, 6 mM G6P, distillated water and tissue homogenate (50 µL).

Glutathione *S*-transferase (GST, EC 1.5.1.18) activity was evaluated with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate in a final reaction mixture containing 0.11 M phosphate buffer (pH 6.5), 30 mM CDNB, 0.1 M GSH, distillated water and tissue homogenate (50 µL) [24]. The activity rate was measured as change in optical density at 340 nm (ε= 9.6 mM^{-1} cm^{-1}).

The level of lipid peroxidation (LPO) was determined by assessment of MDA as total thiobarbituric acid (TBARS)-reactive products by method of Uchiyama and Mihara [25]. The breakdown product of 1,1,3,3-tetraethoxypropane was used as a standard. Tissue homogenates were mixed with phosphoric acid (1 % v/v) and TBA (% 0.6 w/v). Then, the mixture was mixed strongly and incubated in a boiling water bath for 45 min. After cooling the tubes, n-Butanol was added and mixed and the precipitates were removed by centrifugation at 4000 rpm for 10 min. Finally, the MDA concentration in the supernatant fraction was determined spectrophotometrically at 532 nm.

All processes were carried out in the ice or at +4 °C. Specific enzyme activities were defined as enzyme unit (EU) per mg protein and MDA concentrations were expressed as µmol per mg protein. All chemicals used in the present investigation were obtained from Sigma-Aldrich (USA).

2.3. Protein content of samples

The protein content of samples was determined by Bradford method [26] using bovine serum albumin as a standard.

2.4. Data analyses

The statistical analysis was performed with SPSS version 10.0 for Windows [27]. Data were presented as mean ± standard deviation (SD) of the mean. Data were analyzed by one-way analysis of variance (ANOVA). The significant means were compared by Duncan's multiple range tests (P<0.05; n=5).

3. RESULTS

3.1. Environmental parameters

Seasonal variations in water temperature and dissolved oxygen amounts were given in Table 1. The maximum temperature was determined in summer (20 ^{o}C), while the lowest temperature was assayed in winter (4.1 ^{o}C). The maximum and minimum dissolved oxygen amounts were found in the winter, 8.56 mg L^{-1} and summer, 6.03 mg L^{-1}, respectively.

TABLE 1 - Water temperature and dissolved oxygen values of Aras River.

	Temperature (^{o}C)	Dissolved Oxygen (mg L^{-1})
Autumn	6.2	7.29
Winter	4.1	8.56
Spring	16.1	7.52
Summer	20.0	6.03

3.2. Antioxidant defenses in *Silurus glanis* and *Barbus capito capito*

Superoxide dismutase, CAT, GPx, GR, G6PD and GST activities were higher in the liver compared to the gills for both species. The exception were being in *B. capito capito* where GR was higher in the gills compared to the than liver during cold seasons (autumn and winter). In contrast the LPO levels of gills were higher than those of liver (Figures 1-2).

3.3. Hepatic biomarkers in *Silurus glanis*

The highest SOD, (2.74±0.33 EU mg protein^{-1}), CAT (205.72±27.97 EU mg protein^{-1}), GPx (352.71±34.21 EU mg protein^{-1}), GR (1.15±0.18 EU mg protein^{-1}), G6PD (23.77±1.84 EU mg protein^{-1}) and GST (33.00±1.96 EU mg protein^{-1}) activities and LPO level (9.1±1.5 µmol mg protein^{-1}) in the liver were recorded during the summer (Fig. 1a - g). While the lowest liver SOD and G6PD activities and LPO levels were measured in the autumn, 2.17±0.19 and 15.02±1.30 EU mg protein^{-1} and 5.4±1.6 µmol mg protein^{-1}, respectively, it was determined in the winter for CAT (119.17±11.37 EU mg protein^{-1}), GPx (147.85±8.79 EU mg protein^{-1}), GR (0.65±0.07 EU mg protein^{-1}) and GST (27.33±0.88 EU mg protein^{-1}) (Figure 1a - g). The seasonal differences of enzyme activities and LPO level of liver were statistically important (P<0.05).

3.4. Hepatic biomarkers in *Barbus capito capito*

Superoxide dismutase, CAT, GPx, G6PD, GST activities and LPO level of liver in *B. capito capito* also showed seasonal variations (P<0.05), with the highest activities found in the summer, 3.65±0.18, 152.81±13.69, 952.26±47.00, 22.36±0.68, 48.44±2.64 EU mg protein^{-1} and 110±1 µmol mg protein^{-1}, respectively and the maximum GR activity was measured in the spring as 4.53±0.44 EU mg protein^{-1}. The lowest activities were assayed in the autumn for SOD (2.28±0.12 EU mg protein^{-1}), GPx (716.94±28.47 EU mg protein^{-1}) and CAT (62.52±3.77 EU mg protein^{-1}) and in the winter for GR, G6PD, GST and LPO levels (0.66±0.07, 13.24±0.90, 30.45±1.36 EU mg protein^{-1} and 6.7±0.9 µmol mg protein^{-1}, respectively) (Figure 1a - g).

3.5. Branchial biomarkers in *Silurus glanis*

Catalase, GPx, G6PD and GST activities and LPO levels of gills showed similar changes during the investigation, with significantly higher level in the summer (44.34± 6.05, 203.05±12.07, 15.01±1.15 and 13.73±1.75 EU mg protein^{-1} and 141±15 µmol mg protein^{-1}, respectively) and decreases in the cold seasons (P<0.05). However, there were no seasonal differences in the SOD and GR activities (Figure 2a - g)

3.6. Branchial biomarkers in *Barbus capito capito*

The highest SOD (2.56±0.13 EU mg protein^{-1}), CAT (59.75±4.32 EU mg protein^{-1}), GPx (641.53±18.31 EU mg protein^{-1}), GR (1.73±0.10 EU mg protein^{-1}), G6PD (14.51±1.53 EU mg protein^{-1}) and GST (18.15±0.78 EU mg protein^{-1}) activities and LPO (310±20.1 µmol mg protein^{-1}) levels were recorded in the summer (Figure 2a – g). Although the lowest values were determined in the autumn for SOD (2.23±0.21 EU mg protein^{-1}), CAT (48.03±1.77 EU mg protein^{-1}), G6PD (6.68±0.77 EU mg protein^{-1}) and GST (10.75±0.85 EU mg protein^{-1}), it was assayed in the winter for GPx (230.11±14.37 EU mg protein^{-1}), GR (1.09±0.17 EU mg protein^{-1}) and LPO (14.2±2.2 µmol mg protein^{-1}). The seasonal changes in ADS of gills were also statistically important (P<0.05).

4. DISCUSSION

Reactive oxygen species generation, oxidative rates and antioxidant status of a thermoconformer animal are directly related to ambient temperature or metabolic activity. Because, they are not able to display a body temperature, which is distinct from ambient temperature. Higher temperatures generally coincide with high reproductive and/or foraging activities thus favoring greater O_2 and ROS production rates, and consequently, a concomitant higher antioxidant enzyme induction is expected [28]. At the present study, the highest activities of all enzymes and LPO levels in livers of both species were determined in the summer except GR activity of *B. capito capito*; it reached the highest level in the spring (Figure 1a - g). Furthermore; CAT, GPx, G6PD, GST activities and LPO levels of gills in *S. glanis* and all enzymes and LPO levels of gills in *B. capito capito* were determined in the summer. Gorbi et

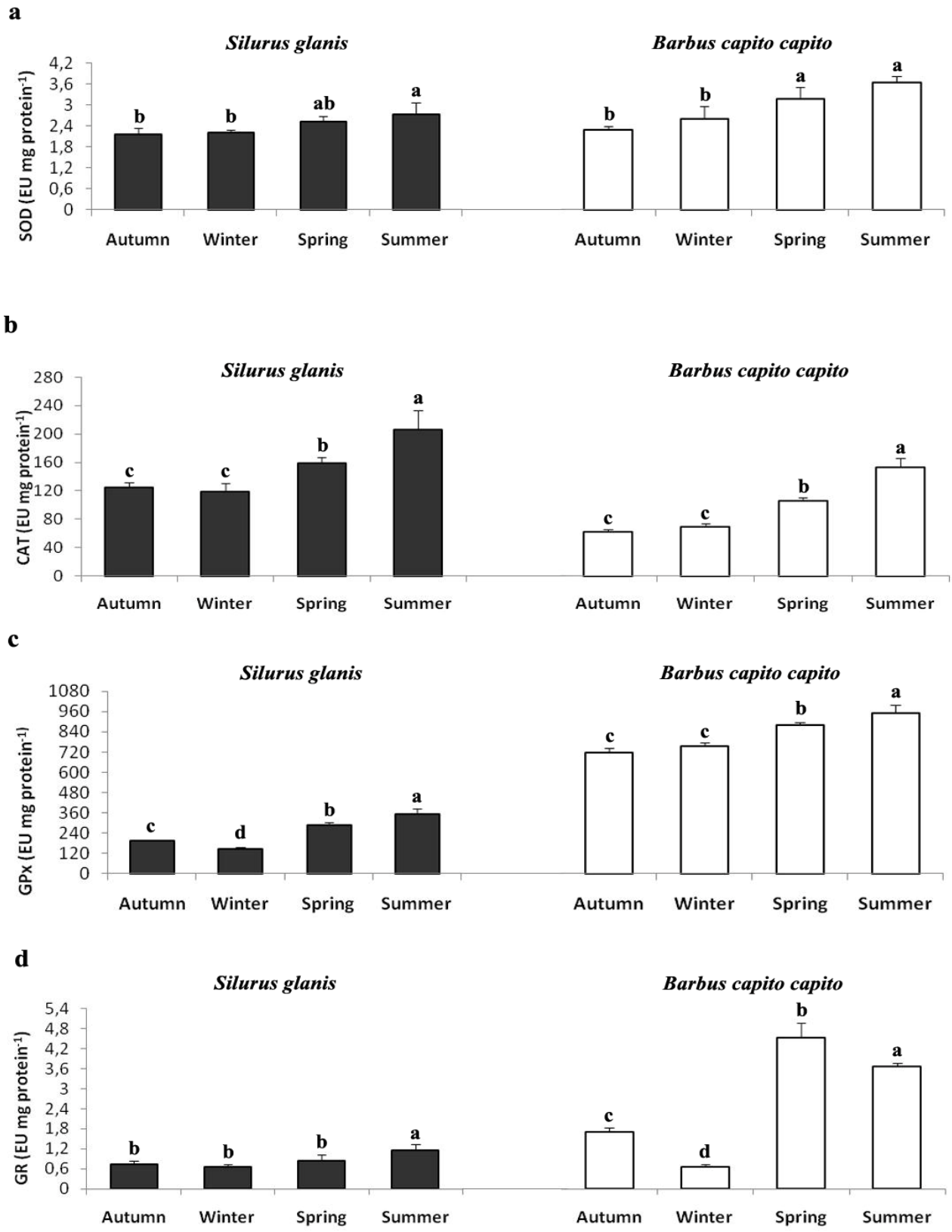


FIGURE 1 - Seasonal changes in antioxidant defense systems of livers of *Silurus glanis* and *Barbus capito capito*. (a): SOD activities; (b): CAT activities; (c): GPx activities; (d): GR activities. Values were presented as mean ± SD (n=5). Data were analyzed by one-way analysis of variance (ANOVA). The significant means were compared by Duncan's multiple range tests. Means with different letters are significantly different ($P<0.05$).

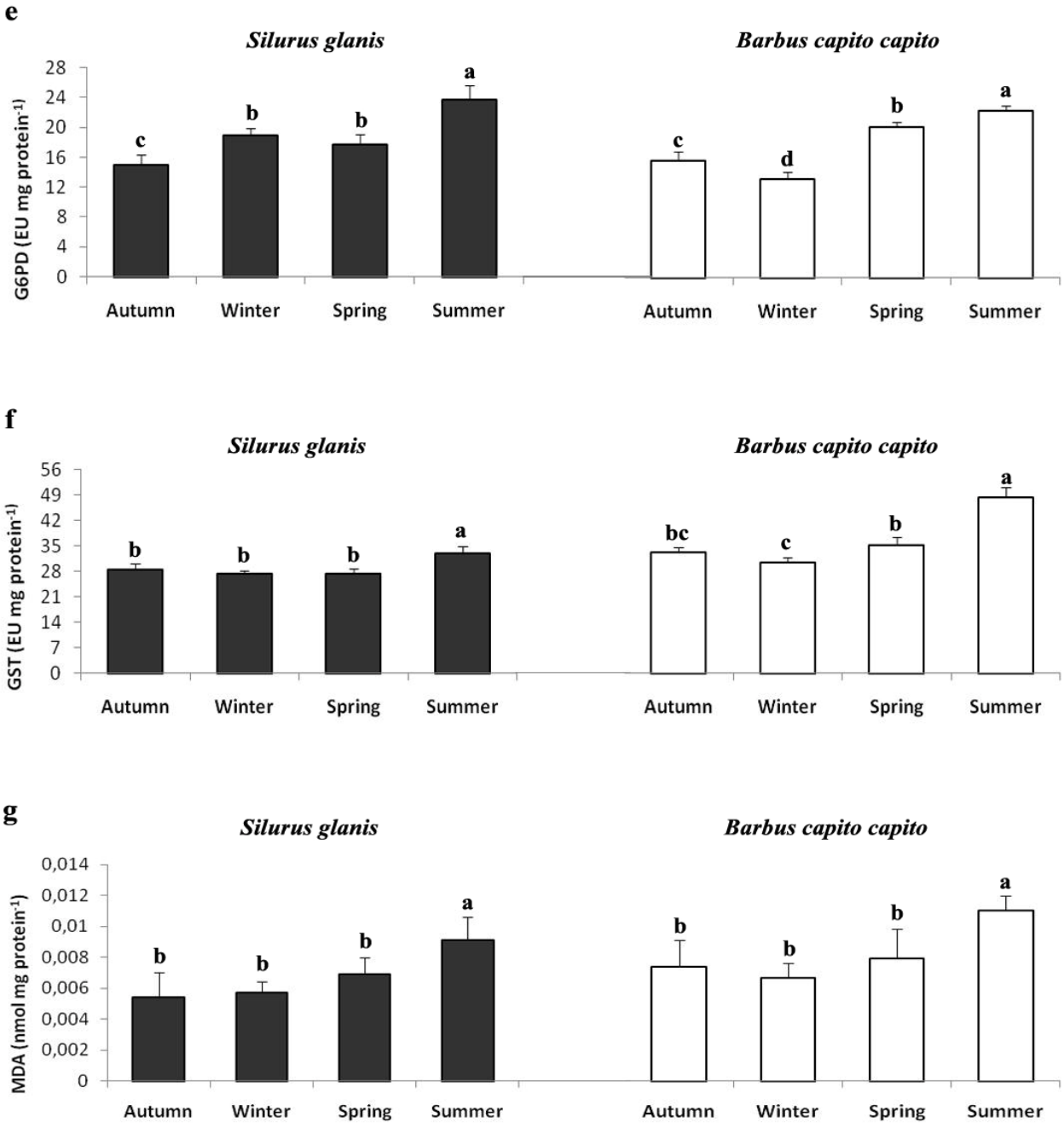


FIGURE 1 (continued) - Seasonal changes in antioxidant defense systems of livers of *Silurus glanis* and *Barbus capito capito*. (e): G6PD activities; (f): GST activities and (g): LPO levels. Values were presented as mean ± SD (n=5). Data were analyzed by one-way analysis of variance (ANOVA). The significant means were compared by Duncan's multiple range tests. Means with different letters are significantly different ($P<0.05$).

al. [29] described the lowest GST activities in January and March and the highest activities in August for *Anguilla anguilla* and *Mugil cephalus*. On the other hand, Rudneva [30] suggested that increasing temperature usually stimulates peroxide oxidation of lipids in the liver of fish. This suggestion is in accordance with results from our study. GST prevents LPO and DNA damage by reducing DNA base hydroperoxides and lipid hydroperoxides [31], therefore the increase in GST activity during the summer suggests that detoxification of toxic products of LPO, such as MDA [32]. Most of earlier studies about seasonal fluctuations of ADS in fish have given similar results with ours; thereof, the increase in the antioxidant status occurring during warm seasons (especially in summer) has been explained as an enhanced susceptibility of fish to oxidative stress.

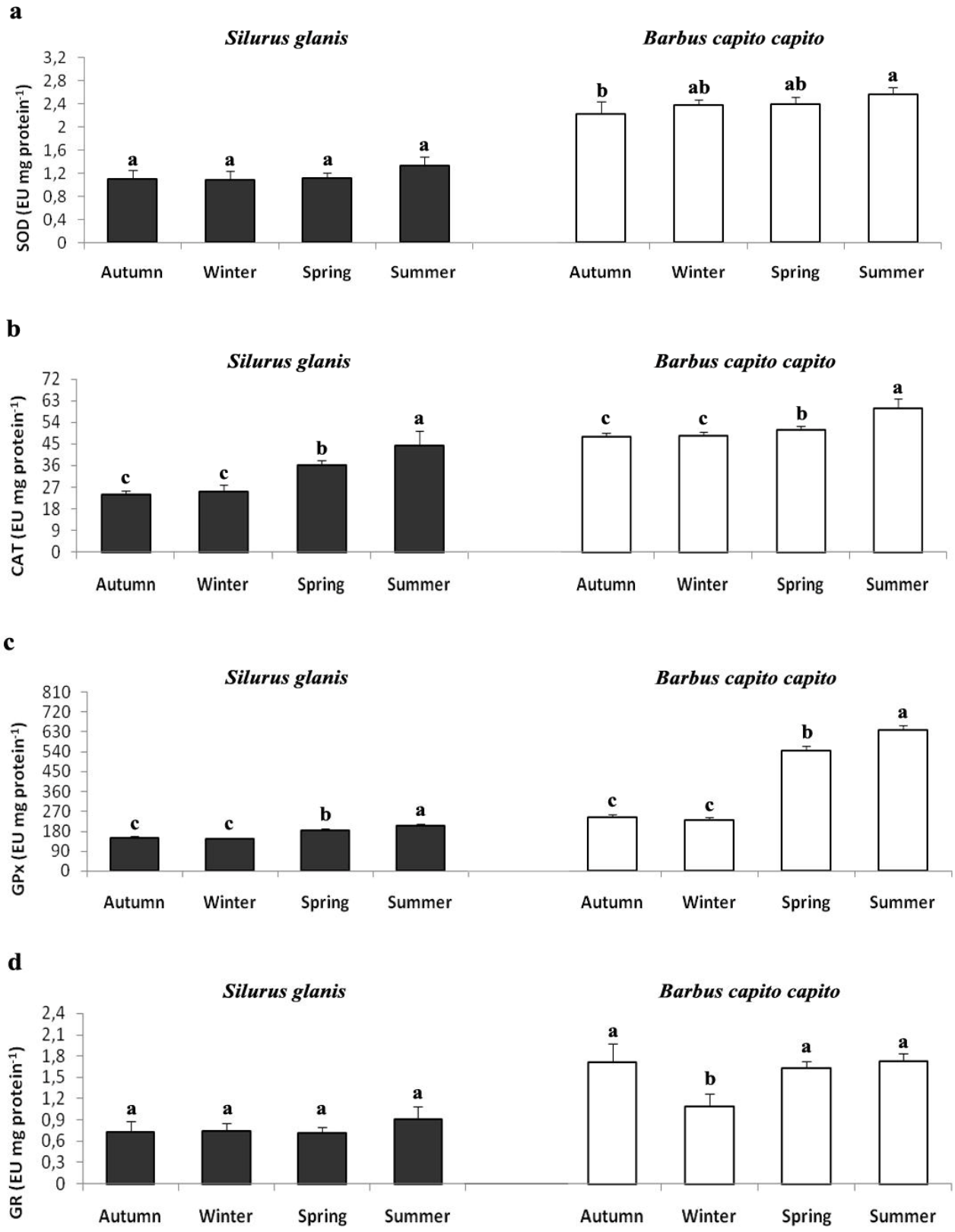


FIGURE 2 - Seasonal changes in antioxidant defense systems of gills of *Silurus glanis* and *Barbus capito capito*. (a): SOD activities; (b): CAT activities; (c): GPx activities; (d): GR activities. Values were presented as mean ± SD (n=5). Data were analyzed by one-way analysis of variance (ANOVA). The significant means were compared by Duncan's multiple range tests. Means with different letters are significantly different ($P<0.05$).

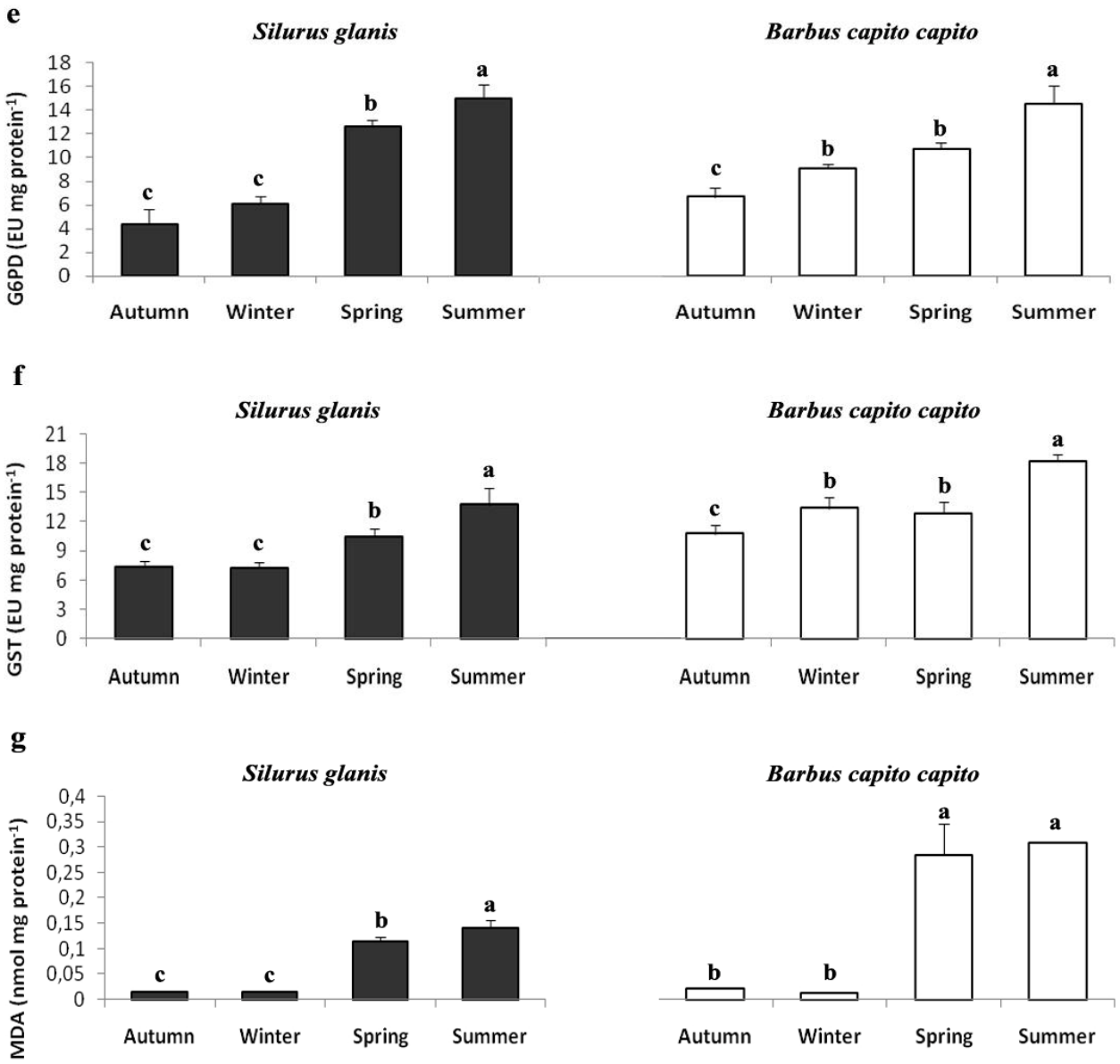


FIGURE 2 (continued) - Seasonal changes in antioxidant defense systems of gills of *Silurus glanis* and *Barbus capito capito*. (e): G6PD activities; (f): GST activities and (g): LPO levels. Values were presented as mean ± SD (n=5). Data were analyzed by one-way analysis of variance (ANOVA). The significant means were compared by Duncan's multiple range tests. Means with different letters are significantly different (P<0.05).

Superoxide dismutase and GR activities in the gills of *S. glanis* were not statistically affected by season, although maximum activities were measured in the summer. These results partly agreed with Ronisz et al. [33] and Solé et al. [14] who reported no correlation between GST, CAT and GPx activities and water temperature in *Pomatoschistus minutus* and *Zoarces viviparus*. However, it is difficult to explain the reason of these seasonal stability and harmony between SOD and GR in *S. glanis*'s gills. Moreover, gill GR activities in *B. capito capito* were higher in the cold seasons than warm seasons. Ritola et al. [2] and Aras et al. [16] reported higher gill GR activities than those of livers in *Oncorhynchus mykiss* and *Salmo trutta labrax*. They suggested that gills are more sensitive than liver against ROS and high GPx and GR activities in the gills may indicate an important role for the glutathione system in the gill epithelial cells as a front-line of antioxidant defense.

The activities of antioxidant and biotransformation enzymes were higher in the liver than gills, whereas the reverse was found for LPO. In thermoconformers vertebrates gut, gills, white muscles and pyloric caeca are less active tissues than red muscles, liver, blood, heart, kidney, roe and

lens, are the main sites of primary $O_2^{\cdot -}$ production and consequently have relatively lower antioxidant enzyme activities [28]. Trenzado et al. [34] demonstrated higher SOD and CAT activities in the liver than other tissues such as gills, heart and digestive tract in rainbow trout and sturgeon. Besides, Martínez-Álvarez et al. [5] reported that liver and erythrocytes usually show higher antioxidant enzyme activities, which could be due to high rates of free-radical generation in these tissues.

Consequently, it was determined that: (1) ADS of *Silurus glanis* and *Barbus capito capito* were affected by many endogenous and exogenous factors. But it was difficult to determine what is the most critical factor affecting ADS in these species because of complex effects of them to the ADS (2) this basic study provides reference values throughout one year of ADS status for these economically valuable and endangered two species and will enlighten to the further investigations on ADS.

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