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Original Research

Antioxidant Status and Lipid Peroxidation in Blood of Common Carp (*Cyprinus carpio* L.)

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Abstract

We estimated the impact of seasonal and anthropogenic contaminants on antioxidant status and lipid peroxidation in common carp. The fish were reared in a power station cooling canal and in a natural pond. The examined blood parameters were: superoxide dismutase (0.91 to 5.01 U·mg^{-1} HGB), glutathione peroxidase (3.13 to 21.03 U·mg^{-1} HGB), bilirubin (0.44 to 1.22 mg·dl^{-1}), urea (1.66 to 6.33 mg·dl^{-1}), malondialdehyde (0.39 to 2.21 nmol·mg^{-1} HGB), cadmium (0.031 to 0.088 mg·kg^{-1} w.w.), and lead (0.035 to 0.131 mg·kg^{-1} w.w.). Antioxidants were measured colorimetrically, and metals by atomic absorption. Fish living in cooling water had higher levels of all the parameters studied.

Keywords: SOD, GPx, MDA, Cd, carp

Introduction

Common carp (*Cyprinus carpio* L.) is a cosmopolitan fish found as native or introduced species in rivers of Europe, North America, and Northern Asia. The aquatic environment is characterized by marked temporal and spatial heterogeneity in the oxygen content due to water features such as temperature, salinity, and flow [1, 2]. Therefore, aquatic organisms are exposed to oxygen levels with daily and seasonal variation.

Free radicals, including reactive oxygen species (ROS), may adversely affect organisms. Oxygen is essential for many metabolic processes that are vital to aerobic life. However, dependence on oxygen forces aerobic life to withstand its considerable toxicity, as increased ROS levels can result in significant damage to cell structures [3, 4]. ROS and other prooxidants are continually detoxified and removed in cells by an antioxidant defense system comprising both antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and small molecular weight free radical scavengers. Metals such as Cd and Pb are pollutants of aquatic and terrestrial ecosystems, adversely affecting the environment and organisms. They may contribute to earlier induction of oxidative stress, similarly as elevated levels of oxygen. The oxygen paradox relies on it, as oxygen bears life and death simultaneously. The antioxidant response in fish can be modulated by natural environmental influences just like seasonal variations: thermoperiod, photoperiod, and oxygen saturation. Therefore, physiological changes in fish can serve as biomarkers of environmental pollution [5]. Molecular biomarkers of oxidative stress have found widespread applications in mechanisms of environmental toxicity and ecotoxicity in aquatic organisms exposed to a variety of chemical pollutants [6]. The aquatic environment daily receives substantial amounts of environmental pollutants that have the potential to cause oxidative stress in aquatic organisms through the free radical mechanism. Aquatic organisms can provide a model system for investigating how ROS damage cellular components, how cells respond, how repair mechanisms ameliorate this damage, and how oxidative stress can lead to diseases. Aquatic organisms are more sensitive to exposure and toxicity compared to terrestrial organisms, including mammals, and in this respect they provide exper-

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Table 1. Number (n) and total length (TL) of fish sampled from the cooling canal (sites I and II) and the breeding ponds (site III), and sampling dates.

Fish age [month]	Total Number of Fish	Sampling site						Significance of differences in TL among sites P>0.001 (a, b, c) P>0.05 (A, B, C)	Sampling date
		I		II		III			
		n	TL (cm) Mean±SD	n	TL (cm) Mean±SD	n	TL (cm) Mean±SD		
3	17	5	19.6±4.8	6	16.2±3.2	6	18.9±3.5	-	October 2006 – Autumn (A)
6	18	6	22.3±3.1↑	6	18.4±2.2↑	6	20.7±2.7↑	-	January 2007 – Winter (W)
9	16	5	26.4±4.6↑	5	23.7±5.4↑	6	24.9±3.9↑	-	April 2007 – Spring (Spr)
12	16	5	29.2±3.6↑	5	25.8±9.7↑	6	26.9±4.4↑	-	July 2007 – Summer (Sum)
15	18	6	31.2±3.9↑	7	28.7±4.3↑	5	29.8±4.7↑	-	October 2007 – Autumn (A)
18	18	7	35.3±4.8↑	6	31.9±5.1↑	5	33.5±3.6↑	-	January 2008 – Winter (W)
21	17	5	38.6±5.6↑	6	34.7±4.6↑	6	37.9±3.4↑	-	April 2008 – Spring (Spr)
24	19	6	41.9±3.8↑	6	38.4±5.3↑	7	39.4±1.8↑	-	July 2008 – Summer (Sum)
Total	139	45		47		47			

a – statistically significant differences among fish length at sites I and II;
b – statistically significant differences among fish length at sites I and III;
c – statistically significant differences among fish length at sites II and III;
"-" differences not found: ↑ – increase in fish total length along with age.

imental data for evaluation of subtle effects of oxidative stress, mutagenicity, and other adverse effects of pollutants [7].

The aim of this study was to estimate the effect of aquatic pollution with cadmium and lead on oxidant status (represented as lipid peroxidation (MDA), as well as on activities of SOD and GPx, and levels of bilirubin and urea in the blood of common carp. We have additionally determined how the season of the year and fish age influenced these parameters.

Experimental Procedures

Fish

To conduct the studies we had the approval of the Polish Local Ethics Committee No. 9/05. The study used one fish species: common carp (*Cyprinus carpio* L.). The fish were sampled 8 times between October 2006 and July 2008 (Table 1). A total of 95 fish specimens were sampled, aged from 3 to 24 months, weighing from 125.7 to 568.4 g (Table 2), and measuring (total length) from 24.3 to 54.5 cm (Table 1). Fish behavior was observed throughout the study.

Water Parameters

The fish were cultured in a canal filled with cooling water from the Dolna Odra Power Station (sites I and II) or in a commercial fish farm, in three ponds supplied by groundwater (site III). Site I was located directly at the cooling water release point, while site II was further downstream in the canal, about 2.5 km from the release point. The commercial fish farm (site III) was located about 20 km from Szczecin in Bukowa Forest, northwestern Poland (Western Pomerania Region), and was chosen as a control for sites situated on the cooling canal (Fig. 1). As all parameters measured in water and fish were similar in all three ponds, the fish from all these ponds were treated as one homogenous control group. Throughout the experiment, water temperature, dissolved oxygen content, pH, hardness and other water parameters were monitored at all three sites (Table 3).

Feed

The fish were fed twice a day with Aller Master extruded feed from Aller Aqua (Table 4), and the daily food ration amounted to 3.3±0.9 g per fish. The feed was designed to meet all the basic nutritional needs of the fish to ensure their health and growth. Table 5 presents the Aller Master feed rationing with regard to fish weight and water temperature.

Blood Sampling

From each individual, samples of the blood were collected for biochemical and chemical assays. Before sampling, the fish were reared in 5 m × 20 m fish breeding ponds at 12-16ºC. Prior to blood collection, the fish were gradually cooled to induce their hibernation. For this, the fish were transferred to a separate tank at 10ºC. After 20 minutes the fish were transferred to a new tank at 4-5ºC. Blood was sampled from the caudal vessel (*a. et. v. cau-*

Table 2. Weight of carp specimens, ages 3 to 24 months, sampled from the cooling canal (sites I and II) and the breeding ponds (site III).

Fish age [month]	Mass fish (g)			Significance of differences among sites P>0.001 (a, b, c) P>0.05 (A, B, C)
	Sampling site			
	Site I	Site II	Site III	
	Mean±SD	Mean±SD	Mean±SD	
3	109.7±12.2	89.7±8.9	115.4±12.9	-
6	187.8±13.4↑	157.8±14.5↑	155.1±18.2↑	a, b
9	248.3±18.4↑	228.3±11.9↑	268.7±11.4↑	b, c
12	391.2±12.4↑	341.2±15.4↑	402.9±14.5↑	a, c
15	633.5±26.6↑	693.5±19.6↑	598.5±28.9↑	C
18	998.9±15.9↑	948.9±26.9↑	954.7±11.4↑	-
21	1362.2±11.9↑	1252.2±12.7↑	1298.7±19.4↑	-
24	1778.3±28.4↑	1738.3±27.4↑	1758.4±20.6↑	-

a – statistically significant differences among fish weights at sites I and II;
b – statistically significant differences among fish weights at sites I and III;
c – statistically significant differences among fish weights at sites II and III;
„-" differences not found; ↑ – increase in fish weight along with age.

Fig. 1. Location of experimental sites: site I – cooling canal of the Dolna Odra Power Station, directly at the cooling water release point; site II – cooling canal of the Dolna Odra Power Station, about 2.5 km downstream of the release point; site III – commercial fish farm about 20 km from Szczecin in Bukowa Forest, northwestern Poland.

Table 3. Comparison of hydrochemical parameters of water from the cooling canal (sites I and II) and the breeding ponds (Site III).

Water parameters	Site I	Site II	Site III	Statistically significant differences among sites, P≤0.001
	Mean±SD	Mean±SD	Mean±SD	
Temperature (ºC)	15.80±4.50	13.80±3.40	14.80±3.89	+
pH	7.88±0.55	7.48±0.95	8.18±1.25	-
Dissolved oxygen ($mg \cdot l^{-1}$)	7.81±0.35	7.94±0.55	7.64±0.85	-
Oxygen saturation (%)	78.21±2.50	79.51±3.48	77.45±3.76	-
Alkalinity ($mmol \cdot l^{-1}$)	1.78±0.84	1.68±0.88	1.87±0.73	-
Hardness of water ($mg \cdot l^{-1}$)	8.25±1.08	7.15±1.18	7.36±1.35	-
Chemical oxygen demand ($mg \cdot l^{-1}$)	1.67±1.32	1.66±1.12	1.76±0.79	-
NH_4-N ($mg \cdot l^{-1}$)	1.18±0.75	1.34±0.48	1.44±0.54	-
NO_3-N ($mg \cdot l^{-1}$)	7.41±1.05	6.11±1.15	6.81±1.34	+
NO_2-N ($mg \cdot l^{-1}$)	0.68±0.16	0.48±0.36	0.64±0.24	+
PO_4^{3+}-P ($mg \cdot l^{-1}$)	0.15±0.07	0.14±0.11	0.17±0.18	-
Ca ($mg \cdot l^{-1}$)	50.51±4.25	41.51±3.75	49.54±3.34	+
Cd ($mg \cdot l^{-1}$)	0.05±0.01	0.04±0.01	0.04±0.02	-
Pb ($mg \cdot l^{-1}$)	0.03±0.05	0.03±0.07	0.03±0.04	-

Results are presented as mean ± SD; "+"significant difference, "-" difference not found

Table 4. Nutritional parameters of Aller master feed for common carp.

Parameter	Value
Size of feed grain [mm]	5.0
Protein [%]	35.0
Fat [%]	9.0
Carbohydrates [%]	43.0
Ash [%]	7.0
Fiber [%]	5.0
All-out energy [Kcal/MJ]	4325/18.1
Energy digestible [Kcal/MJ]	3353/14.0
Nitrogen (N) [d.m.* %]	5.2
Phosphorus (P) [d.m. %]	1.3
Energy in dry mass [Kcal/MJ]	4,701/19.6

*d.m. – dry mass

dalis) with a heparinized syringe. 50 IU of sodium heparin per 1 ml blood was used for stabilization. No anesthetics were used, as they produce inhibitory effects on biochemical parameters [8]. Samples of blood and serum from each fish were frozen and stored at -20ºC until analysis (for bilirubin, urea, metals), or -80ºC (for SOD, GPx, MDA). Directly after blood collection, the fish were decapitated and dissected. When dissecting the fish, anatomical observations of the organs and tissues were made. Fish behavior was observed throughout the study. The fish showed no changes in behavior and external appearance, and their food consumption did not change.

Biochemical Assays

Biochemical assays were performed on the samples of blood and serum from each fish. SOD and GPx activities and malondialdehyde (MDA) concentrations were determined in blood. Blood samples were homogenized (1:10 w/v) using a Potter Elvhjem glass homogenizer in the 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM ethylenediamineteteaacetic (EDTA), and with a few phenylmethanesulfonylfluoride (PMSF) crystals added prior to homogenization to inhibit protease. Next, the homogenates were centrifuged at 4ºC for 15 min at 15,000 g in a SIGMA 3K-15 centrifuge. Supernatants were collected and measured spectrophotometrically (Varian Cary 50 Bio).

SOD

The activity of superoxide dismutase (SOD EC 1.15.1.1) was measured by the adrenaline method [9]. Light absorbance of samples was measured at 560 nm. One SOD activity unit inhibits the rate of nitro blue tetrazolium (NBT) reduction by half. The measure of NBT reduction rate corresponds to 0.0165 absorbance units per minute in a 1-cm cuvette. SOD activity was expressed as $U \cdot mg^{-1}$ hemoglobin (HGB). To test the accuracy of the method applied, Ransod (Superoxide Dismutase) Control SD 126 from RANDOX Laboratories Ltd. was analyzed.

Table 5. Recommended daily rationing of Aller master feed expressed as kg of feed/100 kg of fish/day for common carp.

Water temperature [ºC]	Recommended daily rationing of Aller master feed		
	Fish weight [g]		
	40-100	100-300	300-2000
	Size of feed grain [mm]		
	3	4	5
2	-	-	-
4	-	-	-
6	-	-	-
8	-	-	-
10	-	-	-
12	-	-	1.4
14	2.0	1.8	1.5
15	2.5	2.0	1.5
16	2.5	2.0	1.8
18	3.0	2.5	2.0
20	4.0	3.0	2.5
22	4.5	3.5	3.0
24	5.0	4.0	3.5
>25	4.0	3.5	3.0

GPx

The activity of glutation peroxidase (GPx EC 1.11.1.9) was determined according to Paglia and Valentine [10], and Kraus and Ganther [11]. GPx is a catalyst of glutathione (GSH) oxidation by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione disulphide (GSSG) is converted to its reduced form, with simultaneous oxidation of NADPH to NADP+. The absorbance was measured at 340 nm. The results were reported as $U \cdot mg^{-1}$ HGB. To test the accuracy of the method applied, Ransod (Glutathione Peroxidase) Control SC 692 from RANDOX Laboratories Ltd. was analyzed.

MDA

Lipid peroxidation was measured by determination of malondialdehyde (MDA) concentrations and expressed as nanomol per milligram of hemoglobin ($nmol \cdot mg^{-1}$ HGB). The absorbance was measured at 535 nm. The MDA method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA at 45ºC [12].

Hemoglobin

Blood hemoglobin was determined by Drabkin's cyanmethaemoglobin method [13], using hemoglobin standard from AQUA MED ZPAM-KOLASA SP.J., catalogue No. 1010.2.

BIL_T

Total bilirubin level [BIL_T $mg \cdot dl^{-1}$] was determined by the Jendrassik-Gróf method [14] based on a reaction between bilirubin and diazotized sulfanilic acid. In aquatic solution, bilirubin reacts directly and produces colored azobilirubin. Its absorbance measured at 550 nm is directly proportional to total bilirubin content in the examined sample.

UREA

Urea level [UREA $mg \cdot dl^{-1}$] was determined according to Sampson [15]. The method is based on urease-catalyzed hydrolysis of urea to ammonium ions and CO_2. In the presence of glutamate dehydrogenase (GLDH), ammonium ions react with 2-oxoglutarate and produce glutamate, while NADH is oxidized to NAD. The absorbance was measured at 340 nm and the rate of its change was proportional to urea content in the sample [4]. To test the accuracy of the applied method, we analyzed control sera from Alpha Diagnostics Polska and from AQUA MED ZPAM-KOLAS SP.J.

Chemical Assays

Prior to the actual assay, 1-g wet tissue samples (weighed to the nearest 0.001 g) were mineralized wet in 3 ml concentrated HNO_3 in a CEM MDS 2000 microwave oven. The solution obtained was quantitatively transferred to polyethylene bottles. Total sample weight was brought up to 20 g with the addition of deionized water. Cd and Pb concentrations were determined with flameless graphite furnace atomic absorption spectrometry (GF-AAS) in a ZL 4110 Perkin Elmer spectrometer. The content of heavy metals (Cd, Pb) in blood was calculated from relevant calibration curves after correcting the data with blank results. Elemental blood concentrations are reported in $mg \cdot kg^{-1}$ wet weight ($mg \cdot kg^{-1}$ w.w.). To test the accuracy of the methods applied, the Fish Paste-2 certified reference material (CRM) was analyzed. Cd and Pb recoveries from the Fish Paste-2 CRM were reasonably consistent with the certified values (Table 6). For all of the studied elements the standard deviations were smaller than 5-7%.

Statistical Evaluation

The results are given as arithmetic mean values (mean) and standard deviations (±SD). The statistical analyses were performed using the computer software STATISTICA 6.0. The experimental data were subjected to statistical treatment involving one-way analysis of variance. ANOVA (Duncan's test) was used to test the significance of differences at the significance level of $P \leq 0.05$ (A) and $P \leq 0.001$ (a), and comparison of correlation coefficients (R^2) was per-

Table 6. Metal concentrations (Cd, Pb) in the certified reference material (fish-paste 2) and experimental recoveries (n=10).

Elements	Experimental value ($mg \cdot kg^{-1}$)	Certified value ($mg \cdot kg^{-1}$)	Recovery [%]	Difference between the reference and experimental values [%]
	Mean±SD	Mean±SD		
Cd	0.21±0.03	0.20±0.02	105.00	+5.00
Pb	19.63±1.36	20.81±0.51	94.33	-5.67

Table 7. Ranges of values of blood parameters measured in common carp, aged 3 to 24 months, at the cooling canal (sites I and II) and the breeding ponds (site III).

Blood/Serum Parameter	Carp (*Cyprinus carpio* L.)			Significance of differences P≥0.001
	Sampling site			
	I	II	III	
	min.-max.	min.-max.	min.-max.	
SOD [$U \cdot mg^{-1}$ HbG]	1.11-5.01	0.98-4.01	0.91-3.22	a, c, d, e, g, h
GPx [$U \cdot mg^{-1}$ HbG]	5.84-21.03	4.81-17.16	3.13-15.24	a, b, c, d, e, f, g, h
MDA [$nmol \cdot mg^{-1}$ HbG]	0.53-2.21	0.44-1.65	0.39-1.12	c, d, g, h
Bilirubin [$m \cdot dl^{-1}$]	0.88-1.22	0.55-0.85	0.44-0.71	a, b, c, d, e, f, g, h
Urea [$mg \cdot dl^{-1}$]	2.14-6.33	1.66-2.14	1.95-2.63	a, d, e, h
Cd [$mg \cdot kg^{-1}$ w.w.]	0.052-0.088	0.041-0.073	0.031-0.052	a, b, c, d, e, f, g, h
Pb [$mg \cdot kg^{-1}$ w.w.]	0.066-0.131	0.051-0.099	0.035-0.081	a, b, c, d, e, f, g, h

Statistically significant differences among sites I, II, and III for fish of the same age: 3rd month (a), 6th month (b), 9th month (c), 12th month (d), 15th month (e), 18th month (f), 21st month (g), and 24th month (h).

formed. The results obtained complied with the normal distribution assumption.

Results

Although anatomical and pathohistological examination was conducted in order to eliminate potentially sick individuals, autopsy revealed no disorders or disease symptoms. There were no blood congestions in gill lamellae, and the consistency of internal organs did not indicate any disease symptoms. Positive regression was observed between fish growth rate and experiment duration. Body weight increased by 125.7-568.4 g (R^2=0.952-0.982), and body total length by 24.3-54.5 cm (R^2=0.954-989).

Activity of SOD in fish blood changed throughout the study at each site (I-III), and ranged within 0.91 and 5.01 $U \cdot mg^{-1}$ HGB, averaging from 1.11 to 5.01 $U \cdot mg^{-1}$ HGB at site I, from 0.98 to 4.03 $U \cdot mg^{-1}$ HGB at site II, and from 0.91 to 3.22 $U \cdot mg^{-1}$ HGB at site III (Table 7). Statistically significant differences in SOD activity among sites were revealed for nearly all the studied age groups of fish, except for 6- and 18-month-olds (Table 7). SOD activity increased from spring to summer, and then decreased until winter, when it reached its minimum. The dynamics of changes in blood SOD activity during the growth period from October 2006 to July 2008 showed statistically significant cyclical fluctuations at all three sites: site I (R^2=0.992), site II (R^2=0.962), and site III (R^2=0.976) (Fig. 2a).

GPx activity in fish blood ranged within 3.13 to 21.03 $U \cdot mg^{-1}$ HbG, and underwent cyclical fluctuations at each site throughout the study. Average blood GPx activity varied from 5.84 to 21.03 $U \cdot mg^{-1}$ HGB at site I, from 4.81 to 17.16 $U \cdot mg^{-1}$ HGB at site II, and from 3.13 to 15.24 $U \cdot mg^{-1}$ HGB at site III (Table 7). Statistical significance of differences in GPx activity was confirmed for each site from autumn 2006 to summer 2008 for fish aged 3 to 24 months. GPx activity tended to increase in spring and summer, and to decrease in autumn and winter. This confirms the cyclical nature of seasonal changes during the studied 2 years (Fig. 2b). The dynamics of change in blood GPx activity during the growth period from October 2006 to July 2008 showed statistically significant cyclical fluctuations at all three sites: site I (R^2=0.981), site II (R^2=0.998), and site III (R^2=0.998) (Fig. 2b).

Biochemical parameters of the studied carp were determined on the basis of bilirubin and urea levels in serum without signs of hemolysis. Between October 2006 and July 2008, statistically significant cyclical changes in bilirubin and urea levels were observed in fish from all three sites (Fig. 3a). The average values for carp serum bilirubin (BIL_T) ranged from 0.44 to 1.22 $mg \cdot dl^{-1}$, while at individual sites the ranges were: site I from 0.88 to 1.22 $mg \cdot dl^{-1}$, site II

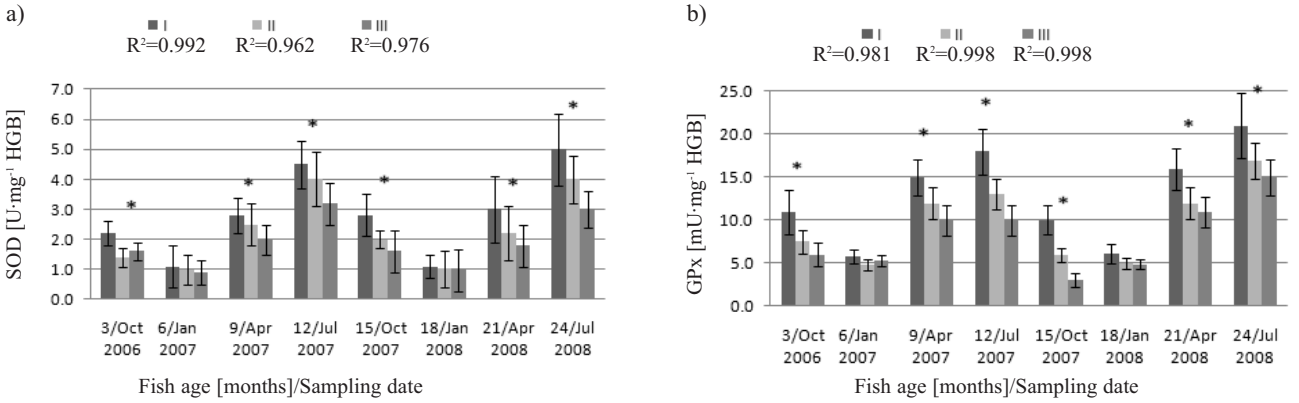

Fig. 2. Comparison of SOD (a) and GPx (b) activities (mean values) in the blood of common carp from the cooling canal (sites I and II) and the breeding ponds (site III);
* – significant difference ($P \leq 0.001$); R^2 regression coefficient.

from 0.55to 0.85 mg·dl^{-1}, and site III from 0.44 to 0.71 mg·dl^{-1} (Fig. 3a). The growth period between the 3rd and 24th months of life was marked by statistically significant bilirubin fluctuations (Fig. 3a, Table 7). The dynamics of changes in serum bilirubin during the growth period displayed statistically significant fluctuations with a slightly increasing tendency at all three sites: I (R^2=0.714), II (R^2=0.513), and III (R^2=0.993).

Average urea levels (UREA) ranged from 1.66 to 6.33 mg·dl^{-1}, while at individual sites the ranges were: site I from 2.14 to 6.33 mg·dl^{-1}, site II (1.66 to 2.14 mg·dl^{-1}), and site III from 1.95 to 2.63 mg·dl^{-1}. On site I, in autumn and summer periods, increased urea levels were detected in fish aged 3, 12, 15, and 24 months. This confirms that site I differed significantly from sites II and III. Urea levels in fish from sites II and III were similar during the whole study. In winter and spring periods, urea level in fish from site I dropped and no statistically significant differences among sites were observed (Fig. 3b, Table 7). The dynamics of change in serum urea levels during the growth period displayed statistically significant cyclical fluctuations at site I (R^2=0.962), and slight fluctuations at sites II (R^2=0.655), and III (R^2=0.619) (Fig. 3b).

Blood levels of MDA ranged from 0.39 to 2.21 nmol·mg^{-1} HGB, while at individual sites the ranges were: from 0.53 to 2.21 nmol·mg^{-1} HGB at site I, 0.44 to 1.65 nmol·mg^{-1} HGB at site II, and from 0.39 to 1.12 nmol·mg^{-1} HGB at site III. Significant differences among the three sites were observed during spring and summer periods, i.e. in fish aged 9, 12, 21, and 24 months (Table 7). In autumn and winter periods, MDA levels decreased, and differences among sites disappeared (Fig. 4). Throughout the whole study, MDA levels oscillated with a slightly increasing tendency. Also, the degree of lipid peroxidation in the examined fish showed significant seasonal or inter-site differences (Fig. 4). The dynamics of changes in blood MDA levels during the growth period showed statistically significant cyclical fluctuations at all three sites: I (R^2=0.999), II (R^2=0.989), and III (R^2=0.971) (Fig. 4).

Cadmium (Cd) levels in carp blood varied throughout this study from 0.031 to 0.088 mg·kg^{-1} w.w., and their fluctuations at individual sites are presented in Table 5. The highest Cd levels were found at site I (0.052 to 0.088 mg·kg^{-1} w.w.), staying lower at the two other sites: II (0.041 to 0.073 mg·kg^{-1} w.w.) and III (0.031 to 0.052 mg·kg^{-1} w.w.). In every season, blood concentrations of Cd differed signif-

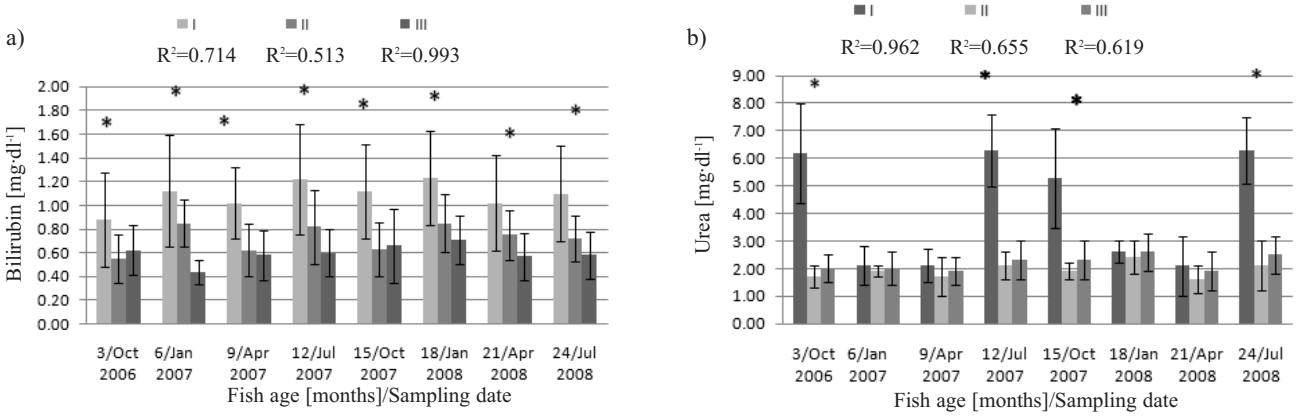

Fig. 3. Comparison of bilirubin (a) and urea (b) levels in the blood of common carp from the cooling canal (sites I and II) and the breeding ponds (site III);
* – significant difference ($P \leq 0.001$); R^2 regression coefficient,

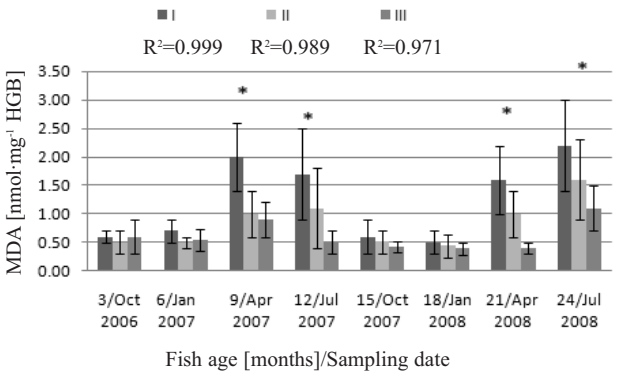

Fig. 4. Comparison of MDA activity (mean values) in the blood of common carp from the cooling canal (sites I and II) and the breeding ponds (site III);
* – significant difference (P≤0.001); R^2 regression coefficient.

icantly among all the examined sites, and the dynamics of changes showed a slightly increasing tendency for each site: I (R^2=0.840), II (R^2=0.845), and III (R^2=0.976) (Fig. 5a).

Lead (Pb) levels in carp blood varied throughout this study from 0.035 to 0.131 mg·kg^{-1} w.w. The highest levels were found in the fish from site I (0.066 to 0.131 mg·kg^{-1} w.w.) and the lowest at site III (0.035±0.081 mg·kg^{-1} w.w.), while at site II they averaged 0.051±0.099 mg·kg^{-1} w.w. (Table 5). Statistically significant differences were found in Pb levels among sites in all the examined seasons (Fig. 5b). Changes of Pb levels during growth showed an oscillating tendency at all three sites: I (R^2=0.894), II (R^2=0.925), and III (R^2=0.874) (Fig. 5b).

Discussion of Results

There is a wide range of oxygen tolerance among fishes. Cyprinid species can survive from nearly full anoxia to hyperoxia [1]. Hyperoxia is the opposite of the anoxia/hypoxia state and stimulates the generation of free radicals. Activities of antioxidant enzymes and the levels of free radical scavengers have been found to correlate with various physiological or pathological conditions, including stress. It is well known that stress leads to a series of biochemical, physiological and behavioral changes, thus altering normal body homeostasis. The generation of ROS in cells impairs antioxidant defense or exceeds the ability of the antioxidant defense system to eliminate oxidative stress. This situation may be associated with an increased influx of free radicals. Fish become more sensitive to diseases and lose adaptation capabilities to different water conditions [14, 16]. The results of our study indicate that average activity of enzymatic (SOD, GPx) and non-enzymatic (BIL_T, UREA) antioxidants in fish from all three sites underwent statistically significant cyclical fluctuations throughout the two-year study period. The highest SOD activity was observed in the fish from site I on the cooling canal, while the lowest was in the fish from the natural breeding pond (site III). Site comparisons revealed that the two parameters displayed a decreasing tendency in the order I>II>III. This indicates that the higher oxygenated cooling water increased activity of antioxidant enzymes. It seems that excess oxygen in water caused oxidative stress, reflected by the degree of lipid peroxidation, and in response carp produced more antioxidants such as SOD, GPx, BIL_T, and UREA to prevent an increase of free radicals. The importance of free radical reactions and ROS in physiological processes of living organisms and in mechanisms of toxicity by exposure to a variety of environmental pollutants stimulated an explosive increase of research and applications into the field of oxidative stress caused by ROS. Wdziaczek et al. [17] studied CAT and SOD activities and lipid peroxidation in erythrocytes and livers of different fish species. They reported that younger fish showed higher antioxidant activity then older fish. Our study confirmed that assumption. Also, Otto and Moon [18], who evaluated the activity of antioxidant enzymes in two teleosts, rainbow trout and black bullhead of age classes 1+ and 3+, reported that glutathione reductase (GR) and SOD activities were significantly higher in hepatic and extrahepatic tissues of younger fish. Ahmad et al. [3] have shown that low lipid peroxidation reflects the protective effects of oxidative enzymes.

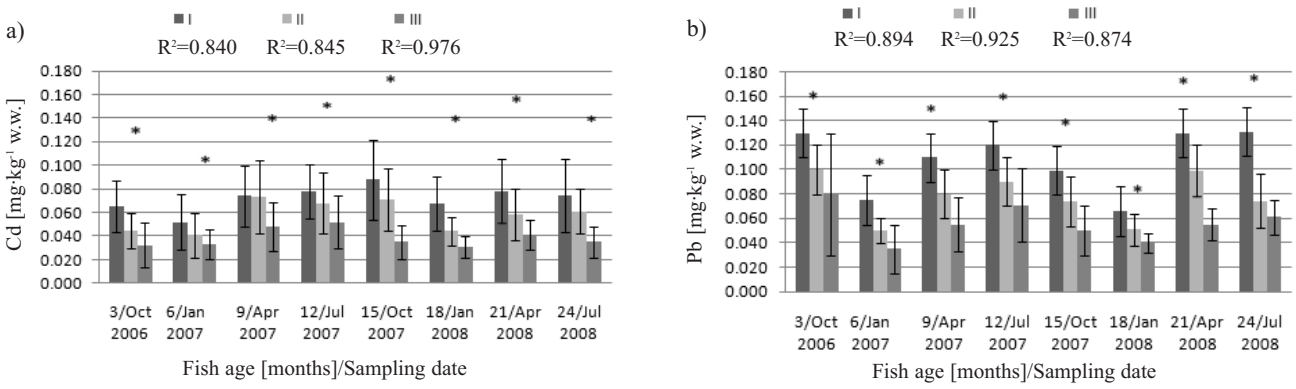

Fig. 5. Comparison of Cd (a) and Pb (b) levels (mean values) in the blood of common carp from the cooling canal (sites I and II) and the breeding ponds (site III);
* – significant difference (P≤0.001); R^2 regression coefficient.

Lipid peroxidation is a well-established mechanism of cellular injury in animals, and is used as an indicator of oxidative stress in cells and tissues. Malondialdehyde is widely used as an indicator of lipid peroxidation [19]. Increased levels of lipid peroxidation products have been associated with a variety of chronic diseases in both humans and other vertebrates [20, 21]. MDA reacts readily with amino groups on proteins and other biomolecules to form a variety of adducts [22], including cross-linked products [23]. MDA also forms adducts with DNA bases that are mutagenic [24] and possibly carcinogenic [25]. The TBARS (thiobarbituric acid) method is commonly used to measure MDA in biological samples [26]. However, this reaction is relatively nonspecific; both free and protein-bound MDA can react.

In our study, statistically significant differences were found in SOD activity, lipid peroxidation (MDA) and Pb content in the examined fish between the seasons. Increased MDA levels in spring and summer periods, as well as decreased levels in autumn and winter periods, were closely related to water oxygenation and oxygen saturation. This corresponded with increased SOD activity in spring, summer and autumn periods, and reduced SOD activity in winter periods. GPx activity was elevated throughout the whole study at site I, compared with sites II and III.

Bilirubin levels ranged from 0.44 to 1.22 mg·dl^{-1}, and each time were the highest at site I (Fig. 3a). The highest urea levels were noted at site I (2.14 to 6.33 mg·dl^{-1}), while at sites II and III they ranged from 1.61 to 2.63 mg·dl^{-1}. Differences between sites I and II were proved to be negligible in winter and spring, while they were significant in summer and autumn ($R^2_{I\ site}$=0.655, $R^2_{II\ site}$=0.619). This indicates that increased levels of free radicals (reactive oxygen species) in warm seasons exerted a negative impact on carp and, simultaneously, induced increased production of antioxidant enzymes.

Oxidative stress and increased lipid peroxidation in blood derived not only from excess oxygen in water in periods from spring to autumn, but also from the presence of heavy metals (Cd and Pb). Slight seasonal changes were observed in the first group compared with groups II and III (Cd I 0.052±0.088, Cd II 0.041±0.073, Cd III 0.031±0.052, Pb I 0.066±0.131, Pb II 0.051±0.099, and Pb III 0.035±0.081 mg·kg^{-1} w.w.). Cadmium exposure reportedly affects antioxidant defenses in fish. In this sense, it has been shown that Cd can compete with essential metals in protein-binding sites, triggering a release of Fe^{2+} and Cu^{2+} ions and causing increased generation of ROS [27]. Risso-de Faverney et al. [28] clearly related cell death to the oxidative stress caused by cadmium in trout hepatocytes. Their study has shown that only the changes of Cd concentration in the examined tissue lead to seasonal differences. Statistically significant differences were observed in the Cd concentration between spring and winter. Andersen et al. [29] reported low muscle concentrations of Cd in fish (roach, perch, and bream) that ranged from 0.000 mg·kg^{-1} to 0.045 mg·kg^{-1} w.w. However, our research has shown that the mean concentration of Cd ranged from 0.031 to 0.088 mg kg^{-1} w.w. in all the examined fish. The obtained research results prove the influence of the season of the year on Cd concentration in the examined blood. Fish are an ectothermic species and temperature significantly influences their metabolism. In summer, water temperature increases on average by 2.32-21.16ºC compared with winter. A slower metabolic rate was observed in winter in comparison to summer. This could inhibit metabolism and promote Cd accumulation in fish blood.

Fish appear to possess the same biochemical pathways to deal with the toxic effects of endogenous and exogenous agents as do mammalian species [30, 31]. It is important to examine toxic effects of metals on fish since they constitute an important link in the food chain, and their contamination by heavy metals (Cd, Pb) may imbalance the aquatic system and pose a health risk to consumers.

Conclusions

Our study revealed significant differences and cyclical fluctuations in the examined carp blood parameters (antioxidant and oxidant markers, metals) during a 21-month period (autumn 2006-summer 2008) at all three sites. We observed that SOD, GPx, BIL_T, Cd, and Pb increased from spring to autumn, and decreased in winter. Differently, urea increased in summer and autumn, and decreased in winter and spring. MDA levels had an increasing tendency in spring and summer and decreasing tendency in autumn and winter. Fish living in cooling water had higher levels of all the studied parameters. This indicates that higher oxygenated cooling water had a significant impact on oxidative status in carp. Carp organisms responded to oxidative stress, producing more antioxidants such as SOD, GPx, BIL_T, and UREA. A site comparison revealed that the parameters displayed a decreasing tendency in the order I>II>III, so the highest values of the parameters were at site I, and the lowest at site III.

This study has explicitly shown that carp reared in cooling water are exposed to a higher number of stress factors (higher water oxygenation, higher levels of Cd and Pb). This results in increased levels of lipid peroxidation biomarkers, and increased activities of antioxidant enzymes, which is an effective response of the organism to neutralize free radicals and prevent the induction of oxidative stress, which might lead to diseases or even death. This study also showed that carp are at greatest risk of intracorporeal disorders in summer, as in this season we consistently observed increases in all the analyzed physiological parameters.

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