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Antioxidant biomarker profile of chironomid larvae from carp ponds: Evaluation of the effects of different fish feeding patterns

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ABSTRACT

A 102-day feeding trial was conducted to evaluate the effects of four different fish feeding patterns on carp pond water quality and antioxidant biomarkers [superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione S-transferase (GST) and Thiol groups (SH)], protein content and biomass of the midge *Chironomus plumosus*. Farmed fish were fed two commercial diets: Soprofish 25/7 Standard (containing 25% protein and 7 % fat) and Soprofish 32/7 Profi Effect (containing 32 % protein and 7 % fat). These feeds were combined during a feeding trial in four different feeding patterns, designated as A, B, C and D. In feeding pattern A, the fish received Soprofish 25/7 Standard throughout the experiment and in feeding pattern D, Soprofish 32/7 Profi Effect. During feeding patterns B and C, a mixed feeding pattern was used, alternating between lower and higher protein diets. The study revealed no significant effects ($P > 0.05$) of the feeding regimens on water quality, so their effects on C. plumosus larvae can be evaluated independently. The activities of the chironomid enzymes CAT and GR showed a clear statistically significant dependence on the feeding pattern ($P < 0.05$), which increased with increasing protein content. On the other hand, the activity of SOD as well as larval biomass were noticeably affected by water quality, the former increasing with its deterioration and the latter with its improvement. The activities of GPx and GST were correlated with each other, but no statistically significant relationship was found with any of the measured abiotic factors ($P > 0.05$). Overall, our results suggest that long-term supplementation with a highly concentrated protein diet may have preventive effects against oxidative stress and support the use of *C. plumosus* as a model for assessing the effects of organic pollution on pond zoobenthic fauna.

1. Introduction

In Central and Eastern European pond aquaculture, carp (*Cyprinus carpio*) is one of the most commonly farmed fish. Carp production in Europe is predominantly based on the use of natural feed with supplemental feeding of cereals (Stanković et al., 2011; Roy et al., 2020). Fish farming in ponds is associated with an increase in ichthyoma, which is achieved through supplemental feeding that affects the ecosystem and the community of aquatic invertebrates living there, especially chironomid larvae, which are the most common inhabitants of the pond bottom (Imanpour et al., 2013; Božanić et al., 2018). The largest percentage of carp growth increment (60–65 %) is achieved by consumption of natural food such as zooplankton and zoobenthos, while the contribution of supplementary feeds containing mainly raw whole grains to carp growth increment is generally around 35–40 % of the total (Hlaváč et al., 2016). Natural feed has advantages over industrially produced feed, such as high digestibility of feed, especially protein, elasticity of natural feed (which easily deforms after ingestion) and high water content (95 %) (Bogut et al., 2007; Živić et al., 2011; Podder et al., 2020). As the predominant type of carp production in Serbia and China,

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which has the largest aquaculture production in the world (Hu et al., 2021), the semi-intensive production system is characterized by two food sources: natural food and supplementary feed (Marković et al., 2005).

Among the different types of natural food, chironomids constitute a considerable part of carp feed. As a natural food, chironomid larvae represent an important link in food chains, where they play a significant role in the metabolic cycle and energy flow of an aquatic ecosystem (Grebenjuk and Tomilina, 2014; Martins et al., 2021; Wonglersak et al., 2021). Chironomid larvae are rich in omega-3 and omega-6 fatty acids, with high concentrations of these fats ranging from 4.48% to 8.22% (Živić et al., 2011) observed in the most abundant species in ponds, *Chironomus plumosus*. According to Bogut et al. (2007) and previous studies by the authors (Živić et al., 2011), the crude protein content in fresh *C. plumosus* larvae is 7.6% and 6.61%, and the fat content is 1.3 % and 0.73 %, respectively, making them a good food source for freshwater fish. In recent decades, chironomid larvae, as aquatic benthic invertebrates, have played an important role as bioindicators of inorganic and organic pollution of aquatic systems (Molineri et al., 2020), providing information on water quality and the effects of natural and anthropogenic processes (Božanić et al., 2019; Nieoczym et al., 2020). Determining antioxidant biomarkers in chironomid larvae can provide information on how certain stressors from their environment affect these organisms, making them ecologically relevant in natural and artificial freshwater bodies (Ding et al., 2021; Planelló et al., 2013). Aquatic animals cannot separate their habitat from their excretory area. This leads to a deterioration of water quality within the production system, resulting in possible pollution of the receiving water body. Waste from aquaculture mainly comes from feed that has not been eaten by the fish and/or from spilled feed as well as excreted faeces. Some of the waste, such as ammonia (NH_3) and phosphorus (P), comes from metabolic products excreted by fish gills and in urine, while some of the waste comes from the decay/suspension of the nutrients themselves (Amirkolaie, 2011). These conditions destabilise the fishpond ecosystem, which in turn affects the zoobenthic fauna of the ponds. For this reason, it is necessary to constantly monitor the effects of the additional food on the pond itself and the organisms living in it. Studies looking at the response of individuals to oxidative stress through the antioxidant defence system should be presented as widely as possible to better understand the effects of different types of anthropogenic influences (Lushchak, 2011). Many environmental stressors induce oxidative stress through excessive production of reactive oxygen species (ROS) such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). To cope with stressful conditions, organisms switch their antioxidant defence system (Dröge, 2002). Therefore, in the present work, antioxidative biomarkers, i.e., i.e. the activities of the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST), as well as the content of total sulphydryl groups (SH), were used as analytical tools to assess the effects of different diets on the antioxidant response of *C. pulmosus* from carp ponds. The aim of our study was to determine the importance of diet composition and its cumulative effect on chironomid larvae. We focused on artificial feed sources, as we assumed that the antioxidant parameters with the strongest interaction with chironomid response would be promising factors for future characterisation of feed quality in fish farming.

2. Materials and methods

2.1. Experimental design and food composition

In order to investigate how carp diets with different mixtures affect the antioxidants of the fourth instar of *C. plumosus*, an experiment was conducted in an experimental fish farm of the Centre for Fisheries and Applied Hydrobiology (CEFAH), Faculty of Agriculture, University of Belgrade (Serbia). The experiment was conducted with four different treatments, including feeding patterns A, B, C and D in a semi-intensive system of carp farming. The trial lasted 102 days. One-year-old juvenile carps were acclimatised for 20 days before the start of the trial. During the adaptation period, the carps were fed once a day (at 9 am CET) with Soprofish 25/7 Standard at a feeding rate of 1.0% of the total fish body mass in the pond. At the start of the experiment, one-year-old juvenile carp (1180 in total) were placed in 12 experimental ponds, each with a rectangular area of about 400 m^2. The average individual weight of the fish was between 72.27 and 77.89 g, and the number of fish varied between 90 and 120 individuals per experimental pond, depending on its surface area, so that the same fish density of 0.3 ind.m^{-2} was achieved. The fish were fed daily at a rate of 2.5 % of the total fish body mass in the ponds. Two mixtures of extruded feed with different components were used for feeding (feeding patterns A, B, C and D), one feed with 25 % protein and 7 % fat (Soprofish 25/7 Standard) and the second feed with 32 % protein and 7 % fat (Soprofish 32/7 Profi Effect), both produced by the Veterinary Institute "Subotica" a.d., Subotica, Serbia (Table 1). Both experimental diets had a granule size of 4 mm. The carp fry in the trial were fed twice a day, at 9 and 13:30 CET. Feeding was done manually every day, always at the same location in each of the experimental lakes, except on days when the farmed carp were caught and measured. In feeding pattern A, the fish were fed only Soprofish 25/7 standard feed throughout the experiment. In feeding pattern B, the fish were fed Soprofish 25/7 standard feed for the first month (the first two periods of 16 feeding days) and Soprofish 32/7 Profi Effect feed for the following two months (the following four periods of 16 feeding days). The fish were fed the Soprofish 25/7 Standard diet for the first two months and the Soprofish 32/7 Profi Effect diet for the third month during feeding pattern C. Finally, in treatment D, the fish were fed the Soprofish 32/7 Profi Effect diet for the entire experimental period. Each feeding pattern was carried out in three replicates (each treatment in three ponds). In order to be able to quantify the possible effects of the different feeding patterns on the biomarkers studied, we assigned them corresponding scores. The scoring system was based on the assumption that the effects of different feeding patterns would have opposite and cumulative effects on the chironomids studied, as the degree of change in detrital composition should differ depending on the type and timing of feeding. This assumption is based on our previous research (Živić et al., 2013), which showed that the type of carp feed affects the fatty

Table 1
Formulation and approximate composition of Soprofish 25/7 Standard and Soprofish 32/7 Profi Effect used for carp receiving feeding patterns A, B, C, and D throughout the experiment at the "Little Danube" Centre for Fisheries and Applied Hydrobiology, University of Belgrade—Faculty of Agriculture, Serbia.

Ingredient, g/100 g of diet Approximate composition (% or kg^{-1} of dry weight)	Soprofish 25/7 Standard	Soprofish 32/7 Profi Effect
Crude protein (%)	25.0	32.0
Crude lipids (%)	7.0	7.0
Crude fibre – cellulose (%)	4.0	6.0
Ash (%)	6. 0	6.0
Water (%)	10.0	10.0
Urease (%)	0.3	0.3
Gross energy (MJ kg^{-1})	17.6	16.5
Digestible energy (MJ kg^{-1})	14.2	13.0
Calcium (%)	0.8	1.0
Phosphorus (%)	0.6	0.7
Vitamin A (IJ kg^{-1})	10,000	10,000
Vitamin D_3 (IJ kg^{-1})	1800	1800
Vitamin E (mg kg^{-1})	60	60
Vitamin C (mg kg^{-1})	50	120
Lysine (%)	1.5	1.5
Methionine + Cystine (%)	0.8	0
Methionine (%)	0	1.0

*Major ingredients: fish meal, fish oil, wheat and by-products of wheat processing, wheat gluten, peeled roasted soy flour, soybean oil, rapeseed oil, and butylated hydroxytoluene (BHT).

acid composition of *C. plumosus* as a result of the deposition of uneaten fish food on the bottom of the fishpond and the change in the composition of detritus, which is the main food source of *C. plumosus*. In accordance with this assumption, each month of feeding with Soprofish 25/7 standard feed is scored − 1 and with Soprofish 32/7 Profi Effect feed + 1, and the total score for each feeding pattern is obtained by adding these monthly scores, as shown in Table 2. As the biochemical marker and biomass measurements were taken at the end of the second and third month of the trial, the total scores were as follows: $A_{II} = -2$, $A_{III} = -3$, $B_{II} = 0$, $B_{III} = 1$, $C_{II} = -2$, $C_{III} = -1$, $D_{II} = 2$ and $D_{III} = 3$ for the nutrition parameter. The values obtained were used to assess the strength of the association between diet and the measured biomarkers or environmental parameters through the Pearson product-moment correlation and in the Co-Inertia analysis. Taking into account the initial assumption of opposing and cumulative effects of the diets used, a significant effect of the diet on any of the measured parameters should result in approximately similar values for diets A and C or B and D at the end of the second month. A similar relationship should be maintained at the end of the month III, but while the values of the measured parameters should continue to change in the same direction for diets A, B and D compared to the control, the direction of change should reverse for diet C due to the change in diet type at the beginning of the third month.

2.2. Monitoring of fish pond water quality

Based on the results of the daily measurements, the following physical parameters were assessed during the feeding trial period: Water temperature (WT), dissolved oxygen concentration (DO), oxygen saturation (DO %), electrical conductivity (Ec) and pH (Table 3). These parameters were measured with a MULTI 340i/ SET instrument (WTW, Germany). For each treatment, water samples were collected for chemical analysis using 1000 ml plastic bottles at a depth of 20 cm below the water surface of the pond, the depth at which most chironomids were sampled. The following chemical parameters were monitored: Total alkalinity - TA (mE L^{-1}), Water hardness - WH (°dH), Chemical oxygen demand - COD (mg L^{-1}), Sulphates - SO_4^{2-} (mg L^{-1}), Total phosphorus - Pt (mg L^{-1}), phosphates - PO_4^{3-} (mg L^{-1}), ammonium - NH^{4+} (mg L^{-1}), ammonia - NH_3 (μg L^{-1}), nitrates NO_3^- (mg L^{-1}), ultraviolet radiation (Uve) and transparency (Tr) (Table 3). Physical parameters were measured and water samples were collected before the start of the experiment (control) and then every 16 days (after every 15 feeding days to determine sampling dynamics and monitor water quality) between 10 and 11 am CET. Analyses were carried out at the Laboratory for Chemical Studies of Water in Fisheries, Faculty of Agriculture, University of Belgrade (Serbia).

2.3. Sample collection

Samples of the chironomid larvae were taken at a depth between 10 cm and 40 cm below the water surface of the pond, before the start of the carp feeding experiment (the control group), in the middle of the experiment (more precisely two months after the start of the fish feeding) and at the end of the experiment (four days after the end of the fish feeding). The fourth stage chironomid larvae were collected with a Van Veen dredge (260 cm^2 area) and manually with tweezers and a sieve. The control group contained 190 larvae. Table 4 shows the number of larvae collected in the different treatments. The individuals were then frozen in foil bags (30 specimens per bag) in liquid nitrogen and taken to the Faculty of Biology, University of Belgrade (Serbia). The labelled samples were stored in a freezer at − 80 °C, where they were kept until biochemical analysis.

Table 2
Summary of the scoring system and the feeding schedule for applied feeding patterns (A, B, C, and D) during three months of the experiment. Feeding with Soprofish 25/7 Standard feed during a month is scored as − 1, and with Soprofish 32/7 Profi Effect feed as + 1, and total scores for each feeding pattern are obtained by adding these monthly scores.

	A	B	C	D
I month	-1	-1	-1	1
II month	-2	0	-2	2
III month	-3	1	-1	3

2.4. Preparation of samples for biomarker analysis

A total of 10 larvae of very similar size were selected from each sample and used for enzyme tests. Larval samples were homogenised using an IKA-Werk Ultra-Turrax homogeniser (Janke and Kunkel, Staufen, Germany) at 4 °C in five volumes of 25 mmol L^{-1} sucrose with 10 mmol L^{-1} Tris-HCl, pH 7.5 (Rossi et al., 1983). The resulting homogenates were sonicated on ice at 10 kHz for 15 s to release enzymes (Takada et al., 1982). The sonicates were centrifuged at 1000g for 90 min (4 °C). The water-soluble fractions were obtained for the determination of protein content and antioxidant parameters. Ten larvae per replicate were used, with a total of three replicates per treatment to ensure representativeness.

2.5. Protein concentration and antioxidant biomarker assays

Total protein content was determined by the Bradford method at 595 nm, using bovine serum albumin as the standard for quantification (Bradford, 1976). Superoxide dismutase activity was determined as the rate of inhibition of autocatalytic adrenochrome formation at 480 nm using the method described by Misra and Fridovich (1972). One unit of SOD activity was defined as the amount of enzyme that causes 50 % inhibition of the autoxidation of adrenaline at 26 °C and was expressed as specific activity (U mg^{-1} protein). Catalase activity was determined by measuring the decomposition of the substrate H_2O_2 (Claiborne, 1985). Absorbance was measured at 240 nm for 3 min. Results were expressed as μmol H_2O_2 /min/mg protein. GPx activity was determined by following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) as substrate with t-butyl hydroperoxide (Tamura et al., 1982) and expressed as nmol NADPH/min/mg protein. Glutathione reductase activity was measured using NADPH and oxidised glutathione (GSSG) as substrates according to the colorimetric method originally described by Glatzle et al. (1974). The activity of GST towards 1-chloro-2,4-dinitrobenzene (CDNB) as substrate was evaluated according to the technique described by Habig et al. (1974). The reaction was recorded at 340 nm and the enzymatic activity was expressed as nmol GSH/min/mg protein. The concentration of total thiol groups (SH) was determined using DTNB according to the method of Ellman (1959) and expressed as nmol SHg^{-1} tissue. All tests were performed using a spectrophotometer (Shimadzu Corporation, model UV-1800 240 V IVDD) equipped with a multiple cell holder and temperature control. Chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA) or Merck (Darmstadt, Germany).

2.6. Data analysis

Data for antioxidant enzyme analyses were reported as mean standard error. To account for local and seasonal variability in antioxidant biomarkers and environmental data, samples were statistically compared using a two-way analysis of variance (ANOVA). Pairwise comparisons were performed using the Holm-Sidak test. The strength of the association between pairs of variables was measured using Pearson product-moment correlation. Sixty independent samples were used when biomarkers were compared for correlation. In all other cases, eight independent samples (four feeding patterns x two seasons) were used. Results were considered statistically significant at $P < 0.05$. Two-way analysis ANOVA and determination of Pearson product-moment correlation were performed using Sigma Plot 12 software (Systat Software

Table 3
Mean values of the physical and chemical water parameters in carp ponds with different feeding patterns (A, B, C, and D) measured at the beginning, in the middle (II month), and at the end (III month) of the experiment.

Water parameter	Control	A_{II}	A_{III}	B_{II}	B_{III}	C_{II}	C_{III}	D_{II}	D_{III} (30th October)	P value
WT (°C)	23.28 ± 0.08	17.60 ± 0.21^{a*+}	13.53 ± 0.27^{a+}	17.43 ± 0.38^{a*+}	11.80 ± 0.91^{b+}	17.53 ± 0.12^{a*+}	11.10 ± 0.49b+	17.37 ± 0.28^{a*+}	11.47 ± 0.19^{b+}	P_{fr}= 0.037 P_s < 0.001
DO_2 (mg L^{-1})	3.13 ± 0.26	2.89 ± 0.32^{a*}	4.13 ± 0.43^{a}	3.53 ± 0.14^{a}	3.66 ± 0.36^{a}	2.88 ± 0.35^{a}	3.80 ± 0.46^{a}	2.64 ± 0.05^{a*}	3.85 ± 0.13^{a}	P_s = 0.001
DO_2% (%)	37.2 ± 3.1	28.6 ± 3.0^{a*}	39.1 ± 3.2^{a}	30.9 ± 3.6^{a}	35.9 ± 4.7^{a}	29.5 ± 3.4^{a}	36.3 ± 4.8^{a}	27.1 ± 0.5^{a}	35.3 ± 1.3^{a}	P_s = 0.006
Ec (μS cm^{-1})	1915 ± 61	2346 ± 117^{a+}	2243 ± 122^{a+}	2162 ± 167^{a}	2143 ± 113^{a}	2134 ± 261^{a}	1902 ± 128^{a}	2290 ± 105^{a+}	2253 ± 117^{a+}	
pH	8.96 ± 0.07	8.82 ± 0.05^{a*}	7.79 ± 0.08^{a+}	8.82 ± 0.09^{a*}	8.07 ± 0.14^{a+}	8.82 ± 0.09^{a*}	8.15 ± 0.29^{a+}	9.030.05^{a*}	8.25 ± 0.30^{a+}	P_s < 0.001
TA (mE L^{-1})	13.38 ± 0.30	13.90 ± 0.31^{a}	13.10 ± 0.50^{a}	13.23 ± 0.09^{a}	13.10 ± 0.40^{a}	13.17 ± 0.48^{a}	12.80 ± 0.17^{a}	13.47 ± 0.71^{a}	12.97 ± 0.41^{a}	
WH (°dH)	6.1 ± 0.2	13.8 ± 0.7^{a}	13.7 ± 0.4^{a}	13.1 ± 1.1^{a}	13.7 ± 0.4^{a}	12.8 ± 0.8^{a}	12.9 ± 0.3^{a}	13.5 ± 0.7^{a}	13.9 ± 0.5^{a}	
COD (mg L^{-1})	66 ± 2	69 ± 3^{a}	77 ± 2^{a+}	80 ± 2^{a+*}	65 ± 5^{a}	77 ± 1^{a+}	71 ± 5^{a}	75 ± 6^{a}	69 ± 6^{a}	
SO_4 (mg L^{-1})	26.49 ± 0.68	37.85 ± 1.7^{a+}	40.22 ± 0.01^{a+}	38.18 ± 0.02^{a+}	39.92 ± 2.53^{a+}	38.16 ± 2.01^{a+}	38.90 ± 2.06^{a+}	37.68 ± 1.33^{a+}	37.65 ± 0.72^{a+}	
P_t (mg L^{-1})	0.497 ± 0.036	0.447 ± 0.014^{a}	0.353 ± 0.032^{a}	0.350 ± 0.020^{a}	0.410 ± 0.050^{a}	0.397 ± 0.041^{a}	0.403 ± 0.018^{a}	0.390 ± 0.055^{a}	0.357 ± 0.009^{a}	
PO_4 (mg L^{-1})	0.42 ± 0.03	0.35 ± 0.02^{a*}	0.25 ± 0.01^{a+}	0.26 ± 0.04^{a+}	0.31 ± 0.04^{a}	0.30 ± 0.04^{a}	0.34 ± 0.02^{a}	0.29 ± 0.03^{a}	0.27 ± 0.03^{a+}	
NH_4 (mg L^{-1})	0.215 ± 0.021	0.393 ± 0.060^{a+}	0.400 ± 0.026^{a+}	0.413 ± 0.034^{a+}	0.423 ± 0.020^{a+}	0.347 ± 0.007^{a+}	0.387 ± 0.029^{a+}	0.413 ± 0.055^{a+}	0.397 ± 0.023^{a+}	
NH_3 (μg L^{-1})	123 ± 33	84 ± 8^{a+}	6 ± 2^{a}	91 ± 15^{a}	12 ± 4^{a}	79 ± 17^{a}	15 ± 7^{a}	145 ± 17^{d}	26 ± 19^{a}	P_{fr}= 0.019 P_s < 0.001
NO_3 (mg L^{-1})	1.39 ± 0.05	1.47 ± 0.03^{a}	1.47 ± 0.03^{a}	1.53 ± 0.03^{a}	1.50 ± 0.00^{a}	1.57 ± 0.03^{a}	1.47 ± 0.07^{a}	1.47 ± 0.12^{a}	1.57 ± 0.09^{a}	
UV	0.20 ± 0.02	0.23 ± 0.08^{a}	0.28 ± 0.03^{a}	0.19 ± 0.03^{a}	0.30 ± 0.03^{a}+	0.23 ± 0.02^{a}	0.26 ± 0.04^{a}	0.34 ± 0.03^{a+}*	0.18 ± 0.03^{a}	
TR	5.3 ± 0.3	5.7 ± 0.9^{a}	8.7 ± 2.0^{a+}	5.5 ± 0.9^{a}	5.5 ± 0.9^{a}	5.3 ± 0.3^{a*}	9.7 ± 2.7^{a+}	5.3 ± 0.3^{a}	6.5 ± 0.5^{a}	P_s = 0.009
d (cm)	77 ± 7	52 ± 10^{a}	56 ± 9^{a}	40 ± 3^{a,b}	63 ± 15^{a}	65 ± 12^{a,b}	75 ± 8^{a}	87 ± 6^{a}	63 ± 3^{a}	

WT - water temperature; DO2 – dissolved oxygen; DO2% - dissolved oxygen saturation; Ec – electroconductivity; TA – total alcality; WH – water hardness; COD –chemical oxygen demand; SO42- - sulfates; Pt - total phosphorous; PO43- - orthophosphates; NH4 + - ammonium ion; NH3 – dissolved ammonia; NO3- - nitrates; UVe – ultraviolet emission; TR – transparency; d–water depth.
+ denotes statistically significant differences to the control feeding pattern ($p < 0.05$, two-way ANOVA)
* denotes statistically significant differences between months of the same feeding pattern
[a, b, c] denote statistically significant differences between four feeding patterns during the same month

Table 4
Number of larvae of *Chironomus plumosus* collected from each treatment (A, B, C, and D) in the middle (II month), and at the end of the experiment (III month).

Treatments	Number of individuals	
	II month	III month
A	135	225
B	145	195
C	125	155
D	100	225

Inc., USA). Environmental data (physical and chemical parameters of water) were analysed together with biomarker data using Co-Inertia Analysis (CIA) (Dolédec and Chessel, 1994). This allowed the simultaneous ordination of two data matrices having the same set of rows. Co-inertia axes were calculated by maximising the covariance of the factorial scores generated in separate ordinations of the two input tables [in this study, the principal component analysis (PCA) correlation matrix for the environmental variables and biomarkers]. Prior to analysis, the enzyme biomarker data were log10(x + 1) - transformed to reduce strong differences in activities. The Monte Carlo permutation test was used to test the significance of the co-structure between the two data sets as indicated by CIA. Co-inertia analysis was calculated using the software ADE − 4 (Thioulouse et al., 1997).

3. Results and discussion

3.1. Feeding patterns

As far as we know, no studies have been conducted on the effects of feeding patterns on the biomass and biochemical responses of chironomid larvae, so the present study is an important contribution. By expressing the given feeding patterns quantitatively, thus allowing quantification of their possible influence on the measured biochemical parameters and on the abundance and biomass of *C. pulmosus* larvae, we hypothesised that chironomid larvae from carp ponds would show biochemical and physiological adaptations to changing environmental conditions. In particular, we hypothesised that the effects of different feeding patterns would have opposite and cumulative effects on the chironomids studied. In view of this, we can reasonably assume a cumulative effect of feeding, as the extent of change in detrital composition will vary depending on when the appropriate feeding pattern is applied.

3.2. Biomarker responses

Despite the increasing use of biomarkers to assess the effects of various environmental factors, there have been no comprehensive studies of Chironomidae comparing the variability of antioxidant

biomarker responses in relation to different dietary compositions. It has already been documented that chironomids are good bioindicators of aquatic habitat condition (Szczerkowska-Majchrzak and Jarosiewicz, 2020). Regardless of their feeding strategy and the quality of the ingested food, they are not able to avoid toxic substances in the water and sediments. Like other benthic organisms of freshwater ecosystems, chironomid larvae depend on sedimentation for their food supply, and seasonal changes affect their physiological and biochemical responses (Nath et al., 2017). The results of the two-way analysis ANOVA, which considered both the effects of different feeding regimes and their length on biomarkers, were very consistent. For all biomarkers except GPx and biomass, ANOVA showed a highly significant statistical difference in the effects of treatment, their length and their interaction (Table 5). For GPx activity, a statistical difference was found between the effects of treatment and the interaction between treatment and its length, while in the case of larval biomass, a difference was only found for the length of treatment (Table 5). Fig. 1A shows the mean values ± SE of the protein concentration in the whole body of the chironomid larvae from the studied carp ponds during the experiment. There were no statistically significant differences in protein concentration between systems A_{II}, A_{III}, B_{III}, C_{II} and C_{III}, with the value in the C_{II} group being significantly higher than in the control group. The chironomid groups in the B_{II} and D_{II} regimes had similar protein concentration, with lower values than the control group. The D_{III} type group had the highest protein concentration compared to the control group and all other feeding patterns. This pattern of changes in protein concentration indicates that the Soprofish 32/7 Profi Effect diet, with a higher protein content than the Soprofish 25/7 Standard diet, had a cumulative effect on chironomid larvae, with the highest values recorded in the D_{III}-type diet, while protein concentrations in the C-type diet were comparable to those recorded in the A-type conditions (Fig. 1A).

Analysis of the data obtained revealed a similar trend in the variation of larval body protein and larval growth, the latter being more pronounced at the end of the third month (Fig. 1A, H). This is consistent with the findings of Nath et al. (2017), who indicated that growth rate and available protein quantity are strongly correlated in chironomid larvae.

The antioxidant biomarkers studied in *C. plumosus* larvae are characterised by considerable and statistically significant variability, both temporally (II months vs. III months of the experiment) and between groups with the type of carp diet (Fig. 1B, C, D, E, F, G). Of all the antioxidant biomarkers measured, two-way analysis ANOVA showed that GPx in C. plumosus had the least statistically significant temporal variability (Fig. 1D), while variability between groups on carp diet ($P < 0.001$) characterised all biomarkers.

Superoxide dismutase is the first line of antioxidant defence against ROS and catalyses the dismutation of $O_2^{\bullet -}$ to oxygen and H_2O_2. The effectiveness of SOD as an antioxidant depends on its cooperation with other antioxidant enzymes, i.e. CAT, GPx and GR (Manduzio et al., 2004). In the present study, the activity of SOD is characterised by a marked seasonal variability in forage types B, C and D, being statistically significantly higher in the second month (with a peak activity in the case of feeding pattern D) than in the third month of the experiment. The exception is forage A, where the situation is reversed. Accordingly, the direction of variation changes so that activity increases statistically significantly from A to D after the second month, while it decreases statistically significantly in the same direction after the third month (Fig. 1B). In addition, activity SOD showed a statistically significant negative correlation with protein concentration ($R = -0.634$, $P < 0.001$). The increase in activity in the month II could be directly responsible for the increased susceptibility of chironomids to excessive $O_2^{\bullet -}$ concentrations during this period. Chironomid larvae have a distinctive red colour and the ability to live in a hypoxic environment, which is due to their high haemoglobin content (Živić et al., 2013). As haemoglobin synthesis is stimulated in an environment with low oxygen concentration (such as a fishpond), this is considered one of the main sources of $O_2^{\bullet -}$ and to a lesser extent H_2O_2 (Nath, 2018). In the presence of Fe^{2+}, H_2O_2 is converted to the harmful hydroxyl radical ($^{\cdot}OH$) by the Fenton reaction. To prevent this phenomenon, CAT decomposes H_2O_2 into molecular oxygen and water, completing the detoxification process mimicked by SOD. The reduction of H_2O_2 to water and of organic hydroperoxides to their corresponding alcohols is performed by GPx by coupling it with the oxidation of reduced glutathione (GSH) (Ighodaro and Akinloye, 2018).

Glutathione reductase catalyses the regeneration of GSH and plays a critical role in maintaining the normal function of other GSH-dependent enzymes. This enzyme reduces glutathione disulphide (GSSG) to the sulphhydryl form GSH, which is also an important cellular antioxidant (Couto et al., 2016). For this reason, the increased activity of GR in larvae from feeding patterns B_{II}, C and D could be due to the formation of oxidised GSH.

Analysis of our data shows that the observed changes in the activities of CAT and GR correspond to those expected under the influence of diet. Catalase activity in the D_{III} pattern (Fig. 2C) also changed significantly compared to the other feeding patterns. The D_{III} pattern significantly increased CAT activity compared to the control and the other feeding patterns. Thus, at the end of the second month, larvae fed with diets B and D had significantly higher CAT and GR activities than larvae fed with diets A and C (Fig. 1C, E). In the month III, the activity of larvae fed with feed D is characterised by a statistically significant maximum for both enzymes, while the values of larvae fed with feed A and C are comparable. Also, the activities of CAT and GR in larvae fed diet C change the trend of their activity compared to control larvae (Fig. 1C, E). The enzymes SOD and CAT are jointly involved in the stepwise oxygen reduction. Since the activity of SOD was increased in months II compared to III in larvae receiving diet patterns B, C and D, we assumed that this increased activity of SOD would lead to increased H_2O_2 concentration and consequently to a further increase in the activity of CAT. However, the activity of CAT decreased, suggesting that during this period the ability to protect against H_2O_2 was reduced and that H_2O_2 was not scavenged by this antioxidant enzyme. Similar results were found in a study with Oreochromis niloticus (Carvalho et al., 2012).

On the other hand, the reduced activity of SOD in larvae fed dietary patterns B, C and D in the months III was associated with an increased activity of CAT compared to II. This is consistent with the results of the study by Choi et al. (2000), suggesting that CAT may have higher

Table 5
Results of two-way ANOVA taking into account both the effects of difference and their length on measured biomarkers in larvae of *Chironomus plumosus*.

		Cp	SOD	CAT	GPx	GR	GST	SH	Biomass
d.f.	length	1	1	1	1	1	1	1	1
	treatment	3	3	3	3	3	3	3	3
	interaction	3	3	3	3	3	3	3	3
F	length	37.97	3043.4	37.18	0.648	39.24	27.54	33.16	17.08
	treatment	6.045	1857.9	16.24	10.95	78.47	47.06	6.45	1.266
	interaction	20.25	611.97	5.564	20.13	19.63	51.43	58.66	1.284
p	length	< 0.001	< 0.001	< 0.001	0.425	< 0.001	< 0.001	< 0.001	< 0.001
	treatment	0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.319
	interaction	< 0.001	< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.314

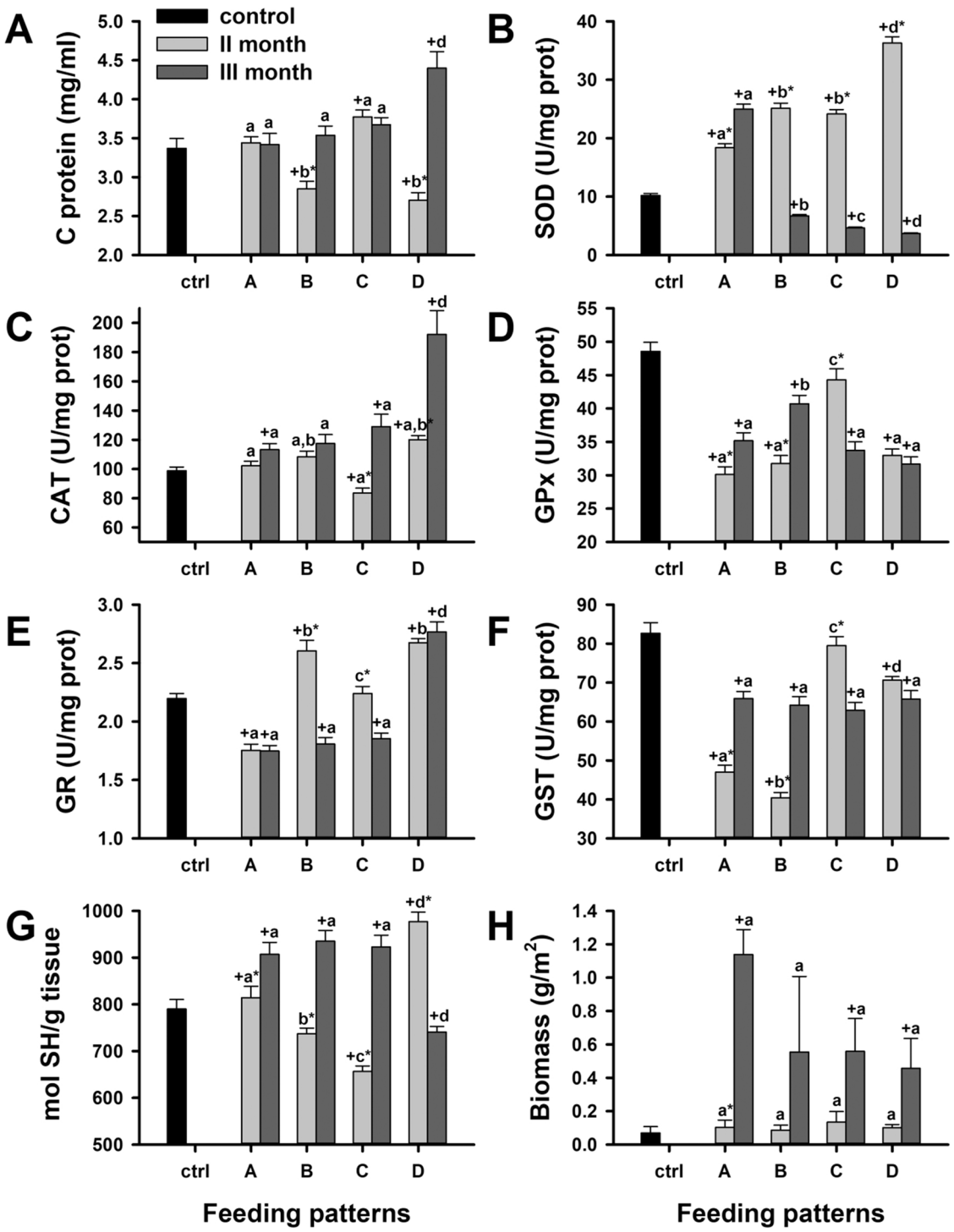

Fig. 1. Activities of antioxidant biomarkers in *Chironomus plumosus* from the carp pond fed with different feeding patterns (A, B, C, and D) measured in control, II month, and III months. (A) Protein concentration (C protein); (B) Superoxide dismutase (SOD) activity; (C) Catalase (CAT) activity; (D) Glutathione peroxidase (GPX) activity; (E) Glutathione reductase (GR) activity; (F) Glutathione-S-transferase (GST) activity; (G) total thiol (SH) groups and (H) biomass of *Chironomus plumosus* (mean ± standard error). Samples with different feeding patterns that are significantly different from the control sample are marked with + . Samples fed with the same feeding pattern at the end of the second month that is significantly different from the samples at the end of the third month of the experiments are marked with * . Samples fed with different feeding patterns sampled in the same month that are significantly different are marked with different letters (a, b, c, d).

activity levels in C. riparius to counteract periodic increases in the production of ROS as observed in C. plumosus (Fig. 1B, C). Insects affected by metabolic generators of superoxide anions can increase the activity of SOD and decrease that of CAT (Łaszczyca et al., 2004).

The greatest deviation from the predicted pattern of changes in the activity of CAT and GR is seen in larvae receiving food B at the end of the month III. The activity of both enzymes is comparable to the activities of larvae receiving food patterns A and C, and statistically significantly lower than in larvae receiving food pattern D (Fig. 1 C, E). The activities of these two enzymes show a statistically significant positive correlation with each other ($R = 0.48$, $P < 0.001$) and a positive correlation with diet ($R = 0.708$, $P = 0.049$, for CAT and $R = 0.708$, $P = 0.049$ for GR).

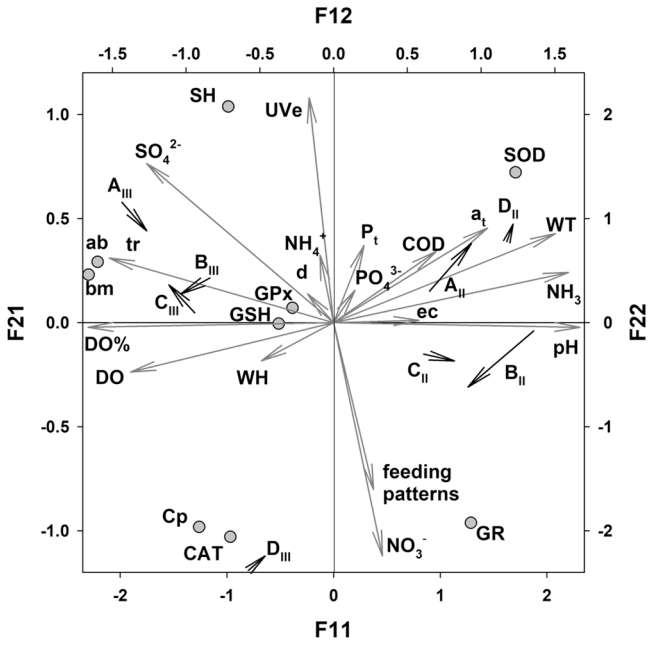

Fig. 2. Effects of abiotic water parameters on antioxidant biomarkers in *Chironomus plumosus* from the carp pond fed with different feeding patterns (A, B, C, and D) measured in control, II month, and III months. Triplot presentation of the co-inertia analysis (CIA) of 20 abiotic water parameters and six antioxidant biomarkers. The ordination diagram of 20 normalized abiotic water parameters (see Materials and methods section for codes) in the CIA is represented by grey arrows starting from the origin and projected on the F13xF23 factorial map. Positions of antioxidant biomarkers (grey circles) are plotted onto the CIA F11xF21 plane (see Materials and methods section for codes). Standardized co-inertia scores of abiotic water parameters and antioxidant biomarkers data for each sample (black arrows) are projected onto the F12xF22 factorial map. The arrow origin locates the sample as ordinated by the abiotic water parameters, and the arrowhead locates it as ordinated by the antioxidant biomarker.

According to these data, Soprofish 32/7 Profi Effect leads to an increase in the activity of CAT and GR compared to Soprofish 25/7 Standard. The increase in the activity of GR confirms its active role in the recycling pathways of the reduced form of glutathione. The activity of GR in insect tissues is expected to decrease proportionally to glutathione deficiency (Łaszczyca et al., 2004).

In addition to the reduction of H_2O_2, GPx also catalyses the reduction of lipid peroxides, the products of lipid oxidation, thus protecting cells from oxidative stress. Lipid oxidation is associated with the formation of H_2O_2, which is neutralised by the action of antioxidant enzymes. These enzymes prevent the formation of H_2O_2, which accelerates the conversion of Fe^{3+} to Fe^{2+} in the Fenton reaction, thus initiating lipid peroxidation (Dröge, 2002). It has already been documented that feeding leads to an increase in lipid content and changes the composition of *C. plumosus* larvae as a result of the deposition of uneaten fish food at the bottom of the fishpond and the change in the composition of detritus, which is the main food source of *C. plumosus* (Živić et al., 2013). The activity of GPx (Fig. 2D) was significantly altered in all feeding patterns, while the activity of the enzyme was most increased in the C_{II} feeding pattern compared to the control (C) and the other feeding patterns.

GST activities differed significantly between all four feeding patterns at the end of the second month (Fig. 1F), while GPx activity showed the lowest degree of variability of all biomarkers measured (Fig. 1D). A common feature of these two enzymes is a statistically significant maximum measured at the end of the second month in larvae fed diet C, but statistically significantly lower than control activity (Fig. 1 D, F). A statistically significant positive correlation is evident between GPx and GST ($R = 0.554$, $P < 0.001$). In feeding pattern C, the pattern of GST activity corresponded to the pattern of GPx and GR activities, with values similar to the control (Fig. 1 D, E, F). In all feeding patterns, GPx and GST are activated in a coordinated manner. One of the possible explanations for this cooperative activity lies in the fact that selenium-dependent GPx activity is not present in *C. riparius* larvae. In general, GST in insects exhibits selenium-dependent GPx activity (Choi et al., 2000). Catalase activity is increased when GPx activity is decreased, especially in D_{III} larvae (Fig. 2C, D). This high level of CAT is necessary to compensate for the lack of peroxidase activity and to protect the larvae from the occasional occurrence of excess ROS (Szczerkowska--Majchrzak and Jarosiewicz, 2020). In the context of the presence of a SH group, the biological importance of thiol compounds is due to their reductive capacity and their ability to react with ROS in antioxidant and detoxification reactions (Włodek, 2002). Fig. 1G shows the expected pattern of dietary change at the end of the month II, namely a concentration of SH groups in diets B and C that is lower than that of the control, but also lower than that in diet A. The greatest depletion of thiol groups was observed in chironomides of feeding pattern C at the end of the second month compared to the control. However, at the end of the month III this pattern changes completely due to the changes in the D diet, where the concentration of SH groups decreases so dramatically that it is statistically significantly lowest (Fig. 1G). There is no statistically significant correlation between this biomarker and diet. The observed decrease in the content of SH -groups in cells at the end of the second month in diets B and C suggests prooxidant effects on proteins. The results also showed that D_{II} chironomides have a significantly increased total thiol content. This increase in total thiol content could be related to a preventive effect against oxidative stress caused by another abiotic factor. We could also assume that the increased thiol content is a mechanism to overcome oxidative stress caused by a proxidative factor in chironomid larvae. The influence of environmental factors on low-molecular components of antioxidant defence such as SH groups has not yet been extensively studied. The concentration of SH groups shows a statistically significant negative correlation with GR activity ($R = -0.391$, $P = 0.002$). In contrast to CAT and SOD, peroxidases use a thiol-based reaction mechanism to detoxify H2O2, and their reduced state must be restored by the GSH/GR or the Trx/TrxR system with consumption of NADPH. These oxidative SH modifications are fully reversible, and their reduction is mediated by thiol-based redox systems, mainly the (GSH)/(GR) and thioredoxin/thioredoxin reductase systems (Brigelius-Flohe and Maiorino, 2013).

Among the population biomarkers, abundance and biomass of the *C. plumosus* population were measured. As these two parameters showed a pronounced positive correlation with each other ($R = 0.985$, $P < 0.001$), Fig. 1H shows only the changes in biomass. Just as in the case of SOD, they are characterised by a pronounced and regular seasonal variability, with biomass (and numbers) significantly higher after the month III than after the month II of the experiment. However, there were no statistically significant differences in the biomass or abundance of *C. plumosus* larvae exposed to different feeding patterns. The lack of a significant correlation with the type of diet suggests that other environmental factors, rather than diet, have a decisive influence on the dynamics of their changes.

3.3. Influence of abiotic water factors on measured biomarkers

Seasonal changes in diet are known to affect the physiological and biochemical parameters of chironomids (Nath et al., 2017), and as a result, seasonal changes occur in their antioxidant defence parameters (Chainy et al., 2016). To the best of our knowledge, the present work is the first integrated study on the combined effects of abiotic factors and diet on different compositions of the antioxidant response of *C. plumosus*.

The physical and chemical water parameters are listed in Table 3. Analysis of the individual parameters using two-way analysis ANOVA shows considerable uniformity of abiotic factors between the different feeding patterns. A statistically significant difference between them is only observed for WT and NH_3. WT was higher in the feeding pattern A_{III}

than in B_{III}, C_{III} and D_{III}. NH_3 concentration was highest in the tanks with feeding pattern D in both months II and III, indicating that increased feed protein content causes a moderate increase of NH_3 in the water column. However, this increase was only statistically significant in the month II, while in the month III significance was lost due to a drastic decrease in NH_3 in all ponds, which could not be attributed to the effects of feeding patterns.

This minimal variability in abiotic factors between different feeding regimes indicates that feeding has a very small effect on abiotic water factors. This is of great importance for the relevance of the results obtained on the influence of feeding regimes on biomarker values and the impact of seasonal changes in abiotic factors on the values of the biomarkers studied. Water temperature, DO (mg L^{-1}), DO %, pH, NH3 and transparency showed statistically significant seasonal variations. The post-hoc test showed that COD was significantly higher in the case of diet B in the second month than after the third month of the experiment (Table 3).

Co-inertia analysis was performed to determine the influence of the described variability in abiotic water factors and feeding patterns on the biomarkers studied (Fig. 2). The results from CIA showed that there was a statistically significant ($P < 0.021$) co-structure between the covariance matrix PCAs of the antioxidant biomarkers and the environmental data. To graphically represent the observed co-structure, two factorial axes were retained to explain most (79 %) of the co-structure (F1 = 63 %, F2 = 16 %). The strength of the co-structure was confirmed by the high values of the correlation coefficients along the F1 axis (0.97) and the F2 axis (0.95).

The analysis of the position of the samples with abiotic factors (origin of the black arrows, Fig. 2) shows their clear seasonal separation along the F1 axis, which explains why most of the structures sampled at the end of the month II of the experiment are associated with their positive part, while those sampled at the end of the months III of the experiment are associated with their negative end. Moreover, all abiotic data that are either closely associated with the positive end of F1 (pH, NH_3 and WT) or its negative end [DO (mg L^{-1}), DO % and TR] are characterised by statistically significant seasonal variations (Table 3). This means that F1 is the axis of seasonal change and that water quality was better in the third month of the experiment than in the second month. Biomass, abundance and SOD activity, which are characterised by considerable seasonal changes (Fig. 1B, H), are closely related to the positive end (SOD) or the negative end (biomass and abundance) of the F11 axis. Consequently, SOD shows a statistically significant correlation with WT ($R = 0.819$, $P = 0.013$), NH3 ($R = 0.741$, $P = 0.035$) and COD ($R = 0.729$, $P = 0.040$), while abundance and biomass show a statistically significant negative correlation with pH ($R = -0.906$, $P = 0.002$ and $R = -0.940$, $P < 0.001$) and NH3 ($R = -0.779$, $P = 0.023$ and $R = -0.814$, $P = 0.014$, respectively) and a positive with DO (mgL-1) ($R = 0.804$, $P = 0.016$ and $R = 0.818$, $P = 0.013$), DO % ($R = 0.797$, $P = 0.018$ and $R = 0.856$, $P = 0.007$, respectively), and transparency ($R = 0.811$, $P = 0.015$ and $R = 0.824$, $P = 0.014$, respectively). This means that the activity of SOD increases in response to the increase in environmental pressure due to an increase in NH_3 and COD, while the population of *C. plumosus* increases when the abiotic factors become more favourable due to an increase in DO (mgL^{-1}), DO % and transparency on the one hand, and a decrease in NH_3 and pH values on the other.

The remains of fish feed and faeces influence the chemical parameters of the receiving environment, leading to an increase in the concentration of total phosphorus and nitrogen compounds and a decrease in the concentration of dissolved oxygen. The changes in physicochemical factors (increase or decrease in the above parameters) lead to a deterioration in water quality and can cause oxidative stress in aquatic organisms (Bartoli et al., 2007; Živić et al., 2009). It has been documented that ammonia exposure can lead to oxidative stress in aquatic organisms. Mirčić et al. (2016) showed that an increase in ammonia ion concentration in receiving waters correlates with an increase in SOD and CAT activities of larvae of *Dinocras megacephala*, in addition to a decrease in dissolved oxygen content. In the study by Zhao et al. (2021), the activity of SOD was upregulated in the freshwater triangular sail mussel *Hyriopsis cumingii* under conditions of acute ammonia exposure. Ultraviolet emission, NO_3 and feeding patterns are most strongly associated with the F2 axis (Fig. 2). However, analysis of Table 3 shows that neither NO_3 nor UVe are characterised by statistically significant variations, neither between feeding patterns nor between seasons, and we therefore assume that the F2 axis is mainly determined by feeding patterns. The activities of GR and CAT, which are positively correlated with feeding patterns, are therefore at the negative end of the F2 axis. Due to the negative correlation with the activity of GR, the concentration of SH groups is at the positive (opposite) end of the F2 axis. In addition, SH groups show a negative correlation with SOD and with the protein concentration (Cp) at the negative end next to CAT, which also shows a negative correlation with SOD (Fig. 2). The DIII sample is very clearly separated at the negative end of the F2 axis from the other samples caught at the end of the third month of the experiment. This separation can be attributed to the fact that the activity of CAT and GR (together with protein concentration) in feeding pattern D reached a statistically significant maximum at the end of the third month of the experiment, while the groups of SH reached a statistically significant minimum (Fig. 1A, C, E, G).

The activities of GST and GPx are near the middle of the CIA level, as they do not correlate statistically significantly with other antioxidant biomarkers or with the measured abiotic factors. It follows that none of the measured abiotic factors are responsible for the observed changes in the activity of these two enzymes. This is consistent with results obtained in C. riparius from the field, where no evidence of significant relationships between physical or chemical parameters and GST activity was found (Olsen et al., 2001). In a previous study of ours with Gammarus dulensis (Vranković et al., 2018), GPx activity showed no statistically significant correlation with the abiotic parameters analysed. No correlation between GPx and GST activities and abiotic parameters on the one hand and other antioxidant biomarkers on the other hand could be detected, so that changes in these enzymes could not be explained by the abiotic factors and the interaction with other measured antioxidants.

In conclusion, *C. plumosus* larvae show biochemical and physiological adaptations that correlate with changing environmental conditions. The studied antioxidant biomarkers of *C. plumosus* are sensitive and respond significantly. They are influenced by a complex interaction between different feeding patterns, season and abiotic factors. Diet-induced changes in antioxidant biomarker profiles appear to be a relatively slow process. The specific combination and interaction of different aquaculture factors with the application of different feeding patterns modulated the ability of biomarkers to cope with excessive ROS, resulting in a significant increase in abundance of *C. plumosus* during the third month and at the end of the experiment. These results provide additional information on the potential consequences of exposure of freshwater invertebrates to aquaculture effluents and highlight the importance of studies to assess the risk of organic pollution.

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CRediT authorship contribution statement

Milenka Božanić, Ivana Živić, and Zoran Marković designed the study. Milenka Božanić, Stefan Marjanović, Vukosav Golubović, and Ivana Živić collected samples in the field. Jelena Vranković determined antioxidant biomarkers. Miroslav Živić and Ivana Živić analyzed obtained data. Jelena Vranković, Milenka Božanić, and Miroslav Živić wrote the paper and are responsible for its final content. Vukosav

Golubović contributed answers to reviewers related to applicable fish food. All of the listed authors contributed sufficiently to the work to be included as authors and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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