ORIGINAL ARTICLE

Resistance of common carp (*Cyprinus carpio* L.) to oxidative stress after chloramine-T treatment is increased by microalgae carotenoid-rich diet

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Abstract

OBJECTIVES: In fish aquaculture, disinfectants are used against bacterial and protozoal infections. These compounds cause oxidative stress that may stimulate the generation of reactive oxygen species, and subsequently the alteration in antioxidant systems of exposed organisms. Antioxidants like carotenoids present in microalgae increase carp resistance to oxidative stress after chemical treatment.
DESIGN: The aim of these experiments was to prove increased resistance of common carp (*Cyprinus carpio* L.) juveniles fed on experimental diets with microalgae biomass supplement (Algadiets) to oxidative stress caused by a disinfectant chloramine-T. In indoor experiments fish were fed on laboratory-prepared extruded diets containing supplement of *Chlorella* spp. (cf. C. vulgaris Beijerinck) biomass which contains antioxidants (carotenoids) like lutein. The young-of-the-year-old fish were acclimatized and fed on basal diet (control group) and the on diets containing 1, 2, 5 and 10% (w/w) of spray-dried *Chlorella* biomass (Algadiet 1, 2, 5 and 10) for 14 days followed by 6 weeks. Consequently, fish were treated daily with chloramine-T (Chl-T) at concentration of 10 $mg.l^{-1}$ for 1 h in three consecutive days. After this treatment, the indices of oxidative stress and antioxidant enzyme activity were assayed in fish gill, muscle and hepatopancreas.
RESULTS: The fish fed on different Algadiets had increased antioxidant enzyme activities of glutathione peroxidase, glutathione reductase and catalase in flesh after the exposure to Chl-T. Higher activities of superoxide dismutase, glutathione peroxidase and glutathione reductase were also observed in the hepatopancreas in all tested concentrations compared to the control group fed on the basal diet. The increased production and activity of antioxidant enzymes confirmed improved protection ability of fish tissues against oxidative damage when microalgae biomass was supplemented to the fish diet which was more pronounced

by higher microalgae supplement in Algadiet 5 and 10 where the content of carotenoids was 105 mg and 214 mg per kilogram of fed, respectively.

CONCLUSION: The results show the positive effect of carotenoids from microalgae biomass to maintain the antioxidant capacity which increases resistance of fish to oxidative stress.

Abbreviations

CAT	- catalase
Chl-T	- chloramine-T
GPx	- glutathione peroxidase
GR	- glutathione reductase
H_2O_2	- hydrogen peroxide
NADPH	- nicotinamide adenine dinucleotide phosphate
ND	- not detectable
ROS	- reactive oxygen species
SGR	- specific growth rate
SOD	- superoxide dismutase
TBARS	- thiobarbituric acid reactive substances

INTRODUCTION

In fish aquaculture, disinfectants like chloramine-T (Chl-T; N-Chloro-p-toluenesulfonamide sodium salt) are used against bacterial and protozoal infections. These compounds cause oxidative stress that stimulates the generation of reactive oxygen species and alteration in antioxidant systems of exposed organisms. Since the early 1900s Chl-T has been used in a wide variety of industries (medical, dental, veterinary, food processing, agricultural etc.) as a mild disinfectant and a biocide (antimicrobial agent) (Haneke 2002). It is effective against a large number of bacteria and viruses without inducing drug resistance (Sanli-Yurudu *et al.* 2007). In aquaculture industry Chl-T is mostly used as a drug against proliferative gill and bacterial gill disease of salmonids (Bullock *et al.* 1991; Haneke 2002; Harris *et al.* 2004; Bowker & Carty 2008; Boran & Altinok 2014), to treatment of trichodiniasis in eel (*Anguilla anguilla*) (Madsen *et al.* 2000), and to disease of American lobsters (*Homarus americanus*) (Speare *et al.* 1996). Some studies suggest that excessive doses of Chl-T may lead to damage to the issues. Exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) to Chl-T (20 mg.l^{-1}) significantly suppressed growth rates compared with controls (Powell *et al.* 1994). However, Bowker and Carty (2011) tested various concentrations of Chl-T on rainbow trout and did not detect fish mortality at low concentrations up to 20 mg.l^{-1}. The most sensitive were juveniles (total length 15.3±1.3 mm) exposed to Chl-T at concentrations at 50-60 mg.l^{-1} and then fingerlings (total length 7.7±1.1 mm). The least sensitive were rainbow trout fry to >100 mg.l^{-1} Chl-T (total length 3.3±0.3 mm) after 1 h exposure. Histopathological changes to gill tissue were observed at concentration of 60 mg.l^{-1} and higher after one hour exposure to Chl-T. In is important to note that Chl-T at concentration of 10 mg.l^{-1} did not have any effect on growth rate, biochemical properties of blood, or to any stress response in fish (Sanchez *et al.* 1997). This concentration does not affect the vital functions of crustaceans (*Astacus leptodactylus*) after 1-h treatment (Kuklina *et al.* 2014). Therefore it is considered that Chl-T at concentrations lower than to 10 mg.l^{-1} for 1 h per day can be safely used to prevent or treat external parasitic and bacterial infections in aquaculture (Powell *et al.* 1994; Sanchez *et al.* 1997; Boran & Altinok 2014).

Chl-T is decomposed in the water releasing a hypochlorite ion OCl^- (a disinfectant), and the sulfonamide moiety (inhibits bacterial growth) which inhibits bacterial growth given the similarity with para-aminobenzoic acid (a bacterial metabolite) (Booth & McDonald 1988; Powell & Perry 1996). Excessive dose of Chl-T may lead to the production of highly oxidative OCl^- anions which results in increased production of reactive oxygen species (ROS) in aquaculture organisms (Maduenho & Martinez 2008; Boran & Altinok 2014) or pollutants in aqueous ecosystems (Stara *et al.* 2012a, 2014). In this respect, some authors describe the positive effects of diets supplemented with microalgae biomass to increase the antioxidant capacity and prevent oxidative stress as well as improvement of coloration of cultured aquatic organisms (Pan *et al.* 2010, 2011; Kouba *et al.* 2013, 2014). Microalgae produced in mass culture, especially *Chlorella* contains high content of protein, lipid, polysaccharide, vitamins, essential amino acids, pigments – carotenoids, minerals and other substances (e.g. Masojidek *et al.* 2011). Those substances have high biological and physiological activity and they are used as feed additives (Becker 2007, 2013; Xu *et al.* 2014).

The common carp (*Cyprinus carpio* L.) is the most extensively cultured freshwater fish species in the world. Besides the global importance in aquaculture, carp is an internationally accepted test organism for biotests (FAO). The aim of these experiments was to prove increased resistance of common carp juveniles fed on experimental diets with microalgae biomass supplement (Algadiets) to oxidative stress caused by exposure to a disinfectant Chloramine-T.

MATERIALS AND METHODS

Experimental animals, diets and experimental design

The young-of-the-year-old common carp (*Cyprinus carpio* L.) juveniles, lineage South Bohemian scaly carp (C73), were obtained from the fish hatchery of the Research Institute of Fish Culture and Hydrobiology in Vodnany. For indoor experiment one hundred eighty carp individuals were divided to five groups for each treatment as to have triplicates (in separate aquaria) of twelve fish. The initial standard body length (measured from the tip of the head to the base hypural plate at caudal flexion) and weight were 67.8±2.3 mm and 10.45±0.7 g, respectively. Fish were held in aquaria containing 45 l of freshwater, in a recirculation system described by Hamackova *et al.* (2009). The flow rate was 0.9 l.s^{-1}. The aquariums were cleaned every morning before the first feeding. Fish were acclimatized for

14 days being fed on basal diet without microalgae supplement. Then, during the experiment, basic physicochemical parameters were recorded daily: temperature (mean of 21.7±1.2 °C), dissolved oxygen concentration (mean of 7.4±1.3 mg.l^{-1}), pH (mean 7.0±0.2). The light:dark cycle was 12:12.

The fish were fed five experimental extruded diets. The home-made basal diet (Control) was composed of fishmeal (40%), wheat flour (38%), soy flour (10%), corn starch (10%), vitamin and mineral premix (Roboran H, Univit Ltd.; 1%), and fish (0.5%) and rapeseed oil (0.5%). The disintegrated and spray-dried *Chlorella* spp. (cf. *C. vulgaris* Beijerinck) biomass was added to the basal diet in the amount of 1% (Algadiet 1), 2% (Algadiet 2), 5% (Algadiet 5) and 10% (w/w) (Algadiet 10) as aliquot replacement of wheat flour.

Fish were fed four times per day (at 08:00, 12:00, 15:00, and 18:00 h) for 6 weeks. Feeding rate of 4% (except 3% during the last week) was adjusted according to weekly batch weighing of fish. After 6 weeks of the feeding trial, all fish groups were exposed to Chl-T (*N*-Chloro-*p*-toluenesulfonamide sodium salt) at concentration of 10 mg.l^{-1} for 1 h during three consecutive days while fish were not fed during these three days. This concentration was used as it did not have any effect on growth rate and biochemical properties of blood (not shown here), similarly as described by Sanchez *et al.* (1997). After the Chl-T treatment, water was drained away, aquaria were gently rinsed and brought to recirculating regime. On the fourth day, eight randomly chosen fish from each aquarium were killed by one stunning blow with a blunt object over the head.. Then, gill, flesh and hepatopancreas were dissected for assays of oxidative stress (TBARS) and antioxidant enzyme activity (SOD, CAT, GR, GPx). Flesh (filets without skin) was also sampled to determine the accumulation of carotenoids. All tissue samples were immediately frozen and stored at −80 °C until analyses. Experimental procedures were performed in compliance with national legislation (Act No. 246/1992 Coll., on the Protection of Animals Against Cruelty, as amended).

Analysis of carotenoids in the feed

The total content of carotenoids in prepared feeds was determined spectrophotometrically after extraction to the 80% of acetone and calculated from equation (Wellburn 1994). The individuals carotenoids were determined by HPLC with a diode array detector (column Luna 3m C8, 100A, Phenomenex company) as described by Gilmore and Yamamoto (1991). We used the lutein to know the retention time, the others carotenoids were determine according this carotenoid and compare to the well known *Chlorella* sp. carotenoids composition.

Analysis of carotenoids in the flesh

For assays, frozen tissue sample was weighed and homogenized 3 min with sea sand at 20 °C in a small volume of 100% acetone using pestle and mortar. The mixture was quantitatively transferred to glass centrifugation tubes, shaker for 20 min and centrifuged (3,000 × *g*/3 min). The supernatant was collected for spectrophotometric and HPLC analyses. Then, the sediment was extracted twice more by 99.5% acetone (used for UV-VIS spectroscopy). The second and third supernatants were also collected and used for spectrophotometric and HPLC analyses. The content of carotenoids was determined spectrophotometrically using absorption maximum at 446 nm (UV2600, Shimadzu) and the amount was calculated from molar extinction coefficient of lutein in acetone (Ex = 2540; 100 ml.g^{-1} cm) (Jeffrey *et al.* 1997; Britton *et al.* 1995). The total amount of carotenoids in the flesh was calculated as the pigment sum in all three extractions.

Individual carotenoids in flesh extracts were determined by HPLC after treatment with 20% of ammonium acetate in methanol (mobile phase) in the first extract). If the turbidity was high to disturb the analysis, the extract was frozen at −20 °C and the pure phase was taken for HPLC analysis. Individual carotenoids were identified according the retention time and spectrum (diode array detector) of the peaks (Gilmore & Yamamoto 1991). As carotenoid standards, beta-caroten and lutein were used to verify the position, spectra and content of important peaks in chromatographic profiles.

Preparation of post-mitochondrial supernatant from tissue samples

For antioxidant and enzymatic activity assays, frozen tissue samples of gill, flesh and hepatopancreas were weighed and homogenized (homogenizer Ultra Turrax, Ika, Germany) using 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA (1 : 10, w/v). The homogenate was divided into two parts, one for measuring of thiobarbituric acid reactive substances (TBARS) and the other was used for other antioxidant analyses.

Determination of oxidative stress and antioxidant enzymatic activities

The TBARS method was used to evaluate lipid peroxidation as described by Lushchak *et al.* (2005). Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined spectrophotometrically using the method of Marklund and Marklund (1974). The catalase (CAT; EC 1.11.1.6) activity assay, based on spectrophotometric measurement of H_2O_2 breakdown at 240 nm, was performed following the method of Beers and Sizer (1952). Glutathione peroxidase (GPx; EC 1.11.1.9) activity was calculated from the rate of NADPH oxidation (at 340 nm) in the reaction with glutathione reductase (GR; EC 1.6.4.2). Glutathione reductase activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm (Carlberg & Mannervik 1975).

Colour evaluation of fish

For comparison, a judge with relevant experience in the culture and trade of ornamental fish was asked

independently to sorte whole body images according to colouration intensity.

Protein assay

Protein content was estimated spectrophotometrically according to Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

The statistical software STATISTICA (version 8.0 for Windows, StatSoft) was used to evaluate results. Prior to analysis, all measured variables were checked for normality (Kolmogorov–Smirnov test) and homoskedasticity of variance (Bartlett's test). If these conditions were met, a one-way analysis of variance (ANOVA) was used to determine differences in measured variables among experimental groups. When a significant difference was detected ($p<0.05$ in all tests), Tukey's multiple comparison test was applied to identify which treatments were significantly different. All data are presented as mean ± standard deviation (SD).

RESULTS

Length, weight and mortality

All fish groups showed normal feeding behaviour and no mortalities were found during the experiment. Fast and well-balanced fish growth was observed in all experimental groups. Fish weight more than doubled during the experiment – from 10.4–10.5 to 24.7–26.3 g. The body length increase by about 25% – from 67.2–68.4 to 84.1–86.5 mm. Specific growth rate (SGR) reached mean values ranging from 1.9 to 2.1% per day. There were no significant differences in fish weight and size as well as growth rates among groups (Table 1).

Content of carotenoids in the diet

The total amount of carotenoids (mg.kg^{-1}) determined in the diets depended on the amount of *Chlorella* biomass supplemented to the feed (Figure 1). Algadiets 1, 2 and 5 contained about 10, 20 and 50%, respectively, of the carotenoid amount of Algadiet 10. It is important to note that the basal diet also contained about 9 mg.kg^{-1} carotenoids which reflects the addition of plant material. Gravimetrically, Algadiets 1, 2, 5 and 10 contained 21, 43, 105 and 214 mg.kg^{-1} carotenoids per which comparatively well corresponded to the amount of *Chlorella* biomass added to diet. The content of individual carotenoids in the basal diet was undetectable or very low except that of lutein which was about 7 mg.kg^{-1} of fresh weight (Table 2).

Content of carotenoids in the muscle

The increased carotenoid content in the flesh corresponded to the supplement amount of microalgae biomass to feed. The most prominent pigment was lutein which represented about 80% of all carotenoids which was 16.5, 33.4, 82.1 and 167.6 mg.kg^{-1} in Algadiet 1 to Algadiet 10, respectively. The other prominent compound corresponding to about 9% was unidenti-

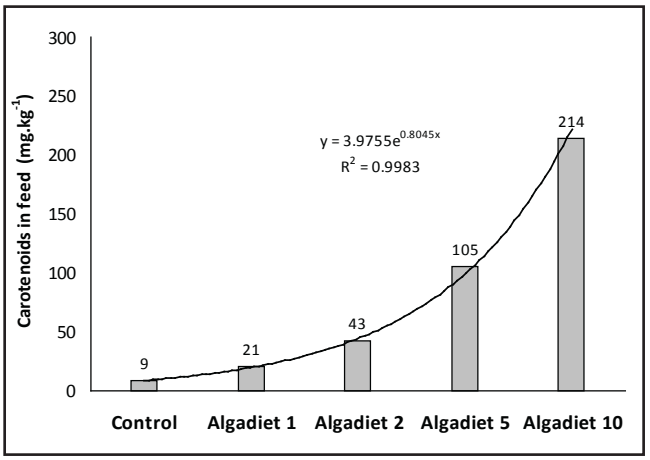

Fig. 1. The total content of carotenoids in the experimental diets. Basal diet (Control), Algadiet 1 (basal diet + 1% of *Chlorella vulgaris*), Algadiet 2 (basal diet + 2% of *Chlorella vulgaris*), Algadiet 5 (basal diet + 5% of *Chlorella vulgaris*) and Algadiet 10 (basal diet + 10% of *Chlorella vulgaris*).

Tab. 1. Physiological changes of common carp (*Cyprinus carpio* L.) in the experiment.

Treatment	Individual weight (g)		Individual body length (mm)		Feeding coefficient	SGR (% per day)
	Start of experiment	End of experiment	Start of experiment	End of experiment		
Control	10.4±0.7^a	24.7±3.2^a	67.6±1.7^a	84.1±3.6^a	1.81±0.02^a	1.93±0.02^a
Algadiet 1	10.4±0.6^a	26.3±3.4^a	68.3±1.9^a	85.5±4.8^a	1.74±0.07^a	2.06±0.10^a
Algadiet 2	10.4±0.8^a	25.3±3.9^a	68.4±2.8^a	85.5±4.8^a	1.81±0.10^a	1.97±0.10^a
Algadiet 5	10.5±0.9^a	25.6±3.6^a	67.6±2.5^a	85.8±4.5^a	1.78±0.05^a	1.98±0.05^a
Algadiet 10	10.4±0.7^a	26.0±3.5^a	67.2±1.9^a	86.5±3.8^a	1.91±0.13^a	1.90±0.07^a

Change of fish weight and body length at the start and the end of 6-week experiment. The experimental groups fed on Algadiet 1 (basal diet + 1% of *Chlorella vulgaris*), Algadiet 2 (basal diet + 2% of *Chlorella vulgaris*), Algadiet 5 (basal diet + 5% of *Chlorella vulgaris*) and Algadiet 10 (basal diet + 10% of *Chlorella vulgaris*) were compared to the Control group (fed on the basal diet). Comparison of Feeding coefficient and specific growth rate (% per day) for the experimental group. SGR specific growth rate (% per day) was calculated as weight increase per day during 6-week trial. Data are expressed as a mean ± SD (n=24). Values with identical superscripts are significantly identical ($p<0.05$, ANOVA).

Tab. 2. Content of carotenoids (mg.kg^{-1}) in individual diets.

Carotenoids	Retention time (min)	Basal diet	*Algadiet* 1	*Algadiet* 2	*Algadiet* 5	*Algadiet* 10
Neoxanthin	10.2	ND	0.8	1.2	2.5	4.7
Violaxanthin	10.8	ND	0.5	1.7	4.5	9.2
Antheraxanthin	14.1	ND	ND	0.3	0.7	1.6
420/445/474	16.2	ND	ND	ND	ND	ND
Lutein	16.6	7	16.5	33.4	82.1	167.6
Cis-lutein	17.6	ND	0.6	1.2	3.1	6.3
416/442/467	18.3	0.8	2.3	4.2	9.8	18.2
Beta-carotene	27.8	0.7	0.3	0.6	2.6	6.0

The description of diets is the same as in Tab. 1. Individual carotenoids were identified according their retention time and absorption spectra. The compounds designated as 420/445/474 and 416/442/467 are unknown carotenoids, the number represent the maxima of the spectral triple peak. ND means not detectable during the analysis.

fied xanthophyll characterised by absorption maxima 416/442/467 nm with the retention time of 18.3 min. Some minor carotenoid compounds (2–4%) detected in the flesh were vioaxanthin, neoxanthin, anteraxanthin and β-carotene.

After the trial, the relation of total carotenoid content in feed to carotenoid content in flesh was not as straightforward (Figure 2). As compared to the control group (0.49 mg.kg^{-1} carotenoid), the significant increase (2.76-times) of carotenoid content in flesh was seen in the fish group fed on Algadiet 1 (1.35 mg.kg^{-1} carotenoid). Then, we did not found adequate increase of carotenoid content in fish groups fed on Algadiet 2 (1.62 mg.kg^{-1} carotenoid) and Algadiet 5 (1.56 mg.kg^{-1} carotenoid) corresponding to twice and five times higher amounts of carotenoids, respectively as compared to Algadiet 1. Only the highest supplement of microalgae biomass (Algadiet 10) doubled the content of carotenoids (2.56 mg.kg^{-1}) in fish flesh as compared to Algadiet 1.

The determination of individual carotenoids in the flesh samples of the control group was undetectable (Table 3). In the experimental groups fed on Algadiets 1–10, the highest content was found in case of lutein between 0.81–1.62. The amount was only doubled when compared Algadiet 1 and Algadiet 10 although the supplement to diet was 10-times higher. The other xanthophylls, anteraxanthin, 420/445/474 and 416/442/467 nm components was low, between 0.22 and 0.32 in all Algadiets.

On the other hand, the effect of *Chlorell*a biomass supplement on the colour of fish skin was rather apparent. The yellowish colour of fish was visible after one week of feeding trial on the sides of the head (gill covers) and abdominal area. The saturation of skin colour continuously intensified with increasing amount of carotenoid content in feed from Algadiet 1 to Algadiet 10 (Figure 3). In the fish group fen on Algadiet 5 and 10, the golden colour was quite distinctive. The colouration increased attractiveness of juvenile carps as ornamental fish.

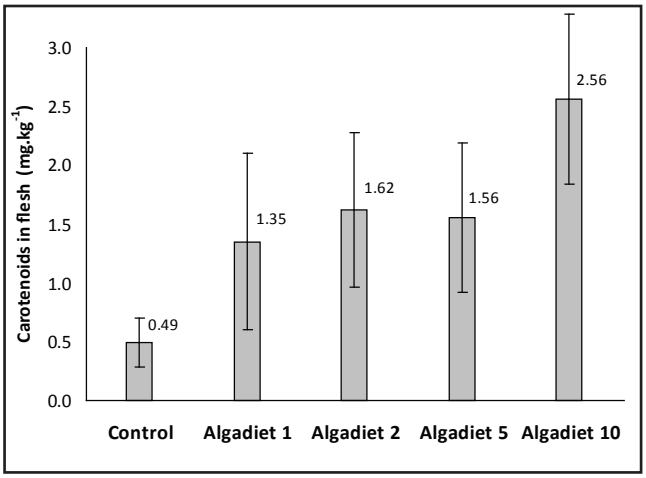

Fig. 2. The total content of carotenoids in the flesh of common carp (*Cyprinus carpio* L.) at the end of the experiment fen on various diets (see description in Fig. 1). For statistical analysis six samples were randomly selected from each group and total amount carotenoids was calculated as mean ± standard deviation.

Oxidative stress and antioxidative response

After the exposure of all fish groups to Chl-T, significant increase (p<0.05) of GR and CAT activities in the muscle were observed in all the groups fed with Algadiets as compared to the control. GPx showed significantly higher activity in the muscle only in groups fed on Algadiets 2, 5 and 10 (Table 4). In the fish groups fed on Algadiet 5 and 10, these enzymatic activities were 2–3-times higher as compared to the control group. The values of the TBARS test and SOD activity in the muscle of fish were not influenced by Algadiets.

In the hepatopancreas of fish groups treated with Chl-T, significant increase (25–35%) of SOD activity was found if fed on Algadiets as compared to the control. Only in the most enriched feed (Algadiet 10) the GPx and GR activity was significantly higher (55–75%) as compared with the control group (Table 5). It is important to note that the SOD, GPx and CAT activ-

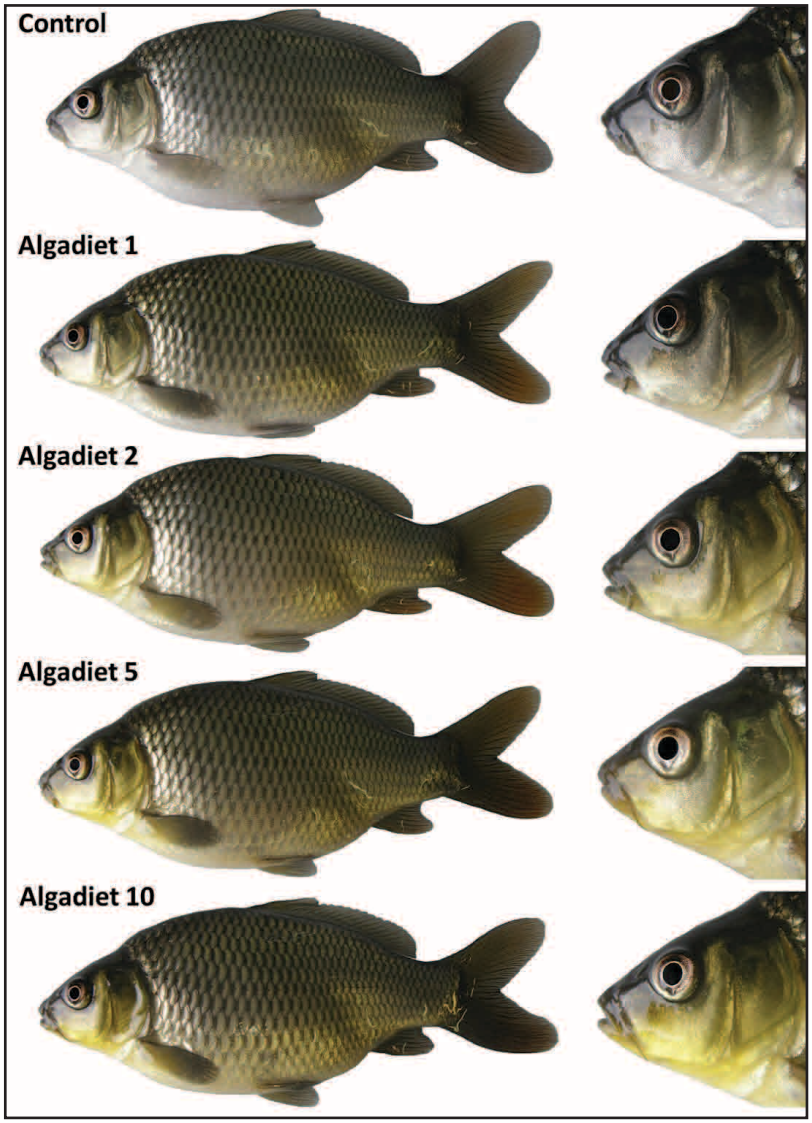

Fig. 3. Changes in the coloration of body (left panels) and head (right panels) of common carp (*Cyprinus carpio* L.) in experimental groups fed on various diets (see description in Fig. 1) at the end of the trial. Diets: Basal diet (Control), Algadiet 1 (1% of *Chlorella* biomass in feed), Algadiet 2 (2% of *Chlorella* biomass in feed), Algadiet 5 (5% of *Chlorella* biomass in feed) and Algadiet 10 (10% of *Chlorella* biomass in feed).

ity in the hepatopancreas was about an order of difference higher then these in the muscle (compare Table 4 and Table 5). There was not significant change in the TBARS tests and CAT activity in the hepatopancreas by Algadiets.

Insignificant changes in the activity of antioxidant enzymes and oxidative stress were observed in the gills when fish were disinfected with Chl-T (Table 6). Except GPx, most activities were at similar level with these found in the muscle of the control group (Table 4).

Although significant changes in the activity of some antioxidant enzymes were observed in the tissues of some fish groups, we did not see any oxidative damage to the cells or alterations in the TBARS test as a consequence of the Chl-T treatment.

DISCUSSION

There has been growing interest in the dietary supplements for fish feed as concerns vitamins, β-carotene, calcium, selenium, omega-3 PUFA, microalgae biomass and many others. Quality feed is important for wellness and fast growth of fish in the aquaculture. Commercial preparations exists containing enough nutrition supplements, such as vitamins, minerals, fibre, fatty acids, or amino acids, which have positive effects on body fitness (Park & Kim 2012). Other dietary components are also added to the food for the prevention and control of fish diseases (Jarmolowicz *et al.* 2012; Loganathan *et al.* 2013). Beneficial effects of dietary arginine and histidine in feed

Tab. 3. Composition of carotenoids (mg.kg^{-1}) in the in flesh of common carp (*Cyprinus carpio* L.) at the end of the experiment.

Carotenoids	Retention time (min)	Control	*Algadiet* 1	*Algadiet* 2	*Algadiet* 5	*Algadiet* 10
Neoxanthin	10.2	ND	ND	ND	ND	ND
Violaxanthin	10.8	ND	ND	ND	ND	ND
Antheraxanthin	14.1	ND	0.32	0.29	0.22	0.24
420/445/474	16.2	ND	0.18	0.13	0.13	0.18
Lutein	16.6	ND	0.81	1.01	0.99	1.62
Cis-lutein	17.6	ND	0.03	0.12	0.15	0.35
416/442/467	18.3	ND	ND	0.06	0.08	0.18
Beta-carotene	27.8	ND	ND	ND	ND	ND

Carotenoids 420/445/474 and 416/442/467 are unidentified compounds, the number represent the maxima of the spectrum detect by diode array detector. ND means not detect during the analysis.

Tab. 4. Changes of oxidative stress and antioxidant biomarkers in the flesh of common carp (*Cyprinus carpio* L.) groups fed on various diets (see description in Tab. 1) at the end of the trial.

Treatment/biomarker	TBARS (nmol.gww^{-1})	SOD (U.mg^{-1} protein)	GPx (mU.mg^{-1} protein)	GR (mU.mg^{-1} protein)	CAT (mU.mg^{-1} protein)
Control	19.2±7.2^{a}	33.5±15.3^{a}	38.9±15.1^{a}	15.6±8.4^{a}	6.3±2.8^{a}
Algadiet 1	21.1±8.8^{a}	26.0±9.3^{a}	39.9±11.6^{a}	16.8±6.2^{ab}	10.5±4.4^{ab}
Algadiet 2	25.9±7.8^{a}	26.4±6.4^{a}	48.3±16.4^{ab}	25.9±12.0^{bc}	14.0±4.0^{bc}
Algadiet 5	22.7±7.0^{a}	28.1±7.4^{a}	59.9±13.7^{b}	35.5±11.7^{cd}	18.2±6.7^{cd}
Algadiet 10	20.7±6.0^{a}	25.1±7.8^{a}	70.6±16.6^{bc}	42.6±11.3^{d}	19.7±6.3^{d}

The following tests to detect antioxidant biomarkers were carried out: TBARS (thiobarbituric acid reactive substances), SOD (superoxide dismutase), GPx (glutathione peroxidase), GR (glutathione reductase) and CAT (catalase). Data are expressed as mean ± SD (n=24). Values with altered superscripts are significantly different (p<0.05, ANOVA).

Tab. 5. Changes of oxidative stress and antioxidant biomarkers in the hepatopancreas of common carp (*Cyprinus carpio* L.) groups fed on various diets (see description in Tab. 1) at the end of the trial.

Treatment/biomarker	TBARS (nmol.gww^{-1})	SOD (U.mg^{-1} protein)	GPx (mU.mg^{-1} protein)	GR (mU.mg^{-1} protein)	CAT (mU.mg^{-1} protein)
Control	23.8±7.9^{a}	269.7±65.6^{a}	645.3±154.1^{a}	40.4±12.9^{a}	168.5±59.1^{a}
Algadiet 1	20.6±7.4^{a}	339.0±54.8^{ab}	728.4±166.2^{a}	46.8±15.1^{a}	175.7±54.4^{a}
Algadiet 2	24.5±6.3^{a}	369.2±89.9^{b}	749.9±160.8^{a}	57.4±21.9^{ab}	160.2±47.6^{a}
Algadiet 5	25.6±7.3^{a}	354.1±80.8^{b}	803.0±157.3^{a}	51.3±21.7^{a}	156.3±43.5^{a}
Algadiet 10	24.0±7.2^{a}	362.1±75.9^{b}	1005.8±191.4^{b}	70.1±19.9^{b}	161.1±37.3^{a}

The following tests to detect antioxidant biomarkers were carried out: TBARS (thiobarbituric acid reactive substances), SOD (superoxide dismutase), GPx (glutathione peroxidase), GR (glutathione reductase) and CAT (catalase). Data are expressed as mean ± SD (n=24). Values with altered superscripts are significantly different (p<0.05, ANOVA).

Tab. 6. Changes of oxidative stress and antioxidant biomarkers in the gill of common carp (*Cyprinus carpio* L.) groups fed on various diets (see description in Tab. 1) at the end of the trial.

Treatment/biomarker	TBARS (nmol.gww^{-1})	SOD (U.mg^{-1} protein)	GPx (mU.mg^{-1} protein)	GR (mU.mg^{-1} protein)	CAT (mU.mg^{-1} protein)
Control	29.4±11.3^{a}	35.0±10.8^{a}	123.3±36.4^{a}	23.9±9.8^{a}	14.2±8.6^{a}
Algadiet 1	32.2±18.6^{a}	27.7±8.8^{a}	118.3±42.5^{a}	26.0±6.9^{a}	16.6±5.3^{a}
Algadiet 2	27.9±9.3^{a}	31.6±15.9^{a}	137.2±52.0^{a}	24.5±8.4^{a}	18.6±9.7^{a}
Algadiet 5	30.0±9.5^{a}	26.9±10.6^{a}	144.0±43.9^{a}	21.5±8.3^{a}	20.5±8.5^{a}
Algadiet 10	26.1±8.8^{a}	28.2±8.7^{a}	151.2±47.1^{a}	24.6±7.2^{a}	19.0±7.6^{a}

The following tests to detect antioxidant biomarkers were carried out: TBARS (thiobarbituric acid reactive substances), SOD (superoxide dismutase), GPx (glutathione peroxidase), GR (glutathione reductase) and CAT (catalase). Data are expressed as mean ± SD (n=24). Values with altered superscripts are significantly different (p<0.05, ANOVA).

have been found in trials with juveniles Japanese flounder (*Paralichthys olivaceus*) (Han *et al.* 2013). Microalgae, for example *Chlorella* have significant protective function against cellular damage for humans and animals (Shein *et al.* 2009), immunostimulatory properties (Mata *et al.* 2010), antiproliferative effect on human colon cancer cells, including inhibition of apoptosis (Lordan *et al.* 2011), reduced oxidative stress (Lim *et al.* 2006). However, *Chlorella* is owing to the taste and flavour adjusting actions of its colouring agent is frequently used as a food additive (Spolaore *et al.* 2006).

Kouba *et al.* (2014) found positive effect of microalgae biomass enriched in selenium on growth of common barbel (*Barbus barbus*) during the 6-week feeding trial. The efficiency of diets with the inclusion of *Spirulina* microalgae in feed was found to promote growth with improved conversion rate for Siberian sturgeon (*Acipenser baeri*) (Palmegiano *et al.* 2005). The diets supplemented up to 2% *Chlorella* dry biomass was tested the gibel carp (*Carassius auratus gibelio*) (Xu *et al.* 2014). In fish groups fed on these diets the increase of body weight and specific growth rate was observed compared with the control group after 60-day feeding trial. *Chlorella* promoted the growth performance, immunostimulation and digestive enzyme activity. It is obvious that *Chlorella* biomass represents an impor-

tant ingredient in the fish feed, due to high content of proteins, pigments, lipids, polysaccharides, vitamins, minerals and other nutritional substances, and those ingredients possess great bioactivity involving in many physiological activity (Xu *et al.* 2014).

Addition of *Chlorella* biomass with 0.3–0.4% of carotenoids in cells to the feed results in carotenoids accumulation in the skin and flesh and generally fortifies immune system of fish against oxidative stress (Meyers 1994). The accumulation of carotenoids is the skin is widely discussed in the literature and closely correlates with microalgae addition to the feed (Zatkova *et al.* 2011; Kouba *et al.* 2013; Sergejevova & Masojidek 2012). In our experiments the results showed that the carotenoid content in flesh did not match the amount added in feed. The accumulation of carotenoids in the flesh was comparable for fish groups fed on Algadiets with 1, 2 and 5% of algae and the content was 3-times higher than that in the control group feed on basal diet without *Chlorella* supplement. It means that Algadiet 1 with the 1% of *Chlorella* biomass had similar effect on the accumulation of carotenoids in flesh as Algadiets 2 and 5. As expected the highest amount of carotenoid in the flesh was found in the group fed on Algadiet 10 which was 5-times higher than that in the control group. There exists studies on the metabolism of carotenoids which refer on transformation carotenoids to the astaxanthin in marine fish (Goodwin 1986; Rajasingh *et al.* 2006; Maoka 2011), but few information is available about transformations of carotenoids in freshwater fish, especially specifically carp.

In our experiments, we found seven carotenoid species in the feed supplemented by *Chlorella* biomass – neoxanthin, violaxanthin, antheraxanthin, lutein, cis-lutein, β-carotene and unidentified carotenoid compound 416/442/467, but only five were detected in the flesh (Table 2 and 3). Carotenoids β-carotene, neoxanthin and violaxanthin were not detected in the flesh. Neoxanthin and violaxanthin could be transformed to antheraxanthin (Latscha 1990; Masojidek *et al.* 1999). The dominant carotenoids in the flesh were lutein (up to 63% of total carotenoids) and antheraxanthin (up to 24% of total carotenoids). A new unidentified carotenoid 420/455/474 was found in the flesh comparing to the feed, with the retention time of 16.2 s, in the position between antheraxanthin and lutein in the HPLC chromatogram.

The inclusion of supplementary antioxidative factors in feed, such as selenium (Kouba *et al.* 2014), lycopene (Sahin *et al.* 2014) astaxanthin (Pan *et al.* 2010; Sheikhzadeh *et al.* 2012), carotenoids (Pan *et al.* 2011), and *Chlorella* biomass (Becker 2007) is expected to increase of the fish resistance against oxidative stress (Sahin *et al.* 2014). This stress occurs in the organism after exposure to xenobiotics in the environment at concentrations, which suppress basic physiological functions of the organism (Stara *et al.* 2012b; Bartoskova *et al.* 2013). It results in imbalance between the production of reactive oxygen species (ROS) and the capacity of antioxidant protective systems which has the ability to deactivate of ROS and regulate their overproduction, but only to a certain extent (Martinez-Alvarez *et al.* 2005; Sheikhzadeh *et al.* 2012). Antioxidant enzymes, SOD, CAT, GPx, GST, are considered as good biomarkers of stress (Roche & Boge 1996). They can directly deactivate harmful ROS and other compounds involved in ROS generation and in this way counteract oxidative damage to cellular components. GR functions as an auxiliary enzyme which helps the above mentioned enzymes by production of reducing equivalents (Lushchak & Bagnyukova 2006). Boran and Altinok (2014) observed enzymatic activity in liver of rainbow trout exposed to Chl-T (in concentration of 10, 20 and 30 $mg.l^{-1}$ for 60 min). The CAT activity was increased at the Chl-T concentrations of 10 and 20 $mg.l^{-1}$. As concerns the SOD activity the opposite effect was observed. The decrease or inhibition of the SOD and CAT observed at the therapeutic concentrations of Chl-T may reflect the damage to this enzyme due to ROS production. Nevertheless, oxidative stress might also be caused by conventional methodological procedures in aquaculture or changes of environmental condition (Lushchak & Bagnyukova 2006; Lushchak 2011). Sahin *et al.* (2014) found mitigation of oxidative stress and increase of antioxidant defence for rainbow trout fed on lycopene enriched diet (200 and 400 $mg.kg^{-1}$ diet) in high-stocking density fish, as compared with basal diet. Dietary effect of β-carotene supplemented feed was beneficial to toxicity reduction modulating the immune and antioxidant functions in Nile tilapia after exposure of mercury chloride (Elseady & Zahran, 2013). Fish feed containing *Haematococcus pluvialis* may effectively enhance the antioxidant system and some biochemical parameters (Sheikhzadeh *et al.* 2012). Fish fed with selenium-enriched *Chlorella* (0.3 and 1.0 $mg.kg^{-1}$ microalgae biomass) had effect on the GR, GPx and CAT in tissues of common barbel (Kouba *et al.* 2014). Antioxidant enzymes were more readily accumulated and biologically active compared to fish fed on basal diet. In our study we observed changes in some antioxidant enzymes (SOD, CAT, GPx, GR) after exposure of common carp to Chl-T. The increased synthesis of antioxidant enzymes led to the adaptation response to prevent lipid peroxidation and cell damage. These results support our hypothesis that the supplement of *Chlorella* biomass has a positive effect due to biologically active substances, especially carotenoids.

CONCLUSION

The study confirmed our hypothesis that a diet containing microalgal biomass supplement enriched in carotenoids protects the fish from the increased production of reactive oxygen species and oxidative damage to the body caused by the exposure to disinfectants, in this case of chloramine-T. Common carp juveniles Feed enriched the algal biomass (*Chlorella*

spp.) with containing carotenoids led accumulation of carotenoids in the body and skin of the carp. This resulted to increased production of antioxidant enzymes. Therefore it seems that food enriched by algal biomass is suitable not only for ornamental or marine fish, but also for common carp.

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