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The Effect of Adding Protein-Xanthophylls Concentrate (PX) from Lucerne (Medicago sativa) on Growth Parameters and Redox Profile in Muscles of carp, *Cyprinus carpio* (L.)

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Abstract

The effect of adding protein - xanthophyll from lucerne (PX) on growth parameters and the redox profile of carp muscle *Cyprinus carpio* (L.) was studied. During a 10-week experiment, carp were kept in two types of tanks: Control, where fish were fed a commercial fish feed, and in experimental tanks (PX+), where the fish were fed the same fish feed with a 5% addition protein - xanthophyll from lucerne. Weekly for all fish the total length (Tl), standard length (Sl) and body weight (W) were measured. After experiment the coefficient of condition, specific growth rate (SGR) was calculated and with the spectrophotometric method oxidative status (MDA, SOD, Vit. C, CAT i H_2O_2) in carp muscle, was estimated. The results of observations have shown that the addition of 5% protein-xanthophyll concentrate (PX) from lucerne, to feed fish resulted in a greater increase ($P>0.05$) of total length and body weight of carp. Moreover, the addition of lucerne caused slightly better coefficients of condition and SGR. The addition of lucerne to fish feed affected the higher values of MDA ($P<0.0001$), SOD and CAT in the muscle of carp, at the same time the fish had a slightly lower value for content of Vit. C ($P<0.0002$) and H_2O_2. The addition of PX to carp feed induced an increase in products of the peroxidation lipid with simultaneous stimulation of the antioxidant enzymatic system in carp muscle.

Keywords: Common carp, feed additives, alfalfa, antioxidant status.

Introduction

Feed additives play an important role in aquaculture. They are added to feed to increase fish growth and production and to improve disease resistance or/and reproductive parameters (Lovell, 1998; Halver and Hardy, 2002). Often the use of additives is to improve the quality of fish meat, to make it more valuable or contain compounds, important in nutrition.

The nutritional value of flesh is determined by the presence of valuable proteins and fats, as well as a number of health promoting bioactive components, such as glutathione fatty acids, vitamins and minerals. Food quality can be affected by many processes, chemical, physical and microbiological as well as and even importantly, through oxidation (Gutteridge and Halliwell, 1990). Although the muscle tissue in the existing peptides, mainly anseryny and carnosine, intravitally runs antioxidant mechanisms involving the chelation of heavy metals and sweeping the free radical, however, often in very advanced oxidation mechanisms, they are not sufficient. Therefore, it is believed that reducing or slowing down the process of oxidation may be obtained by using a feed additive with antioxidant properties (Lovasova and Sesztákova, 2009).

In recent years, protein-xanthophyll concentrate (PX) from lucerne (*Medicago sativa*) has become available, containing approximately 55% crude proteins and 1200 mg of xanthophylls per 1 kg of formulation (Grela *et al.*, 2013). In addition, PX has a lot of valuable bioactive compounds (saponins, flavonoids, vitamins, pro-vitamins and other minerals) (Newall *et al.*, 1996; Ben Aziz *et al.*, 2006; Grela *et al.*, 2013). Since fish are characterized by a high demand for protein, the PX concentrate from lucerne can give an alternative to animal protein source in fish feeding.

Many of these active compounds especially polyphenols, vitamin E and vitamin C,β-carotene next to immunomodulatory properties may have antioxidant properties. Recently, the addition of Lucerne has been used in the production of many animal species (Gaweł and Grzelak, 2012). These characteristics of Lucerne predispose it touse in the

feeding of fish.

Only few studies related to the use of Lucerne in the diet of fish. They are mainly focused on the determination of the impact of lucerne additive on the growth and selected nutritional parameters of fish such as the feed consumption and better palatability (Olvera-Novoa *et al.*, 1990; Jia *et al.*, 1991; Yousif *et al.*, 1994; Yanar *et al.*, 2008) and the effect of substances contained in it, i.e. crude fiber, saponins, protease inhibitors and anti-nutritional factors on fish (Ali *et al.*, 2003). Other studies conducted by Chatzifotis *et al*. (2006) concerned the attempt to replace fishmeal protein by lucerne concentrate, whereas Yanar *et al.* (2008) used lucerne as a natural carotenoid source for goldfish *Carassius auratus*.

The carp, *Cyprinus carpio* (L.) is one of the main farmed species around the world, and its production is a significant part of the many countries aquaculture (Naylor *et al.*, 2000).

In the available literature there is no information on use of concentrate from Lucerne in feeding carp. Hence, the aim of this study was to determine the effect of the adding of protein-xanthophyll concentrate from lucerne (*Medicago sativa*) (PX) on growth parameters of juvenile carp and the oxidative status of their muscles.

Table 1. Experimental design

Item	Experimental groups	
	Control	PX+
Commercialfeed*	+	+
Protein-xanthophylls (PX) from lucerne	-	5%

*Commercial feed – see table 2

Materials and Methods

The experiment was conducted in a 10 week period in 2011 in two repetitions. In each of the four experimental tanks (100 x 50 x 60 cm) ten specimens of juvenile carp were kept. Juvenile carp obtained from a local commercial farm were acclimatized to the experimental conditions for 2 weeks before the start of the experiment.

Throughout the experiment, fish in two of the tanks were fed with commercial feed (Control), whereas experimental group PX+ received the same standard mixture but enriched with 5% of protein-xanthophyll (PX) concentrate from lucerne (Table 1). PX concentrate (Desialis-France Luzerne) (Grela *et al.*, 2013) was dosed in the full-dose mixture balanced for the respective experimental periods (Table 2). During the first five weeks the fish were fed commercial feed 1 and the next five commercial feed 2. The feeding rates in both experimental groups were 3% W day^{-1}. The composition of commercial feeds used in the experiment is shown in Table 2.

In the each tank the water was dechlorinated, well-aerated tap water and fitted with a waste filtration system and a constant temperature through the heating system was maintained. During the experiment the physical and chemical parameters of the water were kept at same level and they were monitored using multiparameter probe YSI 556 MPS.

During the experiment in tanks the water temperature varied in the range from 23.7 to 28.9°C, average 25.8±1.37°C. The average oxygen content of the water was about 5.57 mg dm^{-3}, and the pH of the water in each aquarium of experimental groups reached values 6.8-7.2 pH. Nitrites and ammonia were measured twice a week and they maintained at levels lower than 0.1 and 1 mg L^{-1}, respectively.

Table 2. The composition of commercial feed used in an experiment

Specification	Commercial feed 1	Commercial feed 2
Type of feed	Fry feed	Grower feed
Size mm	2	3
Period of use	1-5 week	6-10 week
Nutrients content:		
Crude protein %	45.0	30.0
Crudefat %	20.0	7.0
Carbohydrates %	16.0	43.0
Crudeash %	8.0	7.0
Crudefibre %	2.0	5.0
Gross energy Kcal/MJ	5171/21.6	4325/18.1
Conv. energy Kcal/MJ	4145/17.3	3353/14.0
Environment		
N in dry matter %	7.8	5.2
P in dry matter %	1.1	1.3
Energy dry matter Kcal/MJ	5682/23.7	4701/19.6
Added per kg of feed		
Vitamin A (IE)	3750.0	2500.0
Vitamin D_3 (IE)	750.0	500.0
Vitamin E (mg)	225.0	150.0

Weekly, the total length (Tl) and standard length (Sl) of all fish was measured (accuracy ±0.1 mm) and body weight was determined (accuracy ± 0.1g). For two groups of fish the growth parameters, survival rate, coefficient of condition (K) and specific growth rate (SGR) was calculated from the formulas: $K=100000\ W/Sl^3$, $SGR = (\ln W_f - \ln W_i \times 100)/t$; where: W – weight of fish (in g), Sl - the standard length of the fish (in mm), $\ln W_f$ - the natural logarithm of the final weight, $\ln W_i$ - the natural logarithm of the initial weight, t - time (days) between $\ln W_f$ and $\ln W_i$.

After the experiment, the fish from all tanks were captured and euthanized, and then above the lateral line of the muscle tissue samples for biochemical analysis were collected. All samples from the each tank were pooled for analysis. The samples of tissue homogenates were prepared in accordance with the methodological recommendations and at the temperature –20°C, the supernatant for analyzes was stored.

The antioxidant enzyme levels in muscles of all individuals of carp were estimated by the spectrophotometric method, i.e. superoxide dismutase (SOD) with the adrenaline assay by Greenwald (1985) modified at 320 nm to increase the selectivity of transient reaction products at this light length (Bartosz, 2004), whereas catalase (CAT) was assayed according to Bartosz (2004). As for the antioxidant status parameters, assays were also taken in vitamin C after Omaye *et al.* (1979). In addition, the biological material was analyzed for levels of lipid peroxidation products: peroxides (H_2O_2) – according to Gay and Gębicki (2000), and malondialdehyde (MDA) as the end product of tissue lipids oxidation – according to Salih *et al.* (1987).

After end of experiment the linear regression parameters were calculated for the increase of total length and body weight in the feeding group of carp, depending on time of measurement. The results of experiment were determined after following formula: Tl/W = aT + b, where: Tl – total length (in cm), W – body weight, T – period of time (week), a i b – parameters describing regression. The statistical differences in total length (Tl), body weight (W), specific growth rate (SGR), coefficient of condition (K) and the parameters ofoxidation -reductionin the muscle of fish from the two feeding groups were tested using one-way ANOVA at a significance level of $P \leq 0.05$.

Results

At the beginning of the experiment, the total length of carp averaged around 9.6±0.5 cm for the control group, and about 9.5±0.5 cm for experimental fish (PX+). The average body weight of fish from two dietary groups was c.a. 15.5±1 g. After 10 weeks, a slightly higher average total length (Tl = 17.01±1.91 cm) was found in the fish fed whose feed included lucerne concentrate (PX+). Statistical analysis showed that the differences of the value of this feature was not statistically significant (ANOVA, P=0.079) (Table 3). Group of fish fed feed with PX + also reached a higher average final body weight W=91.33±25.18 g compared to the control group of fish. However, these differences were not significant (ANOVA, P=0.217) (Table 3). The studies showed, that the coefficient of condition (K) values of juvenile carp ranged from 1.49 to 2.06, but not significantly higher than the average value of features found for PX+ group fish (K=1.81±0.10) (Table 3). The estimated specific growth rate (SGR) was slightly higher for carp PX + (2.60±0.03), with no statistical differences (ANOVA, P=0.280). Simultaneously the fish from this group had a higher survival rate at the level 90%.

The parameters describing the curves of linear regression characterized by an increase of fish total length (Tl) and fish body weight (W) during the experiment, shown in the Table 4. Higher values in this parameter and the characterized feeding of fish with the addition of PX+, showed that these fish had a higher parameter b than fish from the control group.

Analysis of oxidative parameters of muscle tissue of carp showed significantly higher level in the final product of peroxidation of lipid - malondialdehyde (MDA) in PX+ group (ANOVA, P<0.0001). Simultaneously, variability of this parameter in the control group of carp was slightly higher. In carp muscle the level of hydrogen peroxide (H_2O_2) contained in the range from 1.15 to 2.97. But much greater variability of this parameter (SD = 0.63) in tissues of carp fed with lucerne (PX+) was found. Insignificantly higher in mean value of this parameter was found in the muscles tissue of carp in the control

Table 3. The changes of total length (Tl) and body weight (W) of fish during 10 weeks experiment and mean values of growth parameters (SGR, K) and survival rate for two groups of carp

Parameters	Control		PX+		
	1 week Mean ± SD*	10 week Mean ± SD	1 week Mean ± SD	10 week Mean ± SD	p
Total length (Tl) (in cm)	9.60 ± 0.70	16.65 ± 1.53	9.50 ± 0.88	17.01 ± 1.91	0.079
Body weight (W) (in g)	15.50 ± 0.80	83.71 ± 23.70	15.50 ± 0.90	91.33 ± 25.18	0.217
SGR (%)	2.37 ± 0.25		2.60 ± 0.03		0.280
K	1.77 ± 0.14		1.81 ± 0.10		0.320
Survival rate (%)	70.00 ± 2.83		90.00 ± 1.41		

SD – standard deviation

group ($\bar{x}$ = 1.73±0.40 nmol mg^{-1} protein). In addition, in the muscle tissue of this group of fish, the concentration of vitamin C, almost twice higher (mean 0.42 mg/g), has been found. Statistical analysis showed that this difference was statistically significant (ANOVA, P<0.0002) (Table 5). Studies have shown that the addition of the concentrate of lucerne to feed resulted in increased activity of superoxide dismutase (SOD) and catalase, but the differences in average values were statistically insignificant.

Discussion

Lucerne as a dietary supplement has been repeatedly tested in the breeding of many species of farm animals. As a feed additive, lucerne has resulted in increased growth and feed consumption and actively stimulated immunological, antioxidant and hematopoietic systems. Saponins from lucerne enhance the secretion of bile acids while increasing the secretion of steroids (e.g. cholesterol) (Czech *et al.*, 2012; Ognik *et al.*, 2012).

According to other authors (Gaweł and Grzelak, 2012) addition of lucerne is an important source of protein and antioxidants, including vitamins and macro- and micronutrients. Currently, in times of high demand for protein, concentrate from lucerne can be an alternative to animal protein in feed for fish. However, as reported by Chatzifotis *et al.* (2006), this plant cannot promote growth as well as fishmeal.

Studies by Ali *et al.* (2003) showed that the use of lucerne in feed at a rate of more than 5% may be used in the feeding of *Oreochromis niloticus* without decreasing growth, while the replacement of animal proteins in the feeding of tilapia (*Oreochromis mossambicus)* with up to 35% concentrate from the leaves of lucerne resulted in increased growth of fish. In addition, as reported by Jia *et al.* (1991) inclusion of lucerne in practical diets of Chinese blunt snout bream (*Megalobrama amblycephala*) increased palatability and feed intake and improved fish flesh. However, a higher percentage of lucerne leaf proteins inhibited the growth of fish (Olvera-Novoa *et al.*, 1990). Similarly, Yanar *et al.* (2008) found that the addition of 25% or more lucerne in the diet had a negative impact on fish growth in goldfish (*Carasius auratus*).

In the present research, using an application rate of 5% lucerne concentrate, a slightly greater total length and body weight of fish fed with PX+ were obtained. In addition, this group had higher values for the condition factor and SGR (Table 3 and 4). These results confirmthe results obtained byother authors (Olvera-Novoa *et al.*, 1990; Ali *et al.*, 2003; Chatzifotis *et al.*, 2006; Yanar *et al.*, 2008). The low application rate used in the experiment resulted in a slight increase in fish growth, and fish from the PX+ feeding group had greater values of the studied parameters despite there being no statistical differences found. According to Yanar *et al.* (2008) the optimal application rate of lucerne in feed for *Carassius auratus* was found to be 15%.

Differentiation in the efficiency of lucerne for fish feeding is the result of the presence of several compounds in the feed, among which are some that

Table 4. The parameters of linear regression of increase of the total length and body weight of juvenile carp from different feeding group; a - parameter described a linear regression, b - factor refers to the steepness of the curve, R^2- ratio of the sum of squares explained by a regression model

Parameters	Group of fish	a	b	R^2
Total length (Tl)	Control	0.798	8.628	0.994
	PX+	0.888	8.160	0.993
Body weight (W)	Control	7.411	5.155	0.986
	PX+	8.519	0.687	0.981

Table 5. Pro-oxidative and antioxidative parameters in the muscles of carp

Parameters		Group of fish	
		Control (n = 15)	PX+ (n = 18)
Malondialdehyde -MDA	Mean± SD*	0.61^{B*} ±0.15	0.80^{A} ±0.11
nmol mg^{-1} protein	Range	0.38–0.89	0.60–0.99
Vitamin C -Vit. C	Mean± SD	0.42^{A} ±0.18	0.24^{B} ±0.10
mg g^{-1} tissue	Range	0.19–0.71	0.08–0.56
Superoxide dismutase -SOD U	Mean± SD	34.67^{A} ±2.16	35.74^{A} ±2.18
mg^{-1} protein	Range	32.69–37.24	32.40–42.41
Catalase -CAT U	Mean± SD	10.34^{A} ±2.88	11.62^{A} ±2.31
mg protein	Range	2.94–15.16	7.19–14.76
Peroxides -H_2O_2	Mean± SD	1.73^{A} ±0.40	1.64^{A} ±0.63
nmol mg^{-1} protein	Range	1.26–2.52	1.15–2.97

SD – standard deviation

$^{A, B}$ – different letters in the same row indicate significant differences (P<0.05)

stimulate growth and others that may inhibit it, depending on the application rate. In such compounds there are antinutritional factors such as protease inhibitors, saponins, phytoestrogens or antivitamins. Increased proportions of crude fibre in feed accompanying higher proportions of lucerne were observed by Ali *et al.* (2003), and as reported by Dioundick and Stom (1990) and Al-Asgah (1996), the amount of the crude fibre has an effect on nutrient digestibility and can slow down the growth of fish. A study done by Chatzifotis *et al.* (2006) showed that saponins supplied with lucerne used to feed sharp snout sea bream (*Diplodus puntazzo*) had a negative impact on its growth and caused a lower survival rate. On the other hand, as reported by Francis *et al.* (2001, 2002 and 2002a), saponins can have a positive impact on the growth of Nile tilapia and common carp. According to Ali *et al.* (2003) varied conversion of lucerne by different fish may be due to species differences and possibilities of absorption of nutrients contained in lucerne.

In the present study the use of lucerne in feed resulted in differences in survival of juvenile carp. The group of fish that were fed with lucerne (PX+ group) had about a 20% higher survival rate than fish from the control group. Research by Yanar *et al.* (2008) has shown that the addition of lucerne in experiments with goldfish (*Carasius auratus*) did not affect its survival, while Chatzifotis *et al.* (2006) found a much lower survival rate (about 64.6%) of sharp snout bream fed with the highest application rate of lucerne (210g/kg of feed).

The basic biochemical processes in the meat of fish are similar to the processes occurring in the meat of warm-blooded animals. The main difference is the rate of change due to the presence of blood in the tissues, the elevated temperature, the specific factors associated with greater hydration of meat, higher activity of enzymes and the small size of the fish body. Fish lipids are oxidized by auto oxidation and as the result of enzymatic reactions. Their extent and rate depend on the content and composition of lipids in the tissues, the nutritional status of the fish before the catch, the amount of antioxidants taken with the food, the method of treatment prior to freezing, the effectiveness of protection against ice sublimation and air, and the storage temperature.

Lucerne is an excellent source of antioxidants such as polyphenols, vitamin E, vitamin C, β-carotene, saponins, iron, copper, zinc and selenium (Ben Aziz *et al.*, 2006). It is known that antioxidants are required in order to prevent excessive production of free radicals in the body and consequently to prevent the process of oxidation. Much depends on the mode of action of the antioxidant. Antioxidants can directly inhibit free radicals or stimulate natural antioxidant systems inside the cell, for example, activation of glutathione peroxidase genes (GPx) and (SOD).

Enzymatic and non-enzymatic antioxidants are basic compounds that keep a balance between the activity of antioxidants and the amount of reactive oxygen species produced in the cells of fish. They serve as an important biological defence against oxidative processes. Therefore, their levels in the muscle tissue of fish can be a useful indicator for assessing the susceptibility of fish to oxidative stress.

The increase in malondialdehyde levels observed in our study is undesirable (Table 5), as it may indicate oxidative stress inducing lipid peroxidation of muscle tissue in carp. Additionally, the increase in antioxidant enzymes (SOD and CAT) correlated with a decrease in non-enzymatic antioxidants (vitamin C) in muscle tissue of carp fed protein concentrate from lucerne (PX+) may be due to stimulation of antioxidant mechanisms to defend against reactive oxygen species (ROS). In view of the antioxidant properties of protein-xanthophyll concentrate from Lucerne confirmed in studies on other farm animals (Czech *et al.*, 2012; Karwowska *et al.*, 2012; Ognik *et al.*, 2012), the results obtained, especially increased MDA levels, are difficult to interpret, especially as there are reports confirming the fact that the addition of carotenoids to feed for farmed fish may inhibit the oxidation of lipids (Mortensen *et al.*, 2000). The positive effects on antioxidant status in the tissues of fish due to the use of antioxidant supplements in feed were demonstrated by research conducted by Metwally (2009). According to Xie *et al.* (2008) protein from lucerne leaves has the ability to donate electrons or hydrogen and thus possess the ability to neutralize peroxide radicals, hydroxyl radical and DPPH. The high activity of scavenging radicals by lucerne protein is additionally strengthened by antioxidant activity of existing active compounds. Research conducted by Fu (2003) has shown that the leaves of lucerne have proteins that increase the activity of glutathione peroxidase and superoxide dismutase, and decreased the concentration of malondialdehyde. Additionally, there are effects that increase the total antioxidant potential.

The results of the observations have shown that the addition of 5% protein-xanthophyll concentrate (PX) from lucerne (*Medica gosativa*) to fish feed resulted in a greater (though not statistically significant) increase of total length and body weight in carp. Moreover, the addition of lucerne caused higher average increase in length and weight, and also slightly better condition factor and SGR. The addition of PX to the carp feed induced an increase in peroxidation lipid products with simultaneous stimulation of the antioxidant enzymatic system in the muscles of carp.

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