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Evaluation of Growth and Histology of Liver and İntestine in Juvenile Carp (*Cyprinus carpio*, L.) Fed Extruded Diets with or without Fish Meal

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Abstract

Growth and histology of intestine and liver of carp fed diets with or devoid of fish meal (FM) was studied. Carp were fed four experimental diets formulated to contain 38% protein for 90 days. FM was incorporated at 30% in feed A, 15% in feed B and C, and was completely replaced with a mixture of plant proteins in feed D. Feed C and D were supplemented with methionine and lysine. The results showed that carp fed feed D had the lowest weight gain, length and height compared to the other three diets, whereas no differences were observed between A, B and C for the measured morphometric parameters. Inclusion of methionine and lysine tended to improve SGR of carp fed feed C compared to those fed feed B, but growth rate was lower than carp fed feed A. FCR differed for nearly 90 % between the FM rich and solely plant protein diet. No major pathological changes were recorded. At the end of the study shortening of intestinal folds' length was found for all groups, except for fish fed feed D. The height of enterocytes was significantly lower for carp fed diet D compared to other diets. According to the results obtained the best diet is feed A, but feed C with 15% FM and added methionine and lysine represents an acceptable replacement due to its lower price and effect on growth that are the most similar to feed A.

Keywords: Carp, extruded diet, proteins origin, replacement, fish meal.

Introduction

Farm production of carp, mostly semiintensive, in 2002 represented 14% (33 138 962 tonnes) of the total world production of freshwater fish (FAO, 2004). Semiintensive production is based on the use of natural food from the pond environment as the main source of protein and energy, only supplemented with additional feed when the natural production in the pond is deprived (Rahman, 2006; Marković *et al*., 2009a). Different grains or legumes (wheat, corn, barley, triticale, soybean, sunflower) are used as added feed, as well as formulated feed of different composition and level of treatment. Because fish feed comprise the greatest cost in semiintensive and intensive production (De Silva, 2010), it is of extreme importance to select the feed type for carp, which is able to achieve the most profitable production.

Fish meal (FM) is used in semiintensive cultures of carp juveniles, primarily in periods of natural food depression, and in intensive production of juveniles and market size carp in ponds and cage systems. FM price is constantly increasing, so is the price of carp production. Recent research is pointing out possibilities of almost 100 % replacement of FM in diets for carnivorous fish species like Atlantic salmon and Atlantic cod with proteins of alternative origin without adverse effects on growth (Espe *et al*., 2007; Hansen *et al*., 2007). These results suggest that the omnivorous fish, like the carp, also should be able to utilize diets without FM. The limitations of the use of plant proteins in diets for fish are related to imbalances in amino acid composition and the presence of bioactive compounds (Storebakken *et al*., 2000; Francis *et al*., 2001; Gatlin *et al*., 2007). Efficient utilization of protein can only be achieved by feeding a balanced feed where the amino acids can be used for protein growth rather than being excessively catabolized for energy.

Soybean based products represent a major source of protein in diets for monogastric animals, and many have shown great potential as FM replacements in diets for several fish species (Refstie *et al*., 1997; Refstie *et al*., 2000; Storebakken *et al*., 2000; Fagbenro and Davies, 2001; Romarheim *et al*., 2008a; Schuchardt *et al*., 2008; Ali *et al*., 2008). Soybean is low in methionine (Pongmaneerat and Watanabe, 1993; Schwarz *et al*., 1998). Glutens from maize and wheat can be used as a FM replacer. These sources are low in lysine (Gatlin *et al*., 2007).

Supplementation with methionine (Schwarz *et al.*, 1998) and lysine (Hu et al., 2008) should therefore be considered when plant protein ingredients are used as replacement for FM. Bioactive compounds in soybean meal (SBM), mainly in the alcohol soluble fraction (Van den Ingh *et al.*, 1991; Van den Ingh *et al.*, 1996; Knudsen *et al.*, 2007) are shown to cause morphological changes in the distal intestine of several fish species such as rainbow trout (Romarheim *et al.*, 2008b), Atlantic salmon (Baeverfjord and Krogdahl, 1996; Refstie *et al.*, 2000), and common carp (Uran *et al.*, 2008). In addition, lower palatability of feeds with high dietary level of SBM may reduce appetite and induce a drop in feed intake (Francis *et al.*, 2001; Glencross *et al.*, 2005; Skugor *et al.*, 2010).

Replacing expensive FM with cheaper plant protein ingredients is expected to give more profitable production results in semiintensive production system of juvenile carp. However research is, needed to investigate growth performance, feed utilization and histology of vital digestive organs when new feeds are used.

The aim of this study was to evaluate four practical diets with different FM content (0, 15%, and 30%). FM was fully or partly replaced with ingredients of non animal origin and supplemented with metionin and lysisne. Effects on growth and histological structure of liver and intestine were investigated.

Materials and Methods

Feed Preparation

Four experimental feeds were produced to contain the same protein level of 38%. Feed A contained 30 % of fish meal, feeds B and C 15%, while feed D was devoid of fish meal. The mixture of plant proteins used to replace fish meal was: full fat extruded SBM, toasted SBM, yeast, maize gluten, and wheat gluten. Feeds C and D were in addition supplemented with methionine and lysine. The diets were extruded by the Serbian feed company "Sojaprotein" (Bečej, Serbia). Ingredient and chemical composition of the experimental diets is given in Table 1.

Fish, Allotment, Feeding and Growth Parameters

The experiment was carried out with one year old carp (*Cyprinus carpio* L.) originating from the fish farm "Mošorin", Mošorin, Serbia after examination that revealed very good health status. The fish were acclimated to laboratory conditions for 45 days prior to the feeding experiment. During acclimatization fish were fed feed A. The feeding experiment, lasting for 90 days, was carried out in the Laboratory for fish nutrition at the Faculty of Agriculture, University of Belgrade. Carp with start weight varying from 55.75-59.79 g were weighed

Table 1. Ingredient and chemical composition of the experimental diets (%)

Components	Feed			
	A	B	C	D
Fish meal	30.0	15.0	15.0	0.0
Full fat extruded soybean meal	15.0	21.3	21.3	25.4
Wheat	13.5	11.1	10.8	6.5
Maize	13.5	11.1	10.8	6.5
Toasted soybean meal	9.0	12.0	12.0	20.0
Yeast	6.0	8.0	8.0	10.0
Maize gluten	0.0	8.0	8.0	10.0
Wheat gluten	5.0	5.0	5.0	10.0
Soybean oil	5.0	5.0	5.0	6.0
Dicalcium phosphate	1.0	1.5	1.5	2.5
Limestone	0.4	0.4	0.4	0.4
NaCl	0.1	0.1	0.1	0.2
Methionine	0.0	0.0	0.2	0.4
Lysine	0.0	0.0	0.4	0.6
Mineral and vitamin premix*	1.5	1.5	1.5	1.5
Chemical composition				
Dry matter (DM), g kg^{-1}	956.9	955	956.5	955.2
In DM, g kg^{-1}				
Crude protein, g	381.4	377.0	376.5	377.1
Lipid, g	118.2	117.2	117.1	126.8
Crude fiber, g	21.1	25.7	25.6	29.4
Ash g	70.2	57.3	57.2	43.5
Nitrogen-Free Extract (NFE), g	409.2	422.7	423.7	423.3

* Mineral and vitamin premix (made by Sojaprotein, Bečej, Serbia) contained the following components (each kg^{-1} diet): total Ca (%) min. 1.6; total P (%) min. 1.2; Vitamin A (IU-kg) min. 15.000; Vitamin D3 (IU-kg) min. 2.500; Vitamin E (mg-kg) min. 90; Vitamin C (mg-kg) min. 200; Lysine (%) min. 2.3; Methionine + Cystine (%) min. 1.2; Gross energy 18.9 MJ-kg; Metabolic energy 15.0 MJ-kg

individually and stocked in plastic tanks. All tanks were supplied with continuous flow of dechlorinated tap water at the rate of 0.34 L min^{-1}. The experiment was carried out in 12 circular tanks of 0.5 m diameter, and 0.9 m water depth, with 25 carp yearlings/tank. Fish were fed once a day 3.5% of their body mass using semi-automatic feeders with a pendulum (custom made for the University of Belgrade by the company "Plastik", Ljig, Serbia). Water quality and environmental conditions such as temperature, conductivity, and dissolved oxygen, were monitored using the MULTI 340i/SET (WTW, Weilheim, Germany). The water temperature in the tanks ranged between 21.6 to 24.4°C, conductivity was within the interval 483-668 μS cm^{-1}, and dissolved oxygen concentration ranged from 6.30 to 8.20 mg L^{-1}. No mortality occurred during the acclimatization or during the feeding experiment. In 30-day intervals each fish body weight, length and height was measured using a digital CASBEE balance (Model MW 120; Casbee, Samsung, South Korea; accuracy 0.01g). Length and height were measured using the ichthyometer. Following equations were used for calculating growth parameters:

SGR (Specific Growth Rate) = (ln(final weight) – ln(initial weight)) x $days^{-1}$ x 100

BWG (Body Weight Gain) = final body weight (g) – initial weight (g);

FCR (Feed Conversion Ratio) = (feed intake, kg) x (wet weight gain, $kg)^{-1}$;

CF (Condition Factor) = body weight (g) x (fork length, $cm)^{-3}$ x 100;

Feed Intake (g) per fish = (Total feed consumption (g) per tank) / (number of fish per tank)

Histological Analysis

At the start of experiment distal intestine, liver, and gills were sampled from three fish from the initial population. The fish were killed by destruction of the spinal cord, behind the head and a brain, with a needle. At the end of experiment two fish per tank were sacrificed and liver, intestine, and gills were taken for histological analysis. The tissue samples were fixed in 4% formaldehyde and processed by standard histological techniques (dehydrated in ethanol series, embedded in paraffin, serially sectioned at 4–5 μm) and stained with hematoxyline and eosine (HE), (Humason, 1979).

For morphometric analysis height of enterocytes and length of intestinal folds, as well as nuclear area of hepatocytes were measured on microphotographs taken by Leica DM LS microscope (Wetzlar, Germany), with DC 300 camera using Leica IM 1000 program. Enterocytes height was measured on 100 μm of intestinal mucosa by performing 15 to 77 measurements on each histological slide. Length of folds from the proximal to the distal part was measured (2 to 21 measurement on each slide). Nuclear area of 50 hepatocytes per slide was measured.

Statistical Analysis

Statistical analysis of the results obtained in the experiment was carried out using statistical package STATISTICA v.6. Differences in growth and morphometric parameters among the four diets were tested using analysis of variance (ANOVA). Significant differences among treatment means were found by use of LSD test (for calculation of growth parameters), Kruskal-Wallis, and Mann-Whitney U test (for calculation of histological parameters).

Results

Weight gain was significantly affected by the diet (Table 2). At start of the experiment carp had a uniform body weight, length, and height. At the end of the experiment, the fish fed feed D had significantly lower weight gain compared to the other three diets, whereas no differences were observed for the carp fed the other three diets. Length and height of carp fed feed D was significantly lower compared to carp fed the other three diets.

Table 2. Growth parameters, feed and nutrient utilization of *C. carpio* fed the experimental diets. Different letters in superscript denotes statistical difference between diets in the same row. Values are expressed as means±standard deviation.

	Feed A	Feed B	Feed C	Feed D	P-value
Initial weight (g)	56.78±1.03	58.79±0.58	58.60±0.35	58.96±0.83	NSD
Final weight (g)	151.99±64.31^{a}	132.06±52.79^{a}	135.83±64.95^{a}	98.21±41.69^{b}	P<0.001
BWG (g)	95.21±10.63^{a}	73.27±7.48^{a}	77.23±9.19^{a}	39.25±3.3^{b}	P<0.01
Length (cm)	20.55±3.04^{a}	19.74±2.70^{a}	19.58±2.95^{a}	17.61±2.46^{b}	P<0.001
Height (cm)	6.28±1.00^{a}	5.90±0.84^{b}	6.05±1.01^{ab}	5.24±0.83^{c}	P<0.03
CF	1.65±0.13^{ab}	1.64±0.15^{b}	1.71±0.15^{a}	1.71±0.23^{a}	P<0.03
FI (g/ per fish)	229.53±13.78^{a}	226.21±16.65^{a}	229.67±2.60^{a}	196.99±13.81^{b}	P<0.02
FCR (g)	1.49±0.18^{a}	1.89±0.20^{b}	1.81±0.27^{ab}	2.82±0.18^{c}	P<0.05
SGR (% day^{-1})	1.09±0.19^{a}	0.90±0.13^{a}	0.93±0.20^{a}	0.57±0.12^{b}	P<0.03
TGC	2.16±0.33^{a}	1.77±0.26^{a}	1.80±0.19^{a}	1.06±0.10^{b}	P<0.006

Condition Factor (CF) was statistically different in fish fed diet B compared to C and D. Feed Intake (FI) in carp fed feed D was the lowest compared to the other three diets, and no differences were observed for carp fed A, B, and C. Feed Conversion Ratio (FCR) was significantly lowest for carp fed feed A and highest for carp fed feed 4, while fish fed diet B ranged in between. Carp fed diet 3 tended to have a higher FCR than those fed feed A and lower than those fed feed B. Specific growth rate (SGR) ranged from 0.57 to 1.09. At the end of the experiment the significantly highest SGR was shown for carp fed feed A. Fish fed feed D showed the lowest SGR, whereas diets B and C ranged in between.

Histological Analysis

Dietary effects of diets on histology of the distal intestine and the liver were investigated and gill histology was used as monitor of environmental conditions.

Liver

Vacuolated hepatocytes, fatty changes, and focal fibroses (Figure 1) were found on some liver samples regardless of feed and period of sampling (beginning or end of the experiment). Pycnotic nuclei were found in the liver of the carp fed feed D, as well as on one sample from the tank with carp fed feed C. Morphometric measurements of nuclear area of hepatocytes did not differ among the dietary treatments (Figure 2)

Morphometric measurements did not unveil significant differences in average nuclear area of the hepatocytes among the diets (Figure 3). Yet, slide examination unveiled an increased number of pycnotic nuclei in the liver of the fish fed feed D. The largest variability of nuclear area of hepatocytes in fish fed feed D could indicate presence of normal and pycnotic nuclei (Figure 1). In all liver samples examined after 90 days of experiment signs of fatty degeneration of hepatocytes were found compared to the initial group.

Intestine

Normal morphology was observed in carp distal intestine. However, some slides showed leucocytes infiltration in the epithelium, most often accompanied

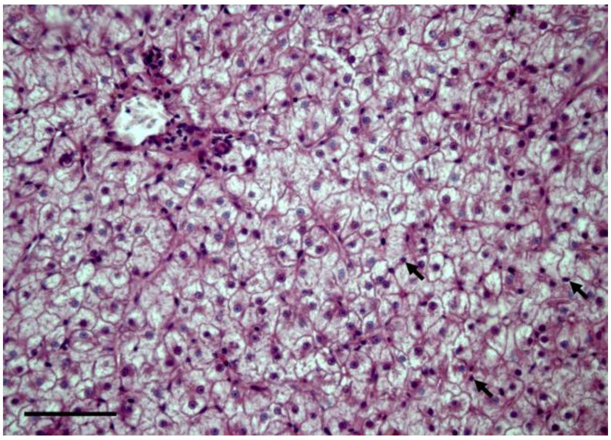

Figure 1. Hepatocytes of experimental carp fed with feed D (HE x40). Note the slight cloudy swelling of hepatocytes and few picnotic nuclei (arrows). Scale bar = 50 µm.

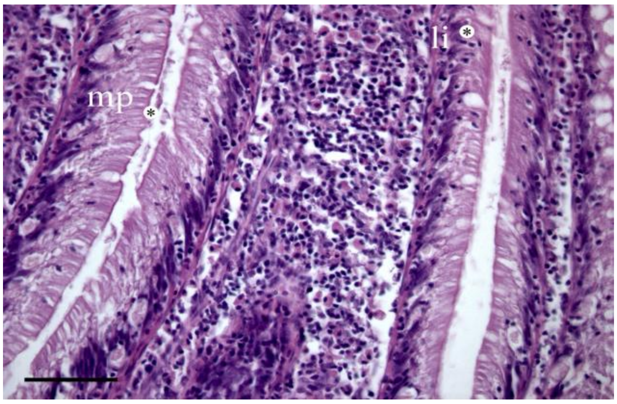

Figure 2. Intestine of experimental carp fed feed D (HE x40). Note slight leucocytes infiltration in the epithelium (li) and mucus production (mp). Scale bar = 50 µm.

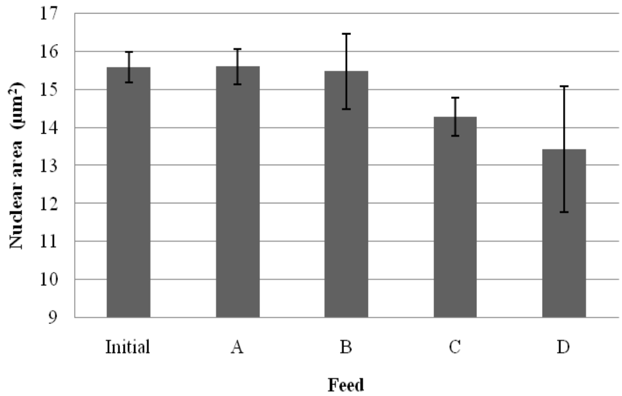

Figure 3. Nuclear area of hepatocytes.

by increased mucous production (Figure 2), regardless of dietary treatment.

At termination of the experiment the height of enterocytes were significantly (P<0.01) longer in fish fed diets A, B and C compared to fish fed feed D (Figure 4). For feed D, however, mucosal enterocytes height was the same at termination of the experiment compared to the start (Figure 4). In carp fed diets A, B and C, the enterocytes appeared to be normal in terms of development and function.

Length of intestinal folds were significantly (P<0.01) higher in fish from the start population than in fish sampled at termination of the experiment (Figure 5). Although not statistically significant, the increased fold length was inversely related to growth rate. Feed D containing solely plant protein sources had the highest fold length and the lowest growth rate.

Gills

All the gills sampled at the beginning and termination of the experiment showed normal morphology. Sporadically lifting of the respiratory epithelium, mild circulatory alterations (hyperemia), and focal hyperplasia of the primary epithelium were observed. These changes were present only locally, regardless of the period of sampling (beginning or end of the experiment) and were not affected by the diets. The gills of carp in this experiment were functionally normal reflecting acceptable water quality.

Discussion

This experiment was carried out to investigate growth performance, feed utilization and digestive organs' morphology of carp yearlings fed diets differing in FM content. The growth performance and feed utilization were associated with the level of FM in the diets (Table 2). Plant-based fish feeds can suffer from an imbalanced indispensible amino acid composition, primarily methionine and lysine (Kaushik, 1995; Watanabe, 2002). In our experiment, inclusion of methionine and lysine improved SGR of carp fed feed C, but still growth rate tended to be lower than carp fed feed A (highest FM content). The lower SGR and weight gain in carp fed feed D is also explained by reduced average daily feed intake (g per fish) in this group (Table 2). The lower feed intake of carp fed feed D may be explained by palatability of soy products, obstruction of digestive processes, and intestinal dysfunctions, as reported for other species (Francis *et al*., 2001; Glencross *et al*., 2005; Skugor *et al*., 2010; Rich and Williams, 2011). Similar results were reported in Gomez-Requeni *et al*. (2004), where weight was depressed up to 30% in the group that had 100% replacement of FM with plant proteins. Hansen *et al*. (2007) found a significant decline in feed intake with complete replacement of FM with plant ingredients in the diet for cod. Reduced appetite may be a consequence of replacement of FM by proteins of plant origin (Gatlin *et al*., 2007).

The results clearly demonstrated that FCR was worsened for carp fed the diets highest in plants. Carp fed diet A had 90% improved FCR compared to those fed diet D devoid of FM. This observation is in accordance with the study of Sevgili *et al*. (2011) who reported that FCR and protein efficiency ratios were significantly lower in carp fed hazelnut meal at a higher inclusion level than 20% of the diet.

Evaluation of histological structure of digestive organs in fish fed new ingredients provide valuable information about digestive capacity and potential health effects of new diets (Caballero *et al*., 2003; Diaz *et al*., 2006), but not many investigations have been carried out with carp (Viola *et al*., 1982; Fontagne *et al*., 1998). Morphological changes in the distal intestine (Van den Ingh *et al*., 1991; Baeverfjord and Krogdahl, 1996; Refstie *et al*., 2000; Krogdahl *et al*., 2003; Uran *et al*., 2008; Knudsen *et al*., 2008) is responsible for the retardation of growth seen in response to SBM-based diets. Normal morphology was predominant in carp intestine in A, B and C dietary groups while a number of inspected slides in group D showed increased leucocytes infiltration in the epithelium, most often accompanied by increased mucous production (Figure 2). Such changes are indicative of intestinal inflammation; SBM induced enteritis most often involves

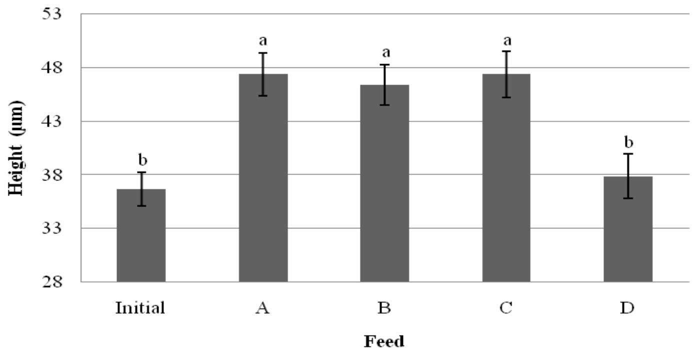

Figure 4. Height of enterocytes ([a, b] different letters denote significant differences among treatment means)

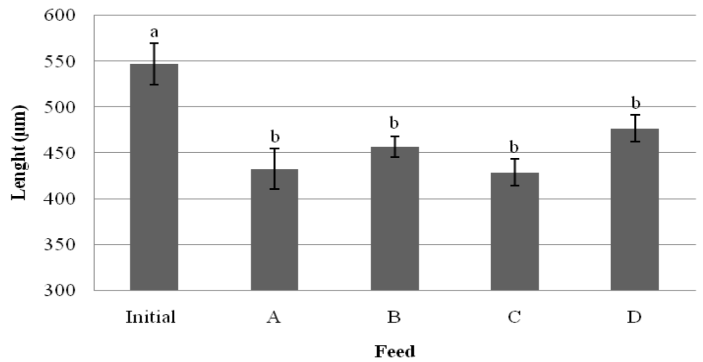

Figure 5. Length of intestinal folds ([a, b] different letters denote significant differences among treatment means)

inflammatory infiltrate (Baeverfjord and Krogdahl, 1996; Uran *et al*., 2008). However, shortening of the length of intestinal folds, a typical marker of SBM induced enteritis, as reported in common carp (Uran *et al*., 2008) and Atlantic salmon (Baeverfjord and Krogdahl, 1996), was found at the end of experimental period for all groups in comparison to the start of the experiment (Figure 5). Interestingly, there appeared to be an inverse relationship between fold length and growth rate in the present experiment. The largest fold length was observed in carp fed diet D that had the lowest growth rate. More likely this was a response to the higher inclusion rate of yeast in this diet. This explanation is supported by a recent finding showing that another single cell protein source, a bacterial protein source, prevented histopathological changes known as SBM-induced enteritis in Atlantic salmon (Romarheim *et al*., 2011). Uran *et al*. (2008) reported that carp show signs of enteritis when fed high levels of soy in the diet. The present findings suggest that inclusion of yeast promoted increased absorptive area and protected fish fed feed D from more severe SBM-induced enteritis.

Morphometric analysis showed significant differences in enterocytes height (Figure 4). At termination of the experiment enterocytes were significantly shorter in carp fed feed D in comparison to all other groups. Mucosal enterocytes' height did not change in the course of the study in this group (Figure 4). Flexibility of the piscine gastrointestinal tract to adjust to food availability is well illustrated; e.g. Atlantic cod with the high feed intake had relatively higher weight of different sections of the gastrointestinal tract compared to cod with the lower feed intake (Refstie *et al*., 2006).

Vacuolated hepatocytes, fatty changes, and focal fibroses (Figure 1) were observed in some liver samples regardless of feed and period of sampling (beginning or end of the experiment). A reduction in nuclear size may be a sign of malnourishment (Fontagne *et al*., 1998; Power *et al*., 2000; Ostaszewska *et al*., 2005). Despite significant reductions in SGR, morphometric analyses of nuclear area of hepatocytes did not detect significant differences in hepatic nuclear area between dietary groups. One reason for this could be the high variability seen in the group D for this parameter, which might partly reflect differential feed intake between individuals in this group. Pycnotic nuclei may have a much better resolution potential to discriminate between underfed fish: while pycnotic nuclei were found in all livers of carp fed feed D, only one out of 12 samples from the group C had pycnotic nuclei. Hepatic apoptosis is a well-established response to reduced feeding, both in mammals and in fish (Skugor *et al*., 2010 and references therein.

Finally, concerning the prices of the feeds used (feed cost decreasing as follows A>C>B>D, according to ingredients' cost) the study has shown that reduced growth of carp fed cheaper feed is nearly proportional to feed price reduction, except for feed D where the percentage of growth reduction is much higher than the percentage of price reduction. Therefore complete replacement of FM is not worthwhile.

Conclusions

The present study showed that replacing fish meal with a mixture of soy ingreidnets, maize gluten, wheat gluten and yeast gave slower growth and reduced feed utilisation, though diets were supplemented with lysine and methionine.

Partial replacement of proteins from fish meal by proteins of alternative origin with added methionine and lysine is acceptable from the growth aspect and in relation to vital organ morphology. Indeed, inclusion of methionine and lysine resulted in SGR of group C being more similar to the SGR in group A in comparison to the situation when no methionine and lysine were supplied (feed B).

The obtained results of partial replacement of fish meal, primarily by SBM with added amino acids are the basis for further investigation aimed to create optimal supplemental carp feed with high protein level. The feed is intended for juvenile carp in semiintensive production system when deficiency of natural food occurs, as well as for juvenile and market size carp in intensive system. Such approach will additionally affect profitability of carp production.

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