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Effect of feeding carp with fat-supplemented pelleted diets on histological appearance of the intestine and hepatopancreas

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Abstract. Sixty two-year-old carps, *Cyprinus carpio* L. were kept in a closed water circulation system for five months. Fish were assigned to five groups (12 fish per group) and fed *ad libitum* on Aller Classic pelleted feed for carp: standard or standard pelleted mixture supplemented with 6% oils. The control group (I_K) received standard pellets, group II_{S+Rz} – sunflower oil + rapeseed oil (50% : 50%); group III_{S+L} – sunflower oil + linseed oil (80% : 20%); group IV_R – fish oil; and group V_{SK} – pork scratchings at the level of 6%. At the end of the experiment, 10 carps from each group were slaughtered and their body length and weight were measured to calculate mean body weight gains and mean individual gains. Fragments of intestine and hepatopancreas were histologically and morphometrically analyzed. The mean weight gains of fish at the end of the experiment, which were the highest for diet III_{S+L}, and the lowest for V_{SK} and I_K diets, were fully confirmed by histological analysis, which showed that the lowest gains in biomass and mean individual weight were due to digestive disturbances associated mainly with lipid metabolism. These disturbances did not occur in groups II_{S+Rz}, III_{S+L} and IV_R.
Key Words: common carp, fat-supplemented diet, histology of intestine and hepatopancreas.

Streszczenie. Sześćdziesiąt dwuletnich karpi *Cyprinus carpio* L. podzielono na 5 grup (po 12 szt.) i przetrzymywano przez 5 miesięcy w zamkniętym obiegu wody. Ryby karmiono *ad libitum* granulatem karpiowym Aller Classic: standardowym lub wzbogaconym olejem (6%). Grupa kontrolna (I_K) otrzymywała standardowy granulat, grupa II_{S+Rz} – olej słonecznikowy + olej rzepakowy (50% : 50%); grupa III_{S+L} – olej słonecznikowy + olej lniany (80% : 20%); grupa IV_R – olej rybi, a grupa V_{SK} – skwarki w ilości 6%. Po upływie 5 miesięcy z każdej grupy zabito po 10 ryb. Zmierzono ich długość całkowitą i zważono masę ciała. Następnie obliczono średnie przyrosty masy i długości ciała. Fragmenty jelita oraz trzustkowątroby poddano analizie histologicznej i morfometrycznej. Średnie przyrosty masy ciała na zakończenie eksperymentu – najwyższe w grupie III_{S+L}, zaś najniższe w gruach V_{SK} oraz I_K – zostały całkowicie potwierdzone w toku analiz histologicznych, które pokazały, że najniższe przyrosty biomasy oraz średniej indywidualnej masy ciała były spowodowane zaburzeniami w trawieniu, związanymi głównie z metabolizmem tłuszczy. Zmiany te nie występowały w grupach II_{S+Rz}, III_{S+L} oraz IV_R.
Słowa kluczowe: histologia jelita i trzustkowątroby, karp, dieta wzbogacana w tłuszcze.

Rezumat. Șaizeci crapi (*Cyprinus carpio* L.), în vârstă de doi ani, au fost crescuți în sistem recirculant pentru o perioadă de cinci luni. Peștii au fost împărțiți în cinci loturi (12 bucăți în fiecare lot) și au fost hrăniți *ad libitum* cu granule Aller Classic pentru crap: de tip standard sau amestec de granule standard suplimentate cu uleiuri 6%. Lotul martor (I_K) a primit granule standard, grupul II_{S+Rz} – ulei de floarea soarelui + ulei de rapiță (50% : 50%), lotul III_{S+L} – ulei de floarea soarelui + ulei de in (80% : 20%); grupul IV_R – ulei de pește, iar lotul V_{SK} – grăsime de porc 6%. La sfârșitul experimentului, 10 crapi din fiecare lot au fost sacrificați, iar lungimea și greutatea corporală au fost înregistrate în vederea calculării sporului mediu corporal. De asemenea, au fost analizate histologic și morfometric fragmente de intestin și hepatopancreas. La sfârșitul experimentului, sporurile corporale medii ale peștilor, cele mai mari în grupul III_{S+L} și cele mai mici în V_{SK} și I_K, au fost deplin confirmate de analizele histologice, care au indicat faptul că cele mai reduse sporuri corporale se datorează unor tulburări digestive asociate în principal metabolismului lipidic. Aceste tulburări nu au fost observate în loturile II_{S+Rz}, III_{S+L} și IV_R.
Cuvinte cheie: crap, dietă îmbogățită cu grăsime, histologie, intestin, hepatopancreas.

Introduction. The basic feed in carp pond culture in Poland is grain of wheat and barley. Studies of the fatty acid profiles in the meat of fish fed in this way show the domination of saturated fatty acid (SFA) over polyunsaturated fatty acids (PUFA), which are the essential unsaturated fatty acids (EUFA) (Sargent 1997; Bieniarz & Kołdras 2000; Bieniarz et al 2000, 2001a,b).

Field experiments with reduced stocking rate in carp ponds and the withdrawal of feeding which means extensive method of culture (natural food only) in the final period of rearing (two-year-old fish reared for commercial purposes) showed an increase of PUFA and a decrease of SFA concentration in carp meat (Bieniarz et al 2001a). However, this solution is unfavourable from the breeding point of view because a reduction in stocking rate, necessary under an extensive rearing system, lowers productivity. This is why the experiments with commercial feed supplements such as vegetable oils and food industry waste products (pork scratchings) were undertaken. These supplements were reported to contain the EUFA (Bartnikowska & Kulasek 1994; Bieniarz & Kołdras 2000). The results of the experiments with a mixture of sunflower and linseed oil, sunflower and rapeseed as well as fish oil gave positive results (Epler et al 2009). However, fish receiving feeds with increased levels of fat show disturbed homeostasis and lipid metabolism, inhibited growth (Ostaszewska & Boruta 2006; Ostaszewska & Sysa 2004) and changes in the histological appearance of the liver and intestine (Ostaszewska & Sawosz 2004; Ostaszewska et al 2003, 2004, 2005a,b). This was the reason why in the present study (carried out as part of the research project no. 311 2454 33) we reared two-year-old carp under controlled conditions using pelleted feed containing vegetable oils, fish oil and pork scratchings to determine the influence of these supplements on the histological appearance of the hepatopancreas and the intestines.

Material and Method. The experiment was conducted on two-year-old common carp *Cyprinus carpio* (Linnaeus, 1758) (age 2+) of equal weight (160 g) which were raised at the Experimental Fish Farm of the University of Agriculture in Kraków. Fish were kept in 800 L basins with controlled water temperature (23-25°C) and oxygen concentration of 5-6 mg O_2 L^{-1}. A closed water circulation system with biofilters was used. Carp were assigned to 5 experimental groups with 12 fish per group and fed *ad libitum* on Aller Classic pelleted feed for carp. The control group (I_K) received standard pellets, and the other groups were fed standard pelleted mixture supplemented with 6% oils: group II_{S+Rz} – sunflower oil + rapeseed oil (50% : 50%); group III_{S+L} – sunflower oil + linseed oil (80% : 20%); group IV_R – fish oil; and group V_{SK} – pork scratchings at a rate of 6%. Fish were fed for 5 months, after which 10 carp were randomly selected from each group, slaughtered and measured for body length (with an accuracy of 1 mm) and for body weight (with an accuracy of 1 g) to determine overall body weight and to calculate mean body weight gains and mean individual gains. Increases in the body weight of particular groups were analyzed statistically using one-way analysis of variance (ANOVA) and Tukey's test.

Fragments of intestine and hepatopancreas were taken for histological analysis from 10 carp in each feeding group. The slices were fixed in Bouin's and Ciaccio's fluids. The samples were subjected to standard histological procedure, embedded in paraffin, cut longitudinally into 4-6 µm sections (Leica RM 2265 Leica Microsystems, Germany) and stained with alcian blue-periodic acid-Schiff reagent (AB/PAS) pH 2.5, 1.0, 0.5 (Gutierrez 1967). Periodic acid Schiff was used to stain for glycogen and mucins (glycoderivatives) with diastase as a control. Sudan black B and Sudan III were used to detect lipids in the samples fixed in Ciaccio's fluid (Martoja & Martoja-Pierson 1967; Pearse 1985). Cells and tissues were measured using a Nikon ECLIPSE 90i microscope coupled with a digital camera Nikon DS5-U1 and NIS – Elements AR microscope imaging software (Nikon Corporation Tokyo, Japan).

Histological tests and morphometric measurements (diameters of hepatocytes) were done to determine morphological differences between individual feeding groups. Morphometric evaluation and image analysis were performed using 20 measurements of each sample at 400x magnification. There were a total of 200 (n = 10 x 20) measurements of hepatocyte diameters per feeding group. The results of morphometric

measurements were analyzed using one-way hierarchical analysis of variance (ANOVA), and the model accounted for the effect of feeding group and variation between the analyzed fish within each group. Group means were compared using Duncan's test. The results are given in tables as the means for groups (mean ± S.D.), with significant differences indicated between the means ($P < 0.05$).

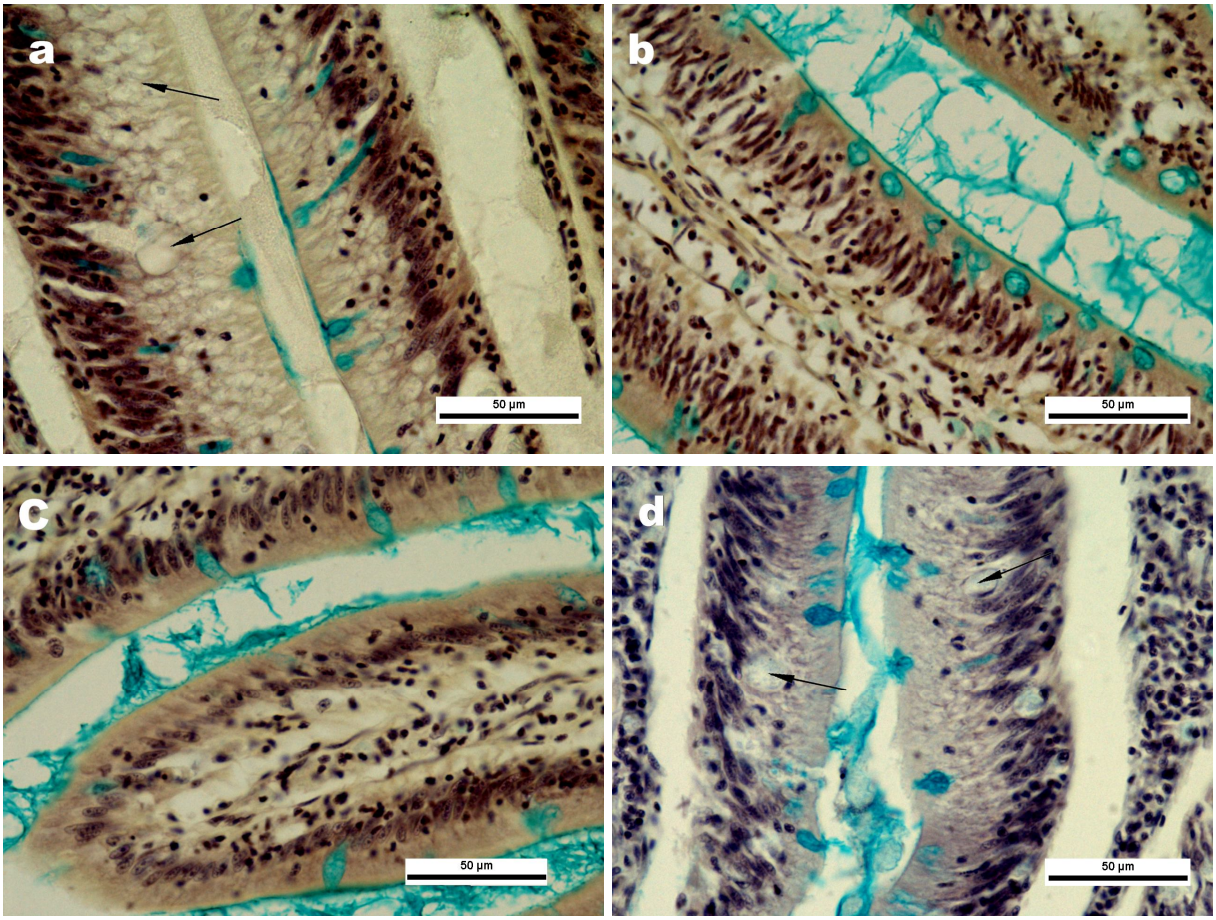

Figure 1. Longitudinal section of mid-intestine enterocytes of carp fed: a, (I_K) standard pellets; b, (II_{S+Rz}) pellets with a mixture of sunflower and rapeseed oils (50:50%); c, (III_{S+L}) pellets with a mixture of sunflower and linseed oils (80:20%); d, (V_{SK}) pellets with pork scratchings. Lipid vacuoles were marked with arrows. AB/PAS staining.

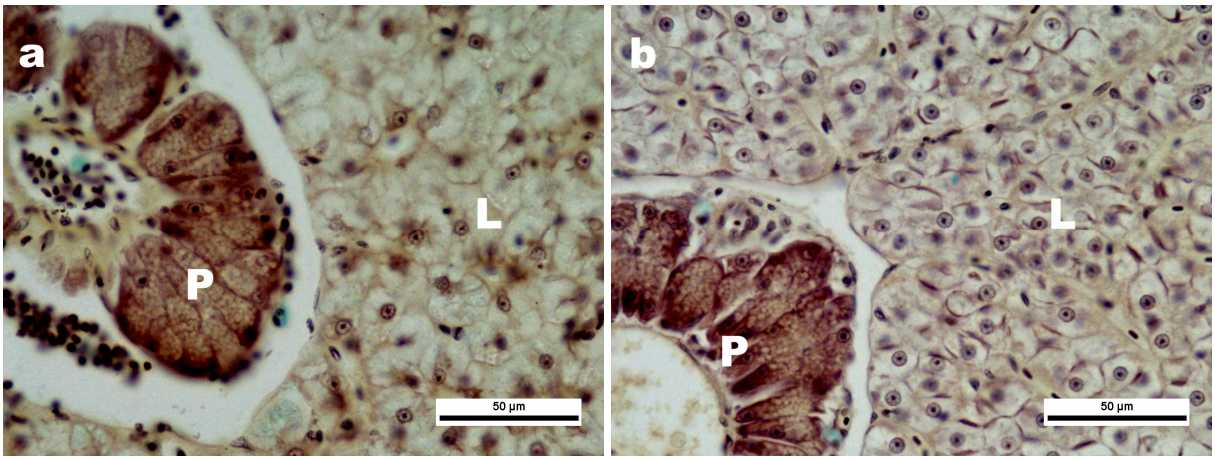

Figure 2. Section through hepatopancreas of carp fed: a, (I_K) standard pellets; b, (V_{SK}) pellets with pork scratchings. P, pancreas; L, liver. AB/PAS staining.

Results. The mean individual body weight of carp in groups I_K, $II_{S+Rz,}$ III_{S+L} and IV_R was significantly higher ($P < 0.05$) than in group V_{SK} (Tab. 1). Body weight of carp from groups $II_{S+Rz,}$ III_{S+L} and IV_R was significantly higher ($P < 0.05$) than in the control group, and in groups III_{S+L} and IV_R it was significantly higher than in group II_{S+Rz}. No significant differences were ascertained between groups III_{S+L} and IV_R.

Histological observations of the intestine and hepatopancreas in two-year-old carp on the last day of the experiment showed the normal development of both organs in all the feeding groups. The plicated intestine was lined with a regular columnar epithelium with nuclei located at the base of the cells. Between enterocytes, along the entire length of the intestine, there were mucous cells producing acid (carboxylated and sulphated) mucins.

Supranuclear enterocyte surfaces in the mid-intestine of carp fed Aller Classic standard pellets were filled with a large number of medium-sized absorptive vacuoles, indicating the accumulation of lipids (Fig. 1a). In fish receiving diets II_{S+Rz}, III_{S+L} and IV_R (Figs. 1b, c), no lipid vacuoles were found on supranuclear enterocyte surfaces. Lipid vacuoles of various size were observed in carp fed pellets with pork scratchings (Fig. 1d).

Statistical analysis showed significant ($P < 0.05$) differences between hepatocyte areas in different feeding groups (Tab. 2). The largest hepatocyte area and the largest cytoplasm area occupied by lipids (Fig. 2a) were found in carp receiving Aller Classic standard pellets (I_K), while the smallest area was found in fish fed pellets (V_{SK}) with pork scratchings (Fig. 2b).

In the other feeding groups, hepatocyte cytoplasm areas occupied by lipids were much larger than cytoplasm areas occupied by glycogen.

Exocrine pancreas cells were pyramid shaped and had clear cell nuclei, with PAS-positive acidophilic zymogen granules (pancreatic enzyme precursors) accumulated in the apical region of the cell. The accumulation of proenzyme granules was found to be similar in all the feeding groups (Figs. 2a, b).

The lack of lipid vacuoles on the supranuclear enterocyte surfaces of fish fed diets II_{S+Rz} and III_{S+L} shows that intracellular digestion and transport of lipids to the circulatory system took a normal course.

The presence of vacuoles on supranuclear enterocyte surfaces of fish receiving diets I_K and V_{SK} suggests the excessive absorption of lipids from the intestinal lumen (excess dietary fat) or disturbances in intracellular digestion (abnormal lipid metabolism).

The largest hepatocyte areas and the presence of lipids in cytoplasm indicated the accumulation of lipids in the liver of fish receiving standard pellets I_K.

In fish fed pellets with pork scratchings (V_{SK}), the presence of lipid vacuoles on supranuclear enterocyte surfaces is evidence of the slowed-down process of intracellular digestion and inhibited transport of lipids to the liver, as indicated by the smallest hepatocyte areas (Tab. 2, Fig. 2b). Disturbances in lipid metabolism were found in groups I_K and V_{SK}.

Discussion. Our recent study (Epler et al 2009) showed a significant effect of dietary oil supplements on reducing the proportion of SFA and increasing the proportion of UFA in carp. $C_{14:0}$ and $C_{16:0}$ decreased in the group of saturated acids, while in the group of unsaturated acids, some acids increased significantly ($P < 0.05$) according to fat supplement used. The use of a sunflower oil and rapeseed oil mixture significantly increased linoleic acid ($C_{18:2}$) n-6 and DHA ($C_{22:6}$) n-3, a sunflower oil and linseed oil mixture increased $C_{20:1}$ n-9, $C_{20:5}$ n-6, $C_{22:1}$ n-9, $C_{22:5}$ n-6 and $C_{22:6}$ n-3, whereas a fish oil supplement increased the level of $C_{18:2}$ n-6 and $C_{18:3}$ n-3 in carp meat.

Changes in the fatty acid content of carp meat fat increased the SFA to UFA ratio, which from the physiological point of view is favourable for animals and for consumers of this meat or other products. In the present study, the n-6 to n-3 ratio was lower than optimal, but this has no negative effect on the nutritive value of the product such as carp meat (Epler et al 2009).

Lipids are transported to the liver by the circulatory system in the form of lipoprotein complexes of chylomicrons and very low density lipoproteins (VLDL), present as small vacuoles. Lipid vacuoles in the enterocytes may serve as a temporary storage

form of re-esterified fatty acids that are accumulated when fatty acid uptake exceeds exporting capacity of enterocytes (Fontagne et al 1998).

Histological analysis clearly showed that after feeding diets I_K and V_{SK}, supranuclear enterocyte surfaces in the mid-intestine of carp were filled with lipid vacuoles, indicating the excessive absorption of fats from the intestinal lumen or abnormal lipid metabolism resulting from disturbances in intracellular digestion. Accumulation of lipids on the supranuclear enterocyte surfaces was the consequence of reduction in reacylation and the rate of lipoprotein synthesis of absorbed lipids (Caballero et al 2002).

The lack of lipid vacuoles in the same cells in fish receiving diets II_{S+Rz}, III_{S+L+} and IV_R indicates that intracellular digestion and transport of lipids to the circulatory system were normal. The changes of intestine cell structure as a response to the diet were also found in other fish species (Fontagne et al 1998; Olsen et al 1999; Caballero et al 2002).

Lipid metabolism is regulated mainly by the liver, both by the synthesis and degradation of fatty acids. The enzymes regulating these pathways showed variable affinity to different fatty acids available in the organ (Kiessling & Kiessling 1993; Henderson 1996). The lack of the equilibrium in dietary fatty acids may modify the function and morphology of the organ. The liver is the main reservoir of the energy, often in the form of the fat fractions such as triacylglycerols (TGs) (Kaushik 1997). In the case of the high lipid content of the diet or during the deposition of energy excess in the form of fat in the liver, the morphological changes and the accumulation of the vacuoles containing TGs in the hepatocytes may occur.

The quality of diets used in carp feeding is also reflected in the measurements of hepatocyte areas, which were the largest in group I_K (this, along with the presence of lipids suggests the accumulation of lipids in the liver), and the smallest in group V_{SK} (indicating the inhibited transport of lipids from the intestine to the liver).

Lower dimension of the hepatocytes shows the limited accumulation of lipids and glycogen in the liver. According to Margulies (1993) the changes in the hepatocytes, which show the suppression of lipid vacuole formation indicate the starvation of fish or incorrect feeding.

These phenomena show disturbances in fat metabolism, which were not observed in groups II_{S+Rz}, III_{S+L+} and $IV_{R.}$

The mean weight gains of fish at the end of the experiment, which were the lowest in groups V_{SK} (185 g) and I_K (230 g), were fully confirmed by histological analysis, which showed that the lowest gains in biomass and mean individual weight were due to digestive disturbances associated mainly with lipid metabolism. These disturbances did not occur in groups II_{S+Rz}, III_{S+L} and IV_R.

Histological analysis suggested disturbances in lipid metabolism for diets I_K and V_{SK}. When $II_{S+Rz,}$ III_{S+L} and IV_R diets were fed, digestion was normal.

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Footnote. The authors have no conflicts of interests.

Table 1

Body weight and weight gains of carp in different groups with an initial individual weight of 160 g

Group	Initial stock (head)	Final stock (head)	Initial weight of stock (g)	Final weight of stock (g)	Men individual weight (g)	Mean individual weight gain (g)*	Survival (%)
I_K	12	8	1920	3120	390	230[a]	66.6
II_{S+Rz}	12	10	1920	4400	440	280[b]	83.3
III_{S+L}	12	10	1920	4680	468	308[cd]	83.3
IV_R	12	10	1920	4690	469	309[d]	83.3
V_{SK}	12	8	1920	2760	345	185[e]	66.6

* Values with different letter are significantly different ($P < 0.05$)

Table 2

Mean hepatocyte area (n = 200) in different feeding groups

Group	Feeding	Mean hepatocyte area (μm^2)*
I_K	Standard pellets	276±70[a]
II_{S+Rz}	Pellets with a mixture of sunflower and rapeseed oils (50% : 50%)	180.5±48[bc]
III_{S+L}	Pellets with a mixture of sunflower and linseed oils (80% : 20%)	187±44[bc]
IV_R	Pellets with fish oil	190±40[b]
V_{SK}	Pellets with pork scratchings	171±45[c]

* Arithmetic mean ± S.D.; values with different letter are significantly different ($P < 0.005$)

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