Biologia (2021) 76:3769–3779
https://doi.org/10.1007/s11756-021-00882-y

ORIGINAL ARTICLE

Effects of different feeds on growth performance parameters, histology of liver, distal intestine, and erythrocytes morphology of common carp (*Cyprinus carpio* L.)

Gavrilo Božić[1] · Božidar Rašković[1] · Marko Stanković[1] · Vesna Poleksić[1] · Zoran Marković[1]

Received: 1 September 2020 / Accepted: 3 August 2021 / Published online: 13 September 2021

Abstract

In the last 60 years, pond farming of common carp (*Cyprinus carpio*) in Europe has gradually intensified, which has increased the stocking density and amount of supplementary feed, resulting in higher yields. Additionally, compound feed has been introduced to semi-intensive farming to increase the growth of farmed fish. Feed quality affects growth directly through conversion efficiency and indirectly by influencing fish physiology. In the present study, juvenile common carp were grown in net cages in a fishpond for 80 days and fed the same amount of different supplementary feed: wheat, pelleted, or extruded feeds or fish relied only on the available natural food (control group). Growth, microanatomy of the liver and distal intestine, and morphology of erythrocytes were evaluated. Compound feed had beneficial effects on the growth and histological parameters compared to the control group and fish fed wheat. This included higher surface areas of erythrocytes and hepatocytes` nuclei, longer intestinal folds, and thicker *tunica muscularis* in the distal intestine. The study showed that increased growth and use of compound feeds did not cause any physiological trade-offs that would be depicted in quantitative histomorphometry, and there was an absence of histopathological changes.

Keywords Pelleted feed · Extruded feed · Wheat · Internal organs · Semi-intensive production

Introduction

Common carp (*Cyprinus carpio* L., 1758) is one of the most important aquaculture species in the world and the most important in Central and Eastern Europe (Horváth et al. 2002; Roy et al. 2020). According to the FAO (2018), it was ranked third, with 4.56 million tons being produced yearly. Its aquaculture is spread worldwide in tropical, subtropical, and temperate regions. Carp is produced in intensive, semi-intensive, and extensive production systems. Semi-intensive systems are dominantly used for carp production in Serbia as in the rest of the world (Marković 2010). Compound feeds are not commonly used in common carp aquaculture (Gyalog et al. 2011), as they are more expensive than cereals. The reason is its financial advantage: fish are fed naturally present food, and additional feeds are supplemented according to fish needs and the presence of natural food. This allows higher yields than those in extensive systems at lower production costs compared to the more intensive production systems. Intensive production systems are characterised by increased profit per unit area but have a decreased profit margin (Naylor et al. 2000).

Common carp are omnivorous fish, and in fish-ponds they dominantly consume macrozoobenthos, zooplankton, detritus, and plant seeds, which are naturally available food (Sibbing 1988; Chapman and Fernando 1994; García-Berthou 2001). Additional feeds used in semi-intensive carp aquaculture are cereals and formulated diets. Formulated diets are mixtures of ground cereals enriched with oils, proteins, and vitamins of various origins (Marković et al. 2016). Some of the widely used ingredients in formulated diets are of plant-derived materials, such as legume seeds, different types of oilseed cakes, leaf meals, leaf protein concentrates, and root tuber meals, which contain a large spectrum of antinutritional factors (Francis et al. 2001). These factors together with toxins that can develop in feeds and ingredients due to mishandling, which are anti-quality factors, interfere

✉ Gavrilo Božić
gajaradnimejl@gmail.com

[1] University of Belgrade - Faculty of Agriculture, Nemanjina 6, Zemun, 11080 Belgrade, Serbia

with food utilisation and negatively affect the health and production of animals (Makkar 1993).

The functional microanatomy of the liver and intestine represent a valuable tool for the assessment of the effects of different feeds on fish nutritional physiology (Rašković et al. 2011). Common carp liver consists of several lobes that are brownish in colour and closely adhered to other organs in viscera, such as the spleen, intestine, and the bladder (Farag et al. 2014). The main building blocks of the liver are hepatocytes, which are polygonal in shape with centrally located nuclei and prominent nucleoli. Their size is dependent on the amount of stored nutrients (Roberts 2012; Caballero et al. 1999) and is often correlated with feed type (Poleksić et al. 2014b). The distal intestine of fish morphologically resembles a simple tube with a wrinkled inner surface. It has distinctive layers, starting from the inner to the outer diameter cross-section: mucosa - mucosal layer containing inner epithelium, lamina propria (a cellular connective tissue), and muscularis mucosae; submucosa - connective tissue; two-layered muscle coat; ending with serosa (Mumford et al. 2007; Genten 2009).

Red blood cell morphology is routinely used in fish physiological surveys, particularly in toxicology (Witeska 2013). Erythrocytes in fish are produced mainly in the spleen and head kidney (Homechaudhuri and Jha 2001). From these organs, immature erythrocytes are released into the bloodstream, where they mature to erythrocytes. Immature erythrocytes are irregularly round to oval, polychromatic, and smaller thanmature erythrocytes, which appear normochromatic (Kondera 2011). An abundance of immature erythrocytes is an indirect measure of erythropoiesis activity in fish (Rios et al. 2005). A reduction in the erythrocyte turnover rate may be a result of disease, malnutrition, or long periods of starvation (Witeska 2015).

This study was designed to follow-up on our previous studies (Rašković el al. 2015, 2016a, b) on the effects of additional diets in the semi-intensive culture system on the growth performance and digestive system histology of the common carp. Unlike previous studies, this experiment was performed in cages placed in a single pond. In addition to previous experiments, a control group of fish fed naturally available food exclusively was included; together with hepatocytes and intestinal fold structure, the morphology of erythrocytes was also evaluated.

Materials and methods

Experimental animals and feeds

Carps used in the experiment originated from the same batch, were one year old, and grown in an earthen pond system at the Centre for Fisheries and Applied Hydrobiology (CEFAH), University of Belgrade, Faculty of Agriculture. No ethical approval was required in order to conduct the trial.

The extruded diet (E) used in the experiment was a commercially available, extruded complete mixture for growing one-year-old juvenile carp and for the intensive growth of two-year-old juvenile carp with 38 % protein (SOPROFISH 38/12 INTENSIVE EFFECT, Veterinary Institute Subotica, Subotica, Serbia). A pelleted feed (P) was obtained from a commercial producer (DTD Ribarstvo, Bački Jarak, Serbia). Wheat (W) was obtained from a local grocery store, and no additional preparations were performed prior to giving it to the fish. Fish feeds' chemical properties were analysed in the Institute of Chemistry, Technology and Metallurgy, Belgrade, Serbia, using accredited methods (Table 1).

Experimental design

The experiment was performed in a 0.216 m^3 (0.6 × 0.6 × 0.6 m) square shaped cages covered with a 1 cm^2 mesh net. The bottom of each cage was paved with a plastic sack to prevent food loss. A total of 12 cages were dipped in a 550 m^2 earthen pond alongside hanging bridge across the pond (Fig. 1). The distance between the nearest cage was approximately 30 cm and between those on opposing sides was approximately 1 m.

Preparation for the experiment included: (i) drying out the pond, disinfection with calcium carbonate (150 g m^{-2}), and three days later, the pond was refilled with water, originating from ground wells at a 125 m depth; (ii) two weeks after the pond was filled, 33 fish, with a total mass of 3.5 kg, were added to the pond to induce normal carp pond turbidity conditions by stirring up the sediment. The experiment started one week after adding the fish.

Carp were randomly chosen and sedated with clove oil. Then, the length, weight, and height of each fish was measured. Fish were randomly allocated in groups and placed in cages. Groups were formed in triplicates, randomly designed with equal initial weight of the fish. In total, 15 fish ($\bar{x}$ = 12.05 ± 0.09 g) were placed in each cage. Four different groups

Table 1 Chemical properties of used feeds

Chemical parameter	Wheat	Pelleted	Extruded
Proteins %	8.24	29.03	38.25
Water %	11.77	8.92	7.79
Total lipids %	3.75	13.43	11.20
Ashes %	1.37	7.60	8.03
Cellulose %	1.82	1.53	3.32
Dry matter %	88.23	91.08	92.21

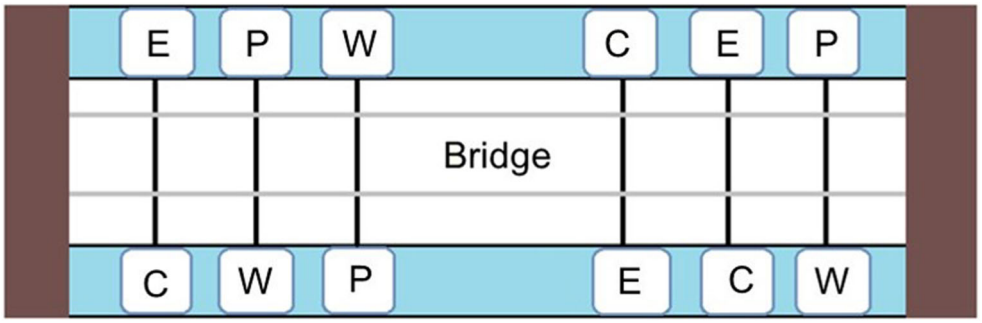

Fig. 1 Scheme of the experiment area. C, control group; W, wheat fed group; P, the group fed pelleted diet; E, group fed extruded diet; brown color, shore; blue color, water

were made: (I) control group (C) fed exclusively on natural feed from the pond; (II) group W fed wheat; (III) group P fed a pelleted diet; (IV) group E fed the extruded diet. The experiment lasted for 80 days from 13th August to 30th October 2016.

Feeding took place every day at 9:00, when fish in each cage were fed 3 % of their total biomass. Feed was dropped above each cage where it slowly sank towards the bottom of a cage. Besides added diets, naturally present invertebrates were able to roam into cages through the mesh net. Immediately after feeding, temperature, pH, conductivity, oxygen concentration, and oxygen saturation were measured using a MULTI 340i/SET apparatus (WTW, Weilheim in Oberbayern, Germany) (Table 2).

The feeding rate was adjusted every 15 days, when body weight, body height, and total length of fish were determined. From these parameters, body weight gain (BWG), specific growth rate (SGR), and conditional factor (CF) were calculated using Eqs. (1), (2) and (3), respectively:

$$\mathrm{BWG}\ (\mathrm{g}) = \text{final body mass}\ (\mathrm{g}) - \text{initial body mass}\ (\mathrm{g}) \tag{1}$$

$$\mathrm{SGR}\left(\%\mathrm{day}^{-1}\right) = \frac{\ln(\text{final body mass in g}) \times \ln(\text{initial body mass in g})}{\text{number of trial days}} \times 100 \tag{2}$$

$$\mathrm{CF}\left(\mathrm{gcm}^{-3}\right) = \frac{\mathrm{weight(g)}}{\text{total length}^3\left(\mathrm{cm}^3\right)} \tag{3}$$

Table 2 Mean values ± SD of physical and chemical parameters of water in the experimental pond

Parameters	Values
Temperature (°C)	18.16 ± 4.64
Oxygen concentration (mg L^{-1})	5.02 ± 1.77
Oxygen saturation (%)	52.42 ± 18.54
pH	8.23 ± 0.19
Conductivity (μS cm^{-2})	1897 ± 64.9

Histological sample preparation and evaluation

Histological samples were collected at the end of the experiment after fish were anesthetised in water containing several drops of clove oil and sacrificed by cervical transection and pithing. Three fish were randomly selected from each cage, for a total of 9 per treatment.

Blood was sampled before the fish were sacrificed by puncturing the caudal vein using a syringe. Blood smears were air dried and stained with Romanowsky stain using a Bio-Diff Kit (BioGnost, Zagreb, Croatia). From each fish, two blood smears were made and stained. Subsequently, 10 microscopic view fields using 400× magnification from each slide were randomly selected using ImageJ software. On each field, three erythrocytes were systematically chosen by superimposing the counting frame at the centre of the image and measuring. The measured parameters were the long and short axes and surface area of each erythrocyte. Circularity was calculated using the following formula:

$$\text{Circularity} = 4\pi \frac{\text{surface area}}{\text{perimeter}^2} \tag{4}$$

Each fish was dissected, and a portion of the liver and distal intestine were sampled and fixed in 4 % formalin solution (Lach-Ner, Czech Republic). After fixation, samples were subsequently dehydrated in an ethanol series and treated with an xylene in automatic tissue processor (TP 1020, Leica, Nussloch, Germany). Samples were then embedded in paraffin wax and sectioned with a sliding microtome SM 2000R (Leica) to 5–7 μm thickness. Sections were fixed on glass slides and stained with haematoxylin and eosin (HE). From each slide, 10 systematically chosen micrographs were taken at random using a 400× magnification. Calculation of the relative volume density was performed using the combination of unbiased counting frames and point counting-intercept methods proposed by Gundersen et al. (1988). The number of points hitting each tissue layer [lamina propria, goblet cells, tunica mucosa, and intraepithelial macrophages (IEM)] was divided by the total number of points present in the grid net (212 in total).

For every intestine cross-section slide, a series of micrographs were taken at 100× magnification. Micrographs were merged into a single cross-section using the MosaicJ plug-in in ImageJ software. The intestinal folds, intestinal muscle layer thickness, and whole cross-section diameter were observed (Fig. 2). Cross-section diameter values are represented by the mean length of $n = 10$ cross-sections for each intestine sample.

Liver samples were collected and processed in a tissue processor using the same histological protocol as intestine samples. Concerning liver slides, 15 microscopic fields per fish were taken using 400× magnification. From each field, three hepatocytes were randomly chosen for measurement, using the same methodology described previously. To avoid bias, only hepatocytes with a nucleolus in the focus were taken for evaluation (Rašković et al. 2019). Cell and nucleus surface area, nucleus circularity, and nucleus distance from the nearest cell membrane (LD) were evaluated for each hepatocyte.

All microscopic slides derived from samples were photographed using a Leica DM LS light microscope (Leica Camera AG, Wetzlar, Germany) with a DC 300 camera (Leica Camera AG). Images were analysed using ImageJ version 1.50e (Schneider et al. 2012).

Statistics

Data were tested for normality with the Shapiro-Wilk W test and analysed with one-way ANOVA and Mann-Whitney-U for non-parametric and Tukey`s pairwise test for parametric analyses. Statistical analyses were carried out using Past 3.17 (Øyvind Hammer, Natural History Museum, University of Oslo, Norway).

Results

Experimental conditions and fish growth

Water physical and chemical properties are shown in Table 2. From the beginning of the experiment in August to the end of the experiment in October, the mean water temperature dropped from 22 to 12 °C, and the mean oxygen concentration rose from 5.41 to 5.62 mg L^{-1}. This change in temperature affected the metabolism of fish and their growth rate, and it was best reflected in a steady drop of SGR throughout the experiment (Fig. 3). After 15 days of the experiment, significant differences in BWG and SGR were noted ($p < 0.05$). As time elapsed, the differences between groups became more prominent. Fish fed extruded and pelleted diets showed no differences throughout the experiment for all three parameters, except BWG in the last 20 days. Meanwhile, the control group and wheat-fed group showed significantly lower performance ($p < 0.05$). Until days 45–60 they expressed similar values for all measured growth parameters. Later, values of SGR and CF of the group fed with wheat became significantly higher than those of the control group ($p < 0.05$). Differences in CF prevailed after one month, where C and E had the lowest and highest values respectively, while the other two groups were positioned intermediately ($p < 0.05$). These differences became wider at the end of the experiment.

Histological evaluation

Histology results in some way correspond to results of fish growth performance, where groups fed pelleted and extruded diets, in most cases, outstands other two groups.

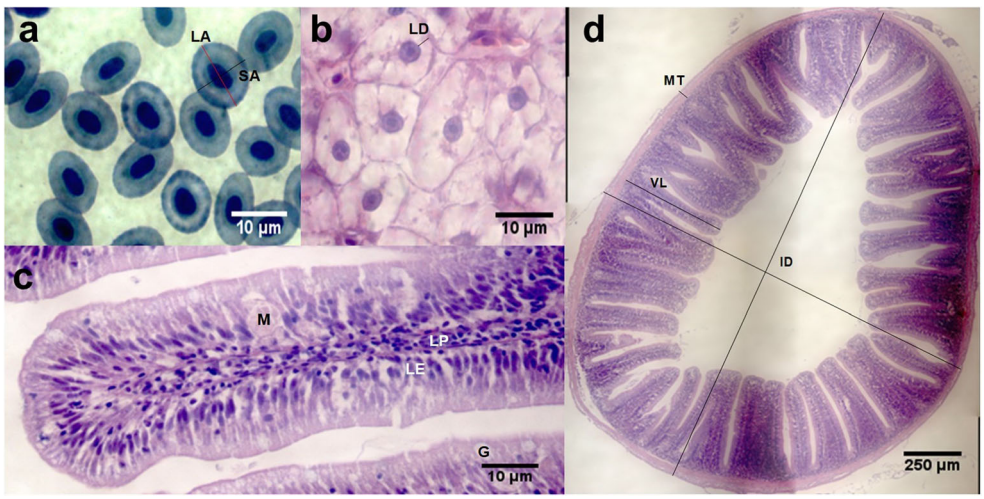

Fig. 2 **a** Erythrocytes from common carp blood smear; **b** hepatocytes from liver cross-section; **c** intestinal folds from intestine cross-section; **d** whole intestine cross-section. LA, longer axis; SA, shorter axis; LD, nucleus distance from the nearest cell membrane; M, intraepithelial macrophage; G, goblet cell; LE, lamina epithelialis; LP, lamina propria; ID, intestine diameter; VL, intestinal fold length; MT, muscle layer thickness

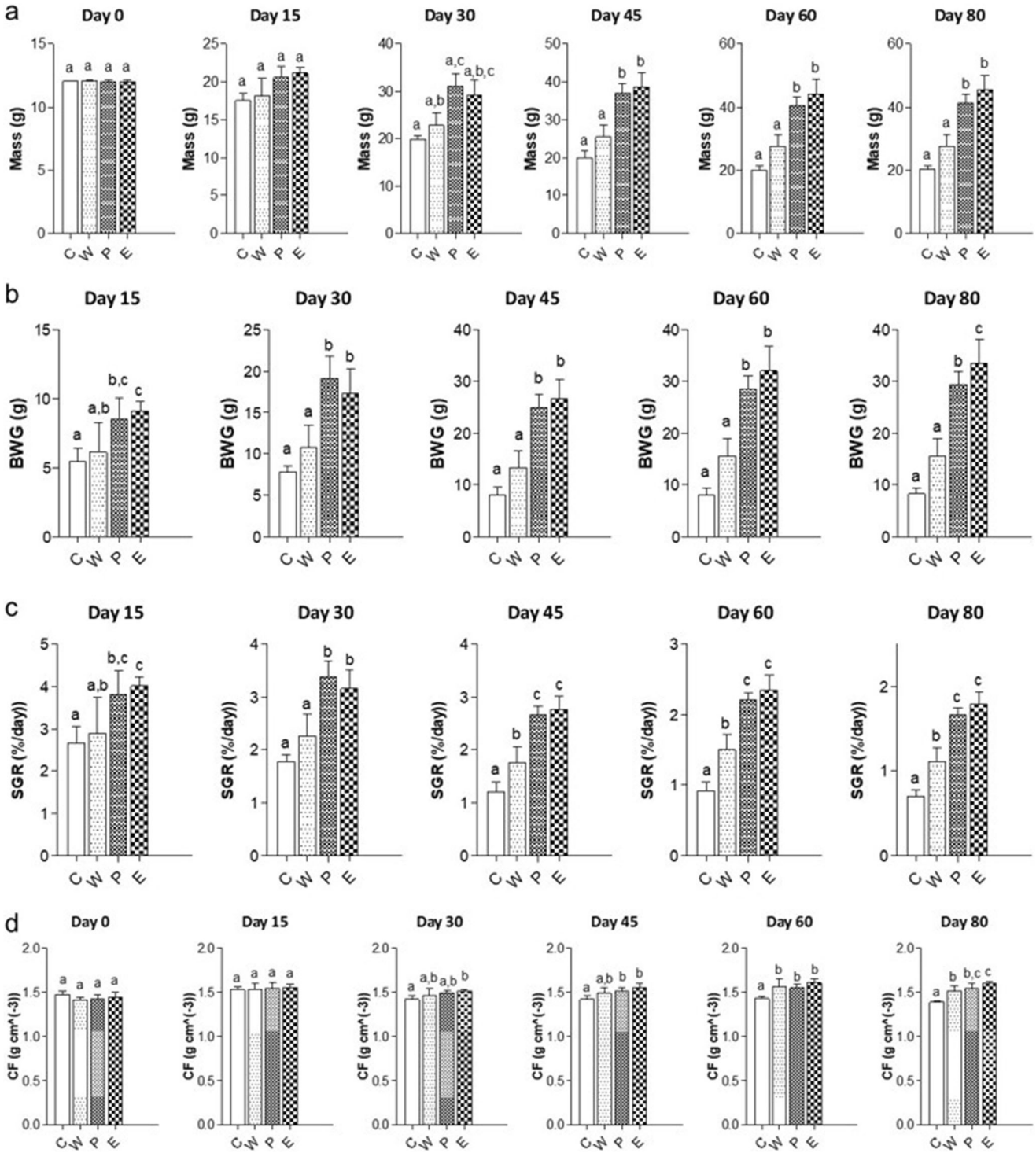

Fig. 3 Growth and fitness parameters dynamics measured at the end of every 15 days. **a** Body mass; **b** BWG, body weight gain; **c** SGR, specific growth rate; **d** CF, Fulton`s condition factor. All values are presented as means ± SD. ^{a,b,c} groups defined by statistically significant differences at $p < 0.05$. C, control group; W, wheat fed group; P, the group fed pelleted diet; E, group fed extruded diet

Erythrocytes morphology

Erythrocytes from fish in the control group had the smallest mean surface area and lowest degree of circularity; their shape was the furthest away from the ideal circle (ideal circle represents a value of 1). On the opposite side are erythrocytes from fish fed extruded diet, and in between erythrocytes from fish fed with wheat and pelleted diet ($p < 0.05$). The longer axis was a major contributor to erythrocyte surface area differences, compared to the shorter axis, which was

significantly larger only in E compared to the other groups (Table 3). Erythroblast count did not show any significant differences between groups.

Morphology of hepatocytes

Hepatocytes showed a similar trend compared to erythrocyte morphology. The smallest mean hepatocyte surface area was present in the control group compared to all other groups ($p < 0.05$). The similar trend was observable in hepatocyte nuclei; nuclei from the control group were shaped in an almost ideal circle with the smallest surface areas. The only discrepancy from this trend was noted in measurements of nuclei distance from the nearest cell membrane. Here, nuclei from W group had a significantly larger distance from the cell membrane, compared to the other groups (Table 3).

Intestine histological morphology

Cross-sections of fish intestines were similar in size, except for those from the control group, which had a 15–20 % shorter intestine diameter ($p < 0.05$). Mean intestinal muscle layer thickness was similar in length in C and W groups and was almost half the thickness of P and E groups ($p < 0.05$). Mean intestinal fold length showed an almost linear increase between groups, in the following order: E > P > W > C.

Volume density, which the lamina propria and goblet cells cover in the intestine cross-sections, was statistically the same in all groups. The tunica mucosa was thicker only in the E group, while IEM volume density differed. C group had the lowest number of macrophages. W had a higher number of macrophages, and P and E were similar (Table 4).

Discussion

Processed cereals, as well as pelleted and extruded formulated diets are well documented to have a high performance on fish growth (Booth et al. 2002; Przybyl and Mazurkiewicz 2004; Venou et al. 2009; Másílko et al. 2014; Ma et al. 2015; Hlaváč et al. 2015, 2016a, b; Marković et al. 2016). They are better balanced to meet carp feeding requirements compared to W and C. In our experiment, P and E diets differed slightly from each other, with a somewhat better performance of the extruded diet, namely higher BWG. In addition to the higher protein content in the extruded diet, there were two reasons that could explain this difference. During the extrusion process, the nutritional value of feed is increased due to the removal of heat-labile secondary compounds of plant-based feed components (Drew et al. 2007; Gatlin et al. 2007), which gives E a higher digestibility over P. The quality and quantity of lipids in feed had a profound effect on fish growth (Watanabe 1982). Various authors reported lower optimal values for common carp lipid requirements: 6 % (Abbass 2007), 8 % (Poleksić et al. 2014b), and 9 % (Ahmad et al. 2012). Thus, higher lipid content in P negatively influenced fish growth performance (Poleksić et al. 2014b). It seems that natural food was

Table 3 The intestine cross-section, erythrocytes and hepatocytes measurements.

Feature	Control	Wheat	Pelleted diet	Extruded diet
	Erythrocytes			
Longer axes (μm)	11.4 ± 0.4^{a}	$11.6\pm0.5^{a,b}$	11.8 ± 0.4^{b}	11.8 ± 0.5^{b}
Shorter axes (μm)	7.9 ± 0.3^{a}	8.2 ± 0.5^{a}	8.2 ± 0.4^{a}	8.5 ± 0.3^{b}
Surface area (μm^2)	72 ± 2^{a}	75 ± 5^{b}	$76\pm5^{b,c}$	79 ± 5^{c}
Circularity	0.83 ± 0.03^{a}	0.84 ± 0.02^{a}	$0.84\pm0.01^{a,b}$	0.85 ± 0.01^{b}
Erythroblasts count (%)	11.7±6.3	11.9±5.5	10.7±4.3	11.8±4.7
	Hepatocytes			
Cell surface area (μm^2)	204 ± 25^{a}	287 ± 27^{b}	280 ± 41^{b}	284 ± 22^{b}
Nucleus surface area (μm^2)	17.4 ± 1.6^{a}	18.8 ± 1.5^{a}	21.8 ± 0.8^{b}	21 ± 1^{b}
Nucleus/Cell surface ratio	0.09 ± 0.01^{a}	0.07 ± 0.01^{b}	0.09 ± 0.02^{a}	$0.08\pm0.01^{a,b}$
Nucleus circularity	0.896 ± 0.005^{a}	0.888 ± 0.004^{b}	0.886 ± 0.007^{b}	0.886 ± 0.006^{b}
Nucleus distance from the nearest cell membrane (μm)	2.2 ± 0.2^{a}	2.8 ± 0.3^{b}	2.3 ± 0.4^{a}	2.3 ± 0.6^{a}
	Intestine			
Whole cross-section diameter (μm)	2221 ± 255^{a}	2591 ± 392^{b}	2658 ± 234^{b}	2732 ± 265^{b}
Intestinal muscle layer thickness (μm)	24 ± 10^{a}	25 ± 5^{a}	41 ± 12^{b}	37 ± 16^{b}
Intestinal fold length (μm)	231 ± 37^{a}	292 ± 34^{b}	438 ± 90^{c}	524 ± 46^{d}

Values are presented as means ± SD; mean values followed by different superscript letters in the same row were significantly different ($p < 0.05$)

Table 4 Results of intestine stereological measurements

Tissue Element	Control	Wheat	Pelleted diet	Extruded diet
Lamina propria (%)	21.4 ± 3^{a}	$20.4\pm4^{a,b}$	$20.9\pm5^{a,b}$	17.9 ± 2^{b}
Goblet cells (%)	2.7 ± 0.3	2.6 ± 0.7	2.4 ± 0.7	2.5 ± 0.7
Lamina Eepithelialis (%)	75.6 ± 3^{a}	$76\pm5^{a,b}$	$76\pm5^{a,b}$	78 ± 3^{b}
Intraepithelial macrophages (%)	0.2 ± 0.1^{a}	0.6 ± 0.3^{b}	0.9 ± 0.4^{c}	1.2 ± 0.5^{c}

Presented values are mean percentages (%) of volume densities of each evaluated tissue element in intestine cross-sections; values are presented as means ± SD; mean values followed by different superscript letters in the same row were significantly different ($p < 0.05$)

sufficient to obtain similar growth in the control group (C) as it was in the group that received wheat as an additional feed (W). There was no statistically significant difference between C and W groups in the total mass and BWG at the end of the experiment, but W had a slightly better SGR and CF. This difference can be explained by considering the reduced availability of zoobenthos, which is the preferred natural food of common carp (Rahman and Meyer 2009), which left fish in the C group mainly dependent on zooplankton as its sole food-source.

Fish that suffered a longer period of starvation tend to have significantly decreased erythropoiesis (Rios et al. 2005; Kondera et al. 2017). Immature erythrocytes in common carp are smaller in size and more round than mature erythrocytes (Witeska et al. 2010), but an opposing situation was noted. In the control group, circularity was the lowest. Similarly, mean cell size had the lowest circularity. Erythroblast frequency did not significantly differ among groups; with $\overline{x} = 11.5 \pm 5.2$ %, they were within the standard values for juvenile common carp (Kondera et al. 2019). These shifts in erythrocyte sizes and circularity can be explained by prolonged erythrocyte life and slowed erythropoiesis in starving fish (Rios et al. 2005; Pronina and Revyakin 2015). On the contrary, the normal erythroblast frequency may represent haemolytic anaemia followed by higher erythropoietic activity in the malnutrition fish (Jain 1993). Judging from the growth performance, fish from the C group were not starving, but we can assume they suffered malnutrition to some lower extent. Their food intake was irregular and less diverse compared to other groups, as it was dependent only on invertebrates that entered cages.

The surface area of hepatocyte nuclei cross-sections were used as indicators of nutritive physiology in fish feeding experiments. This was demonstrated in other experiments where the same feeds were tested on common carp (Rašković et al. 2016a, b). Reduction in hepatocyte size is another indicator of malnutrition in group C, as Rios et al. (2007) and Park (2018) described it as a feature of fish that underwent starvation.

Poleksić et al. (2014b) showed that hepatocyte nuclei size was altered in feeds that contained a fat content higher than 8 %. In general, smaller nuclei in hepatocytes indicate reduced metabolic activity (Rios et al. 2007), which is supported with studies of fish malnutrition or starvation (Strüssmann and Takashima 1990; Margulies 1993).

The nucleus distance from the nearest point of the cell membrane was used as a direct measurement of nuclear displacement, which is an indicator of lipid reserves building up in hepatocytes (Caballero et al. 2004). The only significant higher nuclear distance was observed in hepatocytes from the W group, meaning that nuclei in this group were more centrally positioned, which implicates that built up reserves were lower compared to other groups. This is followed by the lowest nucleus/cell surface ratio coupled with the highest mean cell size in W. At the end of this experiment, we analysed for the presence of fat in the liver. Samples were frozen in liquid nitrogen, cut on a cryotome, stained with oil-red O staining, and examined under the microscope. All samples were negative on lipids. This follows our previous results, where fat disappeared from carp livers in the autumn season (Rašković et al. 2016a). In addition, there were no hydropic degeneration present in the hepatocytes; therefore, we can conclude that the build-up of reserves were most likely glycogen.

Intestine morphology can be modulated by feed ingredients; in that manner, intestine morphology reflects its capability for food digestion and nutrient utilisation (Baeverfjord 1996; Klurfeld 1999; Khodadadi et al. 2018). The length of intestinal folds was positively correlated with nutrient absorption, therefore affecting fish growth (Farhangi and Carter 2001; Zhou et al. 2010). Bakhshi et al. (2018) reported intestinal fold shortening in common carp fed on biofloc with the addition of sugar and corn starch. Rašković et al. (2015, 2016a) observed a positive correlation between common carp body weight and intestinal fold length. Intestinal fold length was E > P > W > C, but the intestinal fold length, together with other size-related features, should be compared only between C and W groups and P and E groups. The reason is that there were no statistically significant differences in mean body mass of fish from C compared to the W group and P compared to the E group. Higher intestinal fold length and higher whole section diameter were observed in fish fed W compared to C

group. In addition, there were no differences between these two groups for the following features: muscle layer thickness, lamina propria, and lamina epithelialis volume densities. When compared, E and P groups had a similar trend. There were no differences in whole section diameter size, muscle layer thickness, lamina propria, and lamina epithelialis volume densities, but intestinal fold length was significantly higher in the E group. Here, we hypothesised that fish from the C group to some extent experienced atrophy of the alimentary tract (smallest intestine diameter and fold length together with reduced muscle layer thickness) due to malnutrition. The rationale for this claim is that despite common carp is primary benthivore fish, in the control group it still could meet some of its nutritional needs by grazing on zooplankton. Lack of mortality increment between groups supports claim that fish did not starve, but malnutrition was possible. In addition, fish experience proteolysis of the intestinal mucosa in the short-term (Ostaszewska et al. 2006) and decrease their body mass in long-term starvation (Rios et al. 2002; Rios et al. 2006), which was not the case in the present study. In terms of histological structure, natural feed is the optimal choice for fish feeding, which was proven in several laboratory trials (Kamaszewski and Ostaszewska 2014; Ostaszewska et al. 2018). However, if fish are fed unsuitable compound feed, it would also show severe pathologies in both the liver and intestine, and as a result, fish would experience atrophy of the intestinal tissue (Kasprzak et al. 2019).

Macrophages are the first line of defence in the fish innate immune system, as they phagocytise foreign particles (Ellis et al. 1976). The highest number of IEM in fish fed an extruded diet corresponds to data reported by Poleksić et al. (2014a). The macrophage number increases in fish intestines, starting from the control group, which may indicate that additional feeds contained an increased number of particles that may be identified as antigens by the fish immune system. These particles may originate from chemicals used in feed production, such as pesticides, or from storage, as protein rich feeds are more prone to the development of microorganisms. In addition, formulated feeds contain soybean meal. Soy contains anti-nutritive factors that are known to induce distal intestine inflammation in fish (Urán et al. 2008). This inflammatory response is characterised by the presence of immune cells common for both a specific and non-specific immune response (Krogdahl et al. 2010).

Shortening of the intestinal folds, together with lamina propria thickening and leucocytes infiltration in the lamina propria and submucosa, are some of the symptoms of distal intestine alterations (Heikkinen et al. 2006; Refstie et al. 2000, 2001). An increase in the number of IEM alone is not enough to claim intestine inflammation but may bear some concerns for a health risk.

Conclusions

Additional feeds have a substantial influence on fish nutritional physiology. Judging from the histological effects on common carp liver and intestine, morphology of erythrocytes, and growth parameters, the extruded feed may be the best choice, but it raises some concerns over the fish physiological status due to the higher number of IEM. In contrast, wheat had no significant effect on the increase of IEM, but all other parameters were low. Growing carp in a cage system is a rare practice and it relies on additional feeds. We can conclude that if common carp feeds on naturally occurring zooplankton, this can contribute to its growth, but without additional feeding on benthos organisms or given feed, fish will probably suffer from malnutrition issues. In conclusion, feeding 3 % of the total mass with pelleted feed daily may be the best choice for common carp yearlings, judging from the perspective of growth performance and fish welfare. In addition to feed price and growth performance, different additional feeds may have different effects on the distal intestine, liver, and red blood cells, which can be important when it comes to feed choice in carp nutrition. More long-term feeding trials are necessary to draw more accurate conclusions on feed choice in carp nutrition.

Acknowledgements We express special thanks to Dalibor Vukojević and Aleksandar Perić who helped with experiment conduction and sample collection.

Author contributions Gavrilo Božić conducted experiment. Gavrilo Božić, Marko Stanković and Božidar Rašković performed sampling, and samples preparation. Gavrilo Božić and Božidar Rašković analyzed samples and results, and drafted manuscript. Zoran Marković and Vesna Poleksić supervised experiment, advised research and proofread manuscript.

Funding This work was supported by the agreement between University of Belgrade - Faculty of Agriculture and Ministry of Education, Science and Technological Development of Serbia [grant number 451-03-9/2021-14/ 200116].

Data Availability The data that support the findings of this study are available from the corresponding author, [G.B.], upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Abbass FE (2007) Effect of dietary oil sources and levels on growth, feed utilization and whole-body chemical composition of common carp, *Cyprinus carpio* L. fingerlings. J Fish Aquat Sci 2:140–148

Ahmad M, Qureshi TA, Singh AB, Manohar S, Borana K, Chalko SR (2012) Effect of dietary protein, lipid and carbohydrate contents on the growth, feed efficiency and carcass composition of *Cyprinus carpio* communis fingerlings. Int J Fish Aquac 4:30–40. https://doi.org/10.5897/IJFA11.080

Baeverfjord G, Krogdahl Å (1996) Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: A comparison with the intestines of fasted fish. J Fish Dis 19:375–387. https://doi.org/10.1046/j.1365-2761.1996.d01-92.x

Bakhshi F, Najdegerami EH, Manaffar R, Tokmechi A, Rahmani Farah K, Shalizar Jalali A (2018) Growth performance, haematology, antioxidant status, immune response and histology of common carp (*Cyprinus carpio* L.) fed biofloc grown on different carbon sources. Aquac Res 49:393–403. https://doi.org/10.1111/are.13469

Booth MA, Allan GL, Evans AJ, Gleeson VP (2002) Effects of steam pelleting or extrusion on digestibility and performance of silver perch *Bidyanus bidyanus* Aquac Res 33:1163–1173. https://doi.org/10.1046/j.1365-2109.2002.00773.x

Caballero MJ, López-Calero G, Socorro J, Roo FJ, Izquierdo MS, Férnandez AJ (1999) Combined effect of lipid level and fish meal quality on liver histology of gilthead seabream (*Sparus aurata*). Aquaculture 179:277–290. https://doi.org/10.1016/S0044-8486(99)00165-9

Caballero MJ, Izquierdo MS, Kjørsvik E, Fernandez AJ, Rosenlund G (2004) Histological alterations in the liver of sea bream, *Sparus aurata* L., caused by short- or long-term feeding with vegetable oils. recovery of normal morphology after feeding fish oil as the sole lipid source. J Fish Dis 27:531–541. https://doi.org/10.1111/j.1365-2761.2004.00572.x

Chapman G, Fernando CH (1994) The diets and related aspects of feeding of Nile tilapia (*Oreochromis niloticus* L.) and common carp (*Cyprinus carpio* L.) in lowland rice fields in Northeast Thailand. Aquaculture 123:281–307. https://doi.org/10.1016/0044-8486(94)90066-3

Drew MD, Borgeson TL, Thiessen DL (2007) A review of processing of feed ingredients to enhance diet digestibility in finfish. Anim Feed Sci Technol 138:118–136. https://doi.org/10.1016/j.anifeedsci.2007.06.019

Ellis AE, Munroe ALS, Roberts RJ (1976) Defence mechanisms in fish: 1. A study of the phagocytic system and the fate of intraperitoneally injected particulate material in the plaice (*Pleuronectes platessa* L.). J Fish Biol 8:67–78. https://doi.org/10.1111/j.1095-8649.1976.tb03908.x

FAO (2018) Fishery and aquaculture statistics. Global aquaculture production 1950–2016 (FishstatJ). In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 2018. www.fao.org/fishery/statistics/software/fishstatj/en

Farag FMM, Wally YR, Daghash SM, Ibrahim AM (2014) Some gross morphological studies on the internal anatomy of the scaled common carp fish (*Cyprinus carpio*) in Egypt. J Vet Anat 7:15–29

Farhangi M, Carter CG (2001) Growth, physiological and immunological responses of rainbow trout (*Oncorhynchus mykiss*) to different dietary inclusion levels of dehulled lupin (*Lupinus angustifolius*). Aquac Res 32:329–340. https://doi.org/10.1046/j.1355-557x.2001.00044.x

Francis G, Makkar HP, Becker K (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199:197–227. https://doi.org/10.1016/S0044-8486(01)00526-9

García-Berthou E (2001) Size-and depth-dependent variation in habitat and diet of the common carp (*Cyprinus carpio*). Aquat Sci 63:466–476. https://doi.org/10.1007/s00027-001-8045-6

Gatlin DM III, Barrows FT, Brown P, Dabrowski K, Gaylord TG, Hardy RW, Herman E, Hu G, Krogdahl Å, Nelson R, Overturf K (2007) Expanding the utilization of sustainable plant products in aquafeeds: A review. Aquac Res 38:551–579. https://doi.org/10.1111/j.1365-2109.2007.01704.x

Genten F (2009) Atlas of fish histology. CRC Press, Boca Raton. https://doi.org/10.1201/9780367803599

Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sørensen FB, Vesterby A, West MJ (1988) Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 96:379–394. https://doi.org/10.1111/j.1699-0463.1988.tb05320.x

Gyalog G, Váradi L, Gál D (2011) Is intensification a viable way for pond culture in Central and Eastern Europe? Aquac Aquar Conserv Legis 4:584–589

Heikkinen J, Vielma J, Kemiläinen O, Tiirola M, Eskelinen P, Kiuru T, Navia-Paldanius D, von Wright A (2006) Effects of soybean meal based diet on growth performance, gut histopathology and intestinal microbiota of juvenile rainbow trout (*Oncorhynchus mykiss*). Aquaculture 261:259–268. https://doi.org/10.1016/j.aquaculture.2006.07.012

Hlaváč D, Másílko J, Hartman P, Bláha M, Pechar L, Anton-Pardo M, Adámek Z (2015) Effects of common carp (*Cyprinus carpio* Linnaeus, 1758) supplementary feeding with modified cereals on pond water quality and nutrient budget. J Appl Ichthyol 31:30–37. https://doi.org/10.1111/jai.12850

Hlaváč D, Másílko J, Anton-Pardo M, Hartman P, Regenda J, Vejsada P, Mráz J, Adámek Z (2016) Compound feeds and cereals as potential tools for improved carp *Cyprinus carpio* production. Aquac Environ Interact 8:647–657. https://doi.org/10.3354/aei00206

Hlaváč D, Anton-Pardo M, Másílko J, Hartman P, Regenda J, Vejsada P, Baxa M, Pechar L, Valentová O, Všetičková L, Drozd B (2016) Supplementary feeding with thermally treated cereals in common carp (*Cyprinus carpio* L.) pond farming and its effects on water quality, nutrient budget and zooplankton and zoobenthos assemblages. Aquac Int 24:1681–1697. https://doi.org/10.1007/s10499-016-0059-0

Homechaudhuri S, Jha A (2001) A technique to evaluate the erythropoietic efficiency in fish. Asian Fish Sci 14:453–455

Horváth L, Tamás G, Seagrave C (2002) Carp and pond culture. 2nd ed. Blackwell Science, Oxford

Kamaszewski M, Ostaszewska T (2014) The effect of feeding on morphological changes in intestine of pike-perch (*Sander lucioperca* L.). Aquac Int 22:245–258

Kasprzak R, Ostaszewska T, Kamaszewski M (2019) Effects of feeding commercial diets on the development of juvenile crucian carp *Carassius carassius*: Digestive tract abnormalities. Aquat Biol 28:159–173

Khodadadi M, Abbasi N, Adorian TJ, Farsani HG, Hedayati A, Hoseini SM (2018) Growth performance, survival, body composition, hematological parameters, intestinal histomorphology, and digestive enzymes' activity in juvenile rainbow trout (*Oncorhynchus mykiss*) fed dietary Immunogen®. J Appl Aquac 30:174–186. https://doi.org/10.1080/10454438.2017.1420515

Jain NC (1993) Essentials of Veterinary Hematology. Lea and Febiger, Philadelphia, pp. 76–250. ISBN-10: 081211437X

Klurfeld DM (1999) Nutritional regulation of gastrointestinal growth. Front Biosci 4:D299–D302. https://doi.org/10.2741/klurfeld

Kondera E (2011) Haematopoiesis in the head kidney of common carp (*Cyprinus carpio* L.): A morphological study. Fish Physiol Biochem 37:355–362. https://doi.org/10.1007/s10695-010-9432-5

Kondera E, Kościuszko A, Dmowska A, Witeska M (2017) Haematological and haematopoietic effects of feeding different diets and starvation in common carp *Cyprinus carpio* L. J Appl Anim Res 45:623–628. https://doi.org/10.1080/09712119.2016.1251926

Kondera E, Witeska M, Ługowska K (2019) Annual changes in hematological parameters of common carp juveniles under laboratory

conditions. Anim Sci Warsaw 58:143. https://doi.org/10.22630/AAS.2019.58.2.15

Krogdahl Å, Penn M, Thorsen J, Refstie S, Bakke AM (2010) Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquac Res 41(3):333–344. https://doi.org/10.1111/j.1365-2109.2009.02426.x

Ma F, Li X, Li B, Leng X (2015) Effects of extruded and pelleted diets with differing protein levels on growth and nutrient retention of tilapia, *Oreochromis niloticus* × *O. aureus* Aquac Int 23:1341–1356. https://doi.org/10.1007/s10499-015-9888-5

Makkar HPS (1993) Antinutritional factors in foods for livestock. In: Gill M, Owen E, Pollot GE, Lawrence TLJ (eds) Animal Production in Developing Countries. Occasional publication No. 16. British Society of Animal Production, pp 69–85

Margulies D (1993) Assessment of the nutritional condition of larval and early juvenile tuna and Spanish mackerel (Pisces: Scombridae) in the Panama Bight. Mar Biol 115:317–330. https://doi.org/10.1007/BF00346350

Marković Z, Stanković M, Rašković B, Dulić Z, Živić I, Poleksić V (2016) Comparative analysis of using cereal grains and compound feed in semi-intensive common carp pond production. Aquac Int 24:1699–1723. https://doi.org/10.1007/s10499-016-0076-z

Másílko J, Hartvich P, Rost M, Urbánek M, Hlaváč D, Dvořák P (2014) Potential for improvement of common carp production efficiency by mechanical processing of cereal diet. Turk J Fish Aquat Sci 14:143–153. https://doi.org/10.4194/1303-2712-v14_1_16

Mumford S, Heidel J, Smith C, Morrison J, MacConnell B, Blazer V (2007) Fish histology and histopathology, US Fish and Wildlife Services (USFWS). National Conservation Training Center (NCTC), Shepherdstown

Naylor RL, Goldburg RJ, Primavera JH, Kautsky N, Beveridge MC, Clay J, Folke C, Lubchenco J, Mooney H, Troell M (2000) Effect of aquaculture on world fish supplies. Nature 405:1017. https://doi.org/10.1038/35016500

Ostaszewska T, Korwin-Kossakowski M, Wolnicki J (2006) Morphological changes of digestive structures in starved tench *Tinca tinca* (L.) juveniles. Aquac Int 14:113–126

Ostaszewska T, Krajnik K, Adamek-Urbańska D, Kasprzak R, Rzepkowska M, Luczynski M, Karczewska AT, Dabrowski K (2018) Effect of feeding strategy on digestive tract morphology and physiology of lake whitefish (*Coregonus lavaretus*). Aquaculture 497:32–41

Park, I.-S. (2018) Effect of starvation on the weight and structure in some tissues of cyprinid loach, *Misgurnus anguillicaudatus*. J. Fish. Mar. Sci. Edu., 30(4), 1170-1181. https://doi.org/10.13000/JFMSE.2018.08.30.4.117

Poleksić V, Stanković M, Marković Z, Relić R, Lakić N, Dulić Z, Rašković B (2014b) Morphological and physiological evaluation of common carp (*Cyprinus carpio* L., 1758) fed extruded compound feeds containing different fat levels. Aquac Int 22:289–298. https://doi.org/10.1007/s10499-013-9654-5

Pronina GI, Revyakin AO (2015) Changes of the morphophysiological parameters of Carp *Cyprinus carpio* at food limitation in aquaculture conditions. J Ichthyol 55:297–301. https://doi.org/10.1134/S0032945215020162

Przybyl A, Mazurkiewicz J (2004) Nutritive value of cereals in feeds for common carp (*Cyprinus carpio* L.). Czech J Anim Sci 49(7):307-314

Rahman MM, Meyer CG (2009) Effects of food type on diel behaviour of common carp *Cyprinus carpio* L. in simulated aquaculture pond conditions. J Fish Biol 74:2269–2278. https://doi.org/10.1093/jas/skab152

Rašković BS, Stanković MB, Marković ZZ, Poleksić VD (2011) Histological methods in the assessment of different feed effects on liver and intestine of fish. J Agric Sci Belgrade 56:87–100. https://doi.org/10.2298/JAS1101087R

Poleksić V, Marković Z, Spasić M, Vukojević D, Dulić Z, Stanković M, Rašković B (2014a) Intraepithelial macrophages in the distal intestine of common carp *Cyprinus carpio* L. fed different added feed in semiintensive system. Poster 410. In: Aquaculture Europe 2014, EAS, Donostia–San Sebastián, Spain. https://www.aquaeas.eu/images/stories/Meetings/AE2014/AE14pink10-7.pdf

Rašković B, Ćirić M, Dulić Z, Grubišić M, Spasić M, Koko V, Poleksić V (2015) Morphometrical study of intestinal folds of carp fed different added feed in semiintensive system. In: Conference Proceedings, 5. International Conference "Aquaculture & Fishery", Faculty of Agriculture, Belgrade-Zemun, Serbia, pp 491-496

Rašković B, Ćirić M, Koko V, Stanković M, Marković Z, Poleksić V (2016a) Effect of supplemental feeds on liver and intestine of common carp (*Cyprinus carpio*) in semi-intensive rearing system: Histological implications. Biologia 71:212–219. https://doi.org/10.1515/biolog-2016-0017

Rašković B, Čičovački S, Ćirić M, Marković Z, Poleksić V (2016b) Integrative approach of histopathology and histomorphometry of common carp (*Cyprinus carpio* L.) organs as a marker of general fish health state in pond culture. Aquac Res 47:3455–3463. https://doi.org/10.1111/are.12795

Rašković B, Cruzeiro C, Poleksić V, Rocha E (2019) Estimating volumes from common carp hepatocytes using design-based stereology and examining correlations with profile areas: Revisiting a nutritional assay and unveiling guidelines to microscopists. Microsc Res Tech 82:861–871. https://doi.org/10.1002/jemt.23228

Refstie S, Korsøen ØJ, Storebakken T, Baeverfjord G, Lein I, Roem AJ (2000) Differing nutritional responses to dietary soybean meal in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Aquaculture 190:49–63. https://doi.org/10.1016/S0044-8486(00)00382-3

Refstie S, Storebakken T, Baeverfjord G, Roem AJ (2001) Long-term protein and lipid growth of atlantic salmon (*Salmo salar*) fed diets with partial replacement of fish meal by soy protein products at medium or high lipid level. Aquaculture 193:91–106. https://doi.org/10.1016/S0044-8486(00)00473-7

Rios FS, Kalinin AL, Rantin FT (2002) The effects of long-term food deprivation on respiration and haematology of the neotropical fish *Hoplias malabaricus* J Fish Biol 61:85–95

Rios FS, Oba ET, Fernandes MN, Kalinin AL, Rantin FT (2005) Erythrocyte senescence and haematological changes induced by starvation in the Neotropical fish traíra, *Hoplias malabaricus* (Characiformes, Erythrinidae). Comp Biochem Physiol A Mol Integr Physiol 140:281–287. https://doi.org/10.1016/j.cbpb.2004.12.006

Rios FSA, Moraes G, Oba ET, Fernandes MN, Donatti L, Kalinin AL, Rantin FT (2006) Mobilization and recovery of energy stores in traíra, *Hoplias malabaricus* Bloch (Teleostei, Erythrinidae) during long-term starvation and after re-feeding. J Comp Physiol B 176:721–728

Rios FS, Donatti L, Fernandes MN, Kalinin AL, Rantin FT (2007) Liver histopathology and accumulation of melano-macrophage centres in *Hoplias malabaricus* after long-term food deprivation and re-feeding. J Fish Biol 71:1393–1406. https://doi.org/10.1111/j.1095-8649.2007.01604.x

Roberts RJ (ed) (2012) Fish pathology. Wiley, Hoboken

Roy K, Vrba J, Kaushik SJ, Mraz J (2020) Nutrient footprint and ecosystem services of carp production in European fishponds in contrast to EU crop and livestock sectors. J Clean Prod 270:122268

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675. https://doi.org/10.1038/nmeth.2089

Sibbing FA (1988) Specializations and limitations in the utilization of food resources by the carp, *Cyprinus carpio*: A study of oral food

processing. Environ Biol Fishes 22:161–178. https://doi.org/10.1007/BF00005379

Strüssmann CA, Takashima F (1990) Hepatocyte nuclear size and nutritional condition of larval pejerrey, *Odontesthes bonariensis* (Cuvier et Valenciennes). J Fish Biol 36:59–65. https://doi.org/10.1111/j.1095-8649.1990.tb03519.x

Urán PA, Gonçalves AA, Taverne-Thiele JJ, Schrama JW, Verreth JAJ, Rombout JHWM (2008) Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol 25:751–760. https://doi.org/10.1016/j.fsi.2008.02.013

Venou B, Alexis MN, Fountoulaki E, Haralabous J (2009) Performance factors, body composition and digestion characteristics of gilthead sea bream (*Sparus aurata*) fed pelleted or extruded diets. Aquac Nutr 15:390–401. https://doi.org/10.1111/j.1365-2095.2008.00603.x

Watanabe T, 1982. Lipid nutrition in fish. Comp Biochem Physiol B Comp Biochem 73:3–15. https://doi.org/10.1016/0305-0491(82)90196-1

Witeska M (2013) Erythrocytes in teleost fishes: A review. Zool Ecol 23:275–281. https://doi.org/10.1080/21658005.2013.846963

Witeska M (2015) Anemia in teleost fishes. Bull Eur Assoc Fish Pathol 35:148–160

Witeska M, Kondera E, Lipionoga J, Jastrzebska A (2010) Changes in oxygen consumption rate and red blood parameters in common carp *Cyprinus carpio* L. after acute copper and cadmium exposures. Fresenius Environ Bull 19:115–122

Zhou QC, Buentello JA, Gatlin DM III (2010) Effects of dietary prebiotics on growth performance, immune response and intestinal morphology of red drum (*Sciaenops ocellatus*). Aquaculture 309:253–257. https://doi.org/10.1016/j.aquaculture.2010.09.003