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Effects of feeding commercial diets on the development of juvenile crucian carp *Carassius carassius*: digestive tract abnormalities

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ABSTRACT: Wild-living populations of the crucian carp *Carassius carassius*, a cyprinid fish of European freshwaters, have decreased in recent years, likely due to increasing competition presented by invasive species. Several initiatives have been launched in an attempt to reintroduce this fish back into its natural habitat, but these approaches require the use of crucian carp fry reared under controlled conditions, and the use of popular and inexpensive commercial diets has been found to result in developmental abnormalities. The aim of the current study was to analyse the impact of feeding juvenile crucian carp either natural food (*Chironomidae* sp. larvae) or 2 commercial diets, with a focus on the intestinal development of these fish. Histological analysis revealed significant pathologies in the alimentary tracts of fish fed both commercial diets; these included lowered hepatocyte and enterocyte proliferation, as well as shortened intestinal folds. Furthermore, the digestive enzyme activity patterns suggested a physiological state close to malnutrition. This study confirmed that basic commercial diets (at least the 2 applied) are not recommended for the rearing of crucian carp juveniles, even though initial growth rates of the fish might suggest otherwise.

KEY WORDS: Commercial diets · Crucian carp · Intestinal abnormalities · Histology · Digestive enzymes

1. INTRODUCTION

The crucian carp *Carassius carassius* is a cyprinid species which was originally autochthonic to the freshwaters of central and northern parts of Europe and Asia (Sayer et al. 2011). In recent years, a sudden decline of natural *C. carassius* populations throughout Europe has been reported (Tarkan et al. 2009, Sayer et al. 2011). The main cause of this phenomenon appeared to be the coinciding growth of wild populations of the Prussian carp *Carassius gibelio* and the common goldfish *Carassius auratus*, both of which are considered invasive in this part of the world (De Giosa et al. 2014, Ribeiro et al. 2015). They occupy the same ecological niche as the crucian carp and thus constitute competition that this species has never faced before (Sayer et al. 2011). Additionally, they disrupt the genetic pool of *C. carassius* due to crossbreeding and are also able to reproduce via gynogenesis (Tarkan et al. 2012, Wouters et al. 2012). In aquaculture, hybrids of the crucian carp with the common carp *Cyprinus carpio* are valued, but they also are a threat to wild-living populations (Hänfling et al. 2005).

Ever since the total extinction of the crucian carp in European waters became a real threat, the demand for these fish reared in aquaculture has grown, and several reintroduction projects have been launched aiming to restore wild populations (Tarkan et al. 2009, 2016, Sayer et al. 2011). However, because of the expansion of invasive species, the aquaculture-reared crucian carps need to be in the best physical condition possible upon release, in order to have higher odds of survival and propagation and to with-

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stand the awaiting environmental challenges. Recently, angling organizations also revealed an increasing interest in the crucian carp, which has only added to the growing demand for cultivated *C. carassius* fry (Żarski et al. 2011, Demény et al. 2012). These 2 different demands (ecological and commercial) are the main reasons that an adequate breeding program should be developed for this species.

Rearing protocols for the crucian carp are yet to be refined, and the selection of a proper starting diet appears to be the most troublesome aspect. Although *C. carassius* larvae and juveniles fed natural food grow quickly and appear to be in a good physical condition (Żarski et al. 2011, Demény et al. 2012), such feeds always generate enormous costs for the producers when used on a large scale. The application of commercial diets would allow producers to overcome this issue, but crucian carp need to be fed natural food (*Artemia* nauplii) for at least 1 mo post-hatching before any dry diets may be introduced (Łączyńska et al. 2016), and feeding some of the popular commercial diets to *C. carassius* juveniles has been found to result in skeletal deformities (Myszkowski et al. 2002, Kasprzak et al. 2019). Other physiological and morphological aspects of juvenile development of this species, however, have not been studied or described.

The overall performance of fish passing early stages of ontogenesis can be assessed by studying both the physiology and morphology of the developing alimentary tract, as these are important indicators of the processes of digestion and absorption of nutrients (Izquierdo et al. 2000, Kamaszewski et al. 2010). One of the most effective and commonly acknowledged methods applied in studies on intestinal physiology of fish larvae or juveniles is the activity analysis of digestive and brush border enzymes (BBEs) (Zambonino-Infante & Cahu 2007), while histomorphometric measurements of enterocytes are helpful in the examination of intestinal morphology (Ostaszewska et al. 2005).

In this study, the development of 1 mo old crucian carp juveniles was examined after feeding them with popular, commercial dry feeds. Feeding experiments usually give precise information about the effects of the nutritional deficiency or overdose of a specific dietary ingredient because they involve the use of formulated diets. To date, however, only a handful of studies have more thoroughly described any of the developmental processes occurring during the early ontogenesis of crucian carp fed dry diets (Łączyńska et al. 2016), which is why 2 commercial feeds, both common in Polish aquaculture, were used in this study as a representation of rearing practices carried out by fish producers. *Chironomidae* larvae were chosen for the control feeding group to act as a representation of natural food, presumably obtained by these fish in the wild.

Activity analysis of digestive enzymes and BBEs was used for the comparison of intestinal development along with histomorphometric measurements of several parameters of the alimentary tract. This paper is a continuation of a previously published article (Kasprzak et al. 2019), which described skeletal deformations in these studied fish.

2. MATERIALS AND METHODS

2.1. Experimental set-up

Crucian carp larvae (3 d post hatching, dph) were stocked in nine 20 l tanks at an initial density of 20 larvae l^{-1}. The tanks were placed in a water recirculation system (water flow through the tanks was constant at 0.4 l min^{-1}) equipped with mechanical and biological filtration and UV irradiation. Water temperature (25 ± 0.5°C) and dissolved oxygen levels (8.3 ± 0.8 mg l^{-1}) were controlled at each feeding. The pH (8.0 ± 0.1; pH-meter: 3110 SET 2; WTW) and concentration of basic organic ions was measured once d^{-1}. Ion concentrations did not exceed the following values (ppm): 0.1 (NH_4^+), 10 (NO_3^-), 0.1 (NO_2^-) and 0.05 (PO_4^-; colorimetric tests: JBL Testlab). Stocking density and water parameters were chosen appropriately for the species (Żarski et al. 2011, Sikorska et al. 2018). Fish were exposed to light for 13 h d^{-1}. Tanks were cleaned twice d^{-1}; dead fish were removed during the maintenance of the tanks, and mortality values were calculated.

All larvae were fed *Artemia* sp. nauplii, ad libitum, 5 times d^{-1} (every 3 h) over the first 4 wk of rearing. After that, 30 sampled fish were weighed (mean body mass = 54.03 ± 13.63 [SD] mg; scale: WPS 60/C/10; Radwag) and measured (mean standard length = 13.31 ± 0.8 mm; caliper: Z22855; Milomex), and classified as the starting-point 'zero' group. The number of remaining fish was unified in each aquarium to account for minor early larval mortality, and the fish were divided into 3 experimental groups; each group was replicated in triplicate (stocking density = 18 larvae l^{-1}). Water parameters remained unchanged and were measured in the same way as before.

Fish in group *N* (natural food treatment) were fed frozen *Chironomidae* sp. larvae, representing a natural diet. Meanwhile, starter diets *X* or *Y* were applied

to experimental food treatment groups *X* or *Y*, respectively. According to their manufacturers, both commercial diets are suitable for many different fish groups, but only diet *X* was recommended for cyprinids (specifically, the common carp).

Feeding frequency remained as it was previously (every 3 h, 5 times d^{-1}). Feeding rations on Day 1 were 25% (group *N*) and 4% (groups *X* and *Y*) of the biomass. The portions were raised slightly each day to account for the nutritional demands of the growing fish. Fish samples were obtained after 2, 4 and 6 wk of the experiment. Body mass measurements after Weeks 2 and 4 were used for recalculating biomass to determine new feeding rations, following the pattern mentioned above and keeping the biomass percentage values unchanged. Intermediate body mass measurements also allowed for calculation of the specific growth rate (SGR, % d^{-1}; Ricker 1979), which was recorded separately for each of the three 2 wk long periods (0–2, 2–4 and 4–6 wk). Basic food composition was analysed according to AOAC methods, while the Ca, P and Mg content was measured with inductively coupled plasma-mass spectrometry (ICP-MS). Carbohydrates were calculated by subtracting the other 4 basic parameters from the dry matter (DM). All parameters are presented in Table 1.

2.2. Sample fixation and body content analysis

Sampled fish were anaesthetized with MS-222 at 4°C and then weighed and measured (mean of 30 fish $group^{-1}$). For whole-body content analysis, fish were frozen and stored at −80°C. For histology, fish were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (24 h, 4°C), flushed with distilled water and stored in 70% ethanol at 4°C. For enzymatic analyses, fish were frozen in liquid nitrogen and preserved at −80°C.

Table 1. Nutritional content of the experimental diets (*N*: natural food [*Chironomidae* sp. larvae]; *X* and *Y*: commercial starter diets) provided to juvenile crucian carp (g kg^{-1} dry matter [DM]). Diet DM: *N*: 163.3; *X*: 925.2; *Y*: 911.4 (g kg^{-1} of wet weight)

	Natural diet	Commercial diets	
	N	*X*	*Y*
Total protein	520.3	643.3	664.1
Total fat	18.0	119.7	101.9
Crude fiber	74.4	11.5	2.5
Carbohydrates	73.3	126.7	112.7
Ash	314.0	98.8	118.8
Ca	32.4	14.7	23.8
P	7.1	15.5	19.7
Mg	6.0	2.2	2.1

For the total body content analysis, the DM, ash, total protein and crude fat were analysed according to AOAC methods. All measurements were conducted in triplicate, in pooled samples (60 fish $group^{-1}$) taken after 6 wk of the trial.

2.3. Digestive tract analyses

Histological analysis of the digestive tract was conducted in non-decalcified fish taken after 6 wk (12 fish $group^{-1}$). They were dehydrated in ethanol, processed through xylene and finally embedded in paraffin, then cut longitudinally (6 µm) on a RM 2265 microtome (Leica Microsystems). General observations and histomorphometric measurements of the digestive tract were made on slides stained with either AB-PAS (AB pH = 2.5), hematoxylin & eosin or immunohistochemically with either a monoclonal mouse antibody directed against the proliferating cell nuclear antigen (PCNA) or a polyclonal rabbit anti-caspase-3 antibody (Bakke-McKellep et al. 2007; adapted to paraffin-embedded slides by Ostaszewska et al. 2008). Incubation with the primary antibody lasted either 1 h (anti-PCNA: dilution 1:300, Clone PC10; Dako) or overnight (anti-caspase-3: dilution 1:200, G7481; Promega), then for 30 min with the secondary polymer (EnVision+ System-HRP; Dako). Visualization with the use of DAB chromogen (peroxidase substrate) lasted 40 s. Slides incubated without the primary antibody were used as a negative control. Harris' hematoxylin was used as a counter-stain.

All histomorphometric measurements were done using the NIS-Elements AR Image Analysis System, with an ECLIPSE 90i microscope and DS5-UI camera (all elements: Nikon Corporation). A single field of view (FOV) area at 400× magnification was 35 000 $µm^2$. The following digestive tract parameters were measured: hepatocyte proliferation and apoptosis (in 60 FOVs $group^{-1}$; Ostaszewska et al. 2016; an example of hepatocyte apoptosis is shown in Fig. 1), nuclear, cellular and lipid area of hepatocytes, as well as nuclear, cellular and zymogenic area of exocrine pancreatic cells (each in 600 cells $group^{-1}$; Ostaszewska et al. 2005, Napora-Rutkowski et al. 2009, Kamaszewski et al. 2010), enterocyte proliferation (in 60 crypts $group^{-1}$; Bakke-McKellep et al. 2007), intestinal fold height (in 180 folds $group^{-1}$; Baeverfjord & Krogdahl 1996) and proportion of acidic, neutral and mixed mucous cells in the intes-

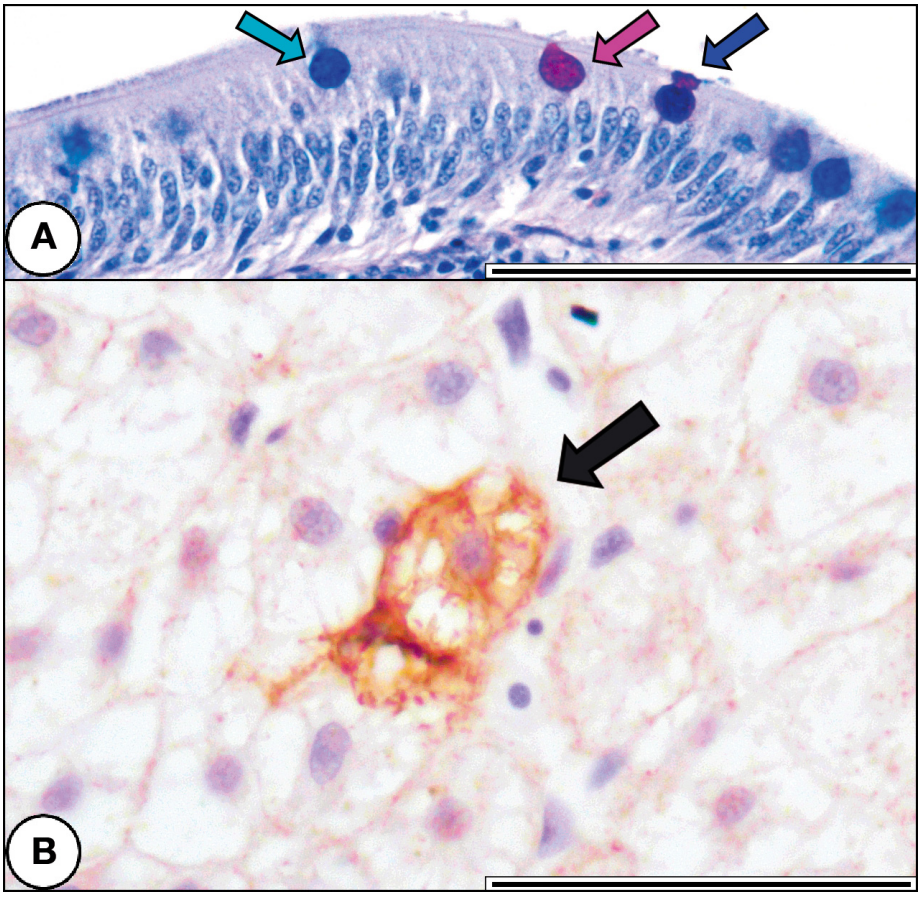

Fig. 1. Exemplary sections of (A) intestinal mucosa and (B) liver of a juvenile crucian carp from group *N* (fed a natural diet), after 6 wk of the feeding experiment. In (A), arrows indicate 3 different recognized and quantified types of mucous cells: acidic (light-blue), neutral (pink) and mixed (dark-blue); in (B), arrow indicates an apoptotic hepatocyte. AB-PAS stain (A) and immunohistochemical stain with anti-caspase-3 polyclonal antibody, counterstained with hematoxylin (B); scale = 100 µm

tinal epithelium (in 60 FOVs group^{-1}, calculated as the percentages of these 3 types of mucous cells separately for each specimen; cells were recognized as in Fig. 1).

For the enzymatic activity analysis, digestive tracts were obtained from rapid frozen fish (12 fish group^{-1} at every sampling point; total n = 120), then homogenised and subjected to spectrophotometric procedures. The following digestive enzymes and BBEs were examined: α-amylase (Foo & Bais 1998), lipase (Winkler & Stuckmann 1979), trypsin and chymotrypsin (Erlanger et al. 1961), acid phosphatase (ACP) (Abbott 1984), alkaline phosphatase (ALP) (Wenger et al. 1984) and leucine aminopeptidase (LAP) (Nagel et al. 1964). All enzymatic activity measurements were conducted at 25°C in triplicate and are presented as U g^{-1} of protein, the quotient of enzyme units (µmol of product min^{-1}) in 1 g of total protein content, which was determined according to Lowry et al. (1951). The activities of ALP and LAP are presented as indexes, with ACP used as the divisor (ALP / ACP and LAP / ACP, calculated for each sample separately). All absorbance measurements were done with a M501 spectrophotometer (Camspec).

2.4. Statistical analyses

Statistical analysis of the numeric data was performed using Statistica 12 (StatSoft). Either 1- or 2-way ANOVAs with Fisher's post hoc test were performed for all calculations, depending on whether the analysed data stemmed from one (final body mass, SGR, histomorphometry) or multiple (enzymatic analysis) time points. Differences were considered significant at $p < 0.05$ (SGR) or $p < 0.01$ (other measurements). Average values are displayed ±SD.

3. RESULTS

3.1. Basic parameters and body content analysis

Final survival rate, fish body mass, standard length and the results of whole-body content analysis are provided in Table 2. At the end of the experiment, the survival rate in group *Y* was significantly lower than in groups *N* and *X*. No statistically significant differences in body mass were recorded among the groups (even at $p < 0.05$), but the standard length of

Table 2. Survival, measurements and body content analysis of juvenile crucian carp after 6 wk of experimental treatment (*N*: natural diet; *X* and *Y*; commercial diet). Dry matter of the fish groups: *N* = 205; *X* = 208.3; *Y* = 195 (g kg^{-1} of wet weight). Superscript letters indicate significant differences among treatment groups (1-way ANOVA, $p < 0.01$). Means ± SD are shown

	Feeding treatment groups		
	N	*X*	*Y*
Survival (%)	$97.8^{B} \pm 1.4$	$96.6^{B} \pm 1.0$	$90.9^{A} \pm 2.0$
Mean body mass (mg)	$583.5^{A} \pm 280.5$	$620.2^{A} \pm 189$	$582.9^{A} \pm 156.1$
Mean standard length (mm)	$26.86^{A} \pm 3.63$	$27.12^{A} \pm 3.72$	$25.39^{B} \pm 2.01$
Body content analysis (g kg^{-1} of wet weight)			
Total protein	$147^{B} \pm 4.5$	$132.6^{A} \pm 0.7$	$129.4^{A} \pm 1.8$
Crude fat	$31.7^{A} \pm 1.3$	$55^{C} \pm 1.5$	$45.9^{B} \pm 1$
Ash	$2.3^{C} \pm 0.6$	$1.65^{B} \pm 0.4$	$1.43^{A} \pm 0.1$

fish from group *Y* was significantly lower than in the other 2 groups. In the whole-body content analysis, group *N* was characterised by significantly higher protein and ash values and a lower fat content than groups *X* and *Y*, while fish from group *X* had the significantly highest body fat values. SGR differed significantly among groups only during the first 2 wk period, where group *N* was characterised by a lower SGR ($p < 0.05$) than group *X* (Fig. 2). Further information about other basic fish body parameters is presented and discussed extensively in Kasprzak et al. (2019).

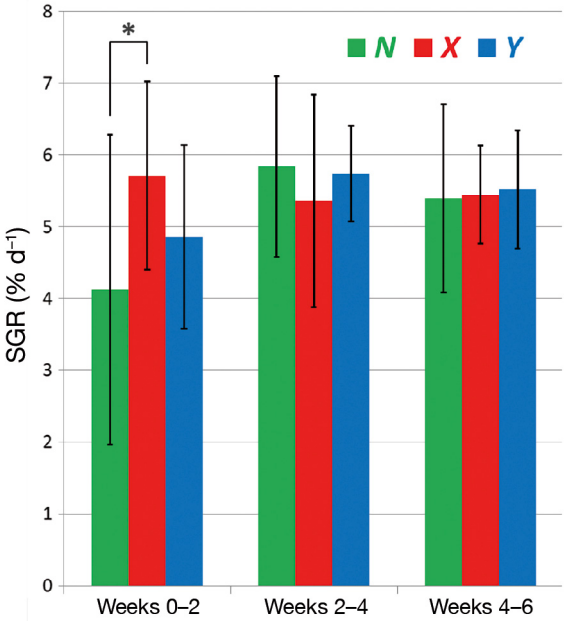

Fig. 2. Specific growth rates (SGR) of juvenile crucian carp during three 2 wk intervals of the experimental feeding treatment (*N*: natural diet group; *X* and *Y*; commercial diet groups; see Table 1); asterisk indicates significant difference among treatment groups (1-way ANOVA, $p < 0.05$). Error bars are ± SD

3.2. Histological analysis of the digestive tract

3.2.1. Intestinal epithelium

PCNA-positive enterocyte nuclei were found, only in the basal area of the mucosal folds, in all experimental groups (Fig. 3). Meanwhile, the anti-caspase staining did not provide satisfactory results and therefore apoptosis was not quantified in the intestine. Fish from group *N* had the significantly highest mucosal folds and the highest proliferation percentage of enterocytes. Group *N* also had different proportions of acidic and neutral mucous cells than groups *X* and *Y* (Table 3).

3.2.2. Liver parenchyma

Hepatocytes of fish from group *N* were characterised by a significantly higher nuclear area and proliferation than in the other 2 groups, and the significantly lowest lipid content of the cytoplasm (Table 3, Fig. 4). Group *X* had the lowest nuclear area, nucleo-cellular area index, proliferation and the highest lipid content, while hepatocytes in group *Y* had the smallest total cell area. Apoptotic hepatocytes were scarce and their number did not differ significantly among the groups.

3.2.3. Exocrine pancreas

A distinct trend was observed in the cells of fish from group *N*, as they were significantly larger, had larger nuclei and a larger nuclear-cytoplasmic index, as well as a larger zymogen-filled area than the cells of fish from groups *X* and *Y*. However, the zymogen-cytoplasmic index did not differ among any of the experimental groups (Table 3). In fish fed commercial diets, an overflow of adipose tissue (steatosis) among the tissues of the pancreas led to the dissipation of groups of exocrine secretory cells (Fig. 5).

3.2.4. Skin fat

During other observations of the longitudinal sections of fish, an excess of adipose tissue was noted in

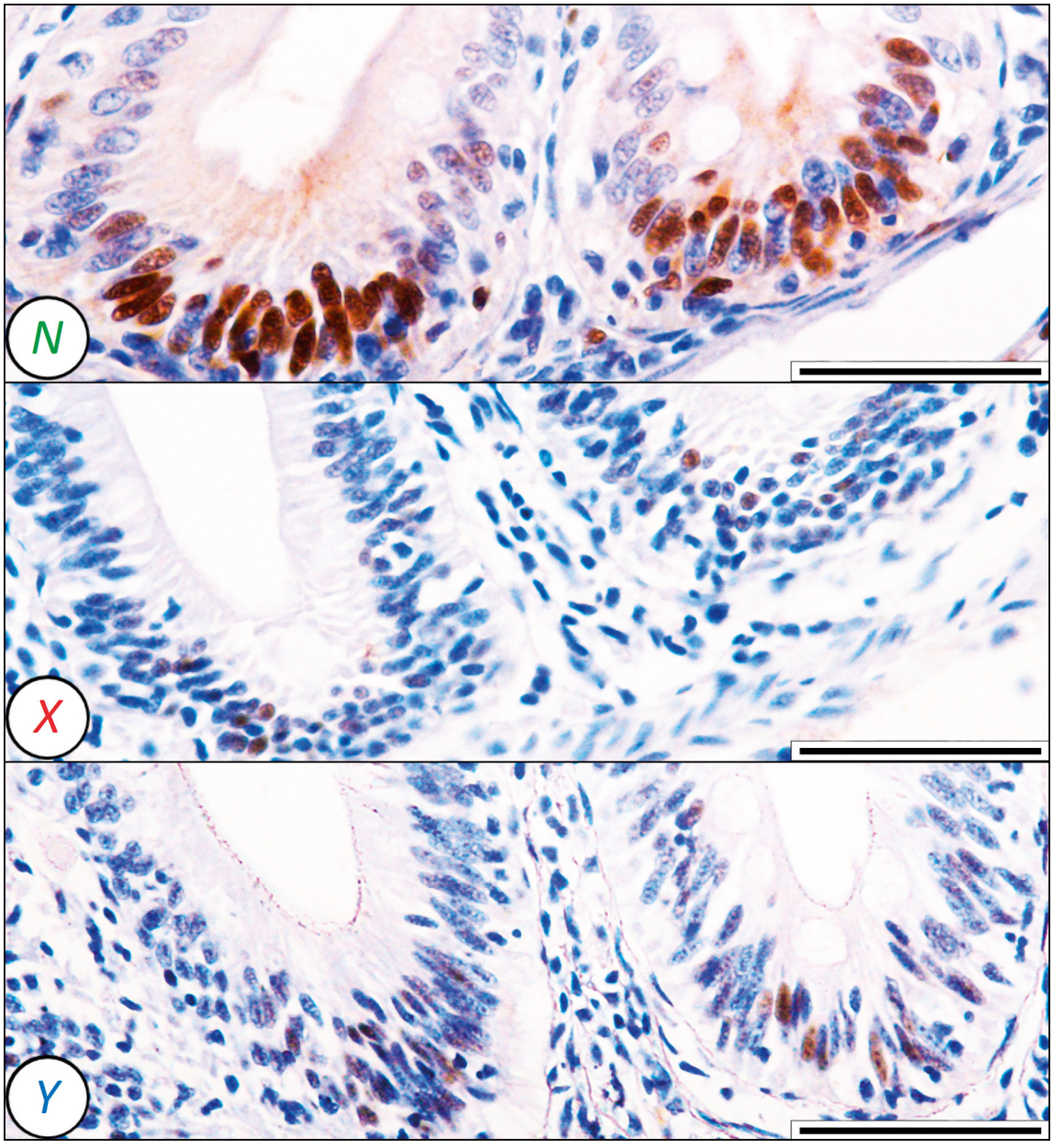

Fig. 3. Sections of intestinal folds of juvenile crucian carp after 6 wk of experimental treatment with natural food (*N*) or commercial diets (*X* and *Y*) (see Table 1). Proliferating cell nuclear antigen (PCNA)-positive nuclei of enterocytes are stained brown. Immunohistochemical stain with anti-PCNA monoclonal antibody, counterstained with hematoxylin; scale = 100 µm

the skin of fish from groups *X* and *Y*, on the bottom side of the abdominal cavity (Fig. 6).

3.3. Digestive enzyme activity analyses

The activities of amylase, lipase, trypsin and chymotrypsin are presented in Fig. 7. After 6 wk, the highest amylase activity was in group *Y*. The activity of lipase was significantly higher in *N* than in the other 2 groups. On the contrary, both trypsin and chymotrypsin revealed identical activity patterns, with both *X* and *Y* groups characterised by approximately 3 times higher results than in group *N*.

The 2 graphs presenting ALP and LAP are similar (Fig. 8), because the activity indexes did not change after 2 and 4 wk; however, after 6 wk one of the groups distinguished itself from the others with a significant activity growth. In the case of ALP it was the *X* group, while in LAP it was group *Y*.

Table 3. Histomorphometric measurements of the intestine, hepatocytes and exocrine pancreas cells of juvenile crucian carp after the 6 wk feeding experiment (*N*: natural diet; *X* and *Y*; commercial diet; see Table 1). Superscript letters indicate signifcant differences among treatment groups (1-way ANOVA [2-way ANOVA for quantity of mucous cells], $p < 0.01$). FOV: field of view

		Feeding treatment groups		
		N	*X*	*Y*
Intestine				
Height of folds (µm)		$276.97^{C} \pm 156.25$	$188.33^{A} \pm 74.14$	$231.59^{B} \pm 94.78$
Enterocyte proliferation (%)		$48.41^{C} \pm 13.34$	$33.27^{B} \pm 9.62$	$22.67^{A} \pm 11.72$
Quantity of mucous cells (%)	Acidic	$47.33^{B} \pm 23.73$	$34.54^{AB} \pm 17.35$	$33.78^{AB} \pm 13.81$
	Neutral	$19.61^{A} \pm 11.49$	$29.73^{AB} \pm 14.65$	$32.03^{AB} \pm 13.0$
	Mixed	$33.06^{AB} \pm 15.8$	$35.73^{AB} \pm 9.72$	$34.19^{AB} \pm 5.15$
Hepatocytes				
Nuclear area, S_n (µm^2)		$20.99^{C} \pm 5.14$	$14.41^{A} \pm 4.00$	$15.28^{B} \pm 3.80$
Total cell area, S_c (µm^2)		$307.05^{B} \pm 67.13$	$308.38^{B} \pm 91.65$	$230.35^{A} \pm 60.19$
Nucleo-cytoplasmic area index, $S_n \times (S_c - S_n)^{-1}$ (%)		$7.68^{B} \pm 2.47$	$5.18^{A} \pm 1.66$	$7.55^{B} \pm 2.57$
Cytoplasmic lipid area, S_l (µm^2)		$152.17^{B} \pm 69.09$	$188.94^{C} \pm 72.53$	$124.92^{A} \pm 49.70$
Lipid-cytoplasmic index, $S_l \times (S_c - S_n)^{-1}$ (%)		$52.53^{A} \pm 17.53$	$64.18^{C} \pm 14.06$	$57.90^{B} \pm 14.64$
Proliferation (%)		$29.61^{C} \pm 12.24$	$5.33^{A} \pm 5.89$	$12.12^{B} \pm 9.78$
Apoptosis (cells FOV^{-1})		$0.93^{A} \pm 1.72$	$1.07^{A} \pm 1.05$	$0.63^{A} \pm 0.85$
Exocrine pancreas cells				
Nuclear area, S_n (µm^2)		$11.30^{B} \pm 5.20$	$6.82^{A} \pm 4.22$	$6.51^{A} \pm 3.90$
Total cell area, S_c (µm^2)		$109.27^{B} \pm 42.91$	$89.12^{A} \pm 29.53$	$92.14^{A} \pm 37.28$
Nucleo-cytoplasmic area index, $S_n \times (S_c - S_n)^{-1}$ (%)		$12.17^{B} \pm 5.47$	$8.45^{A} \pm 4.54$	$7.82^{A} \pm 3.77$
Cytoplasmic zymogen area, S_z (µm^2)		$46.88^{B} \pm 26.22$	$39.36^{A} \pm 18.94$	$40.16^{A} \pm 24.51$
Zymogen-cytoplasmic index, $S_z \times (S_c - S_n)^{-1}$ (%)		$47.35^{A} \pm 13.63$	$47.62^{A} \pm 15.46$	$46.12^{A} \pm 14.45$

4. DISCUSSION

4.1. General remarks

The obvious diversity of the experimental feeding did not translate into any significant differences among the groups regarding the main production parameter — total body mass — and the SGR was almost identical after the first 2 wk period. Therefore, this study evaluated the small details and nuances in morphology and physiology of the digestive tract, to determine whether the tested diets had a predominantly positive or negative effect on the development of crucian carp. The aim was to examine if such knowledge could be extrapolated for a feeding period longer than the duration of this experiment.

Fish fed diets *X* and *Y* had a lesser total-body content of ash and protein, but this was likely offset by their higher fat content; thus, no differences in total body mass were found. However, it has already been determined that the lower ash content in fish fed the 2 commercial diets was caused by skeletal demineralization, and the shorter standard length and lower survival of fish in group *Y* was mostly related to skeletal deformities (Kasprzak et al. 2019). The results of whole-body content analysis are further discussed in Sections 4.2 and 4.3.

At the time of yolk sac absorption, the alimentary tract of many fish species, including the agastric cyprinids, is not fully developed (Dabrowski 1984). It was once thought that live food was essential for the development of fish larvae because this food source contains not only a balanced source of nutrients, but was also believed to be the origin of exogenous digestive enzymes which were needed to overcome early developmental deficiencies in the digestive tract (Lauff & Hofer 1984). The importance of this foreign enzymatic activity in larval ontogeny was later discounted (Cahu & Zambonino-Infante 2001). Nevertheless, live food does contribute to the course of digestion and absorption, likely due to specific nutrient fractions acting as activators, precursors or modulators of these processes (Kolkovski 2001, Zambonino-Infante & Cahu 2007), and therefore it is still utilised in rearing programmes for many fish species (Kolkovski et al. 2009). Furthermore, the use of regular commercial diets shortly after hatching may cause diverse developmental alterations, as severe as skeletal deformities (Boglione et al. 2013), but also a variety of physiological and morphological defects in the digestive organs (Ostaszewska et al. 2005). Fish larvae tend to have very different nutritional requirements to those demanded by juveniles (Cahu & Zambonino-Infante 2001), and it is very difficult to provide them with appropriately composed dry diets, which also need to be formulated separately for each species. In the case of *Carassius carassius*,

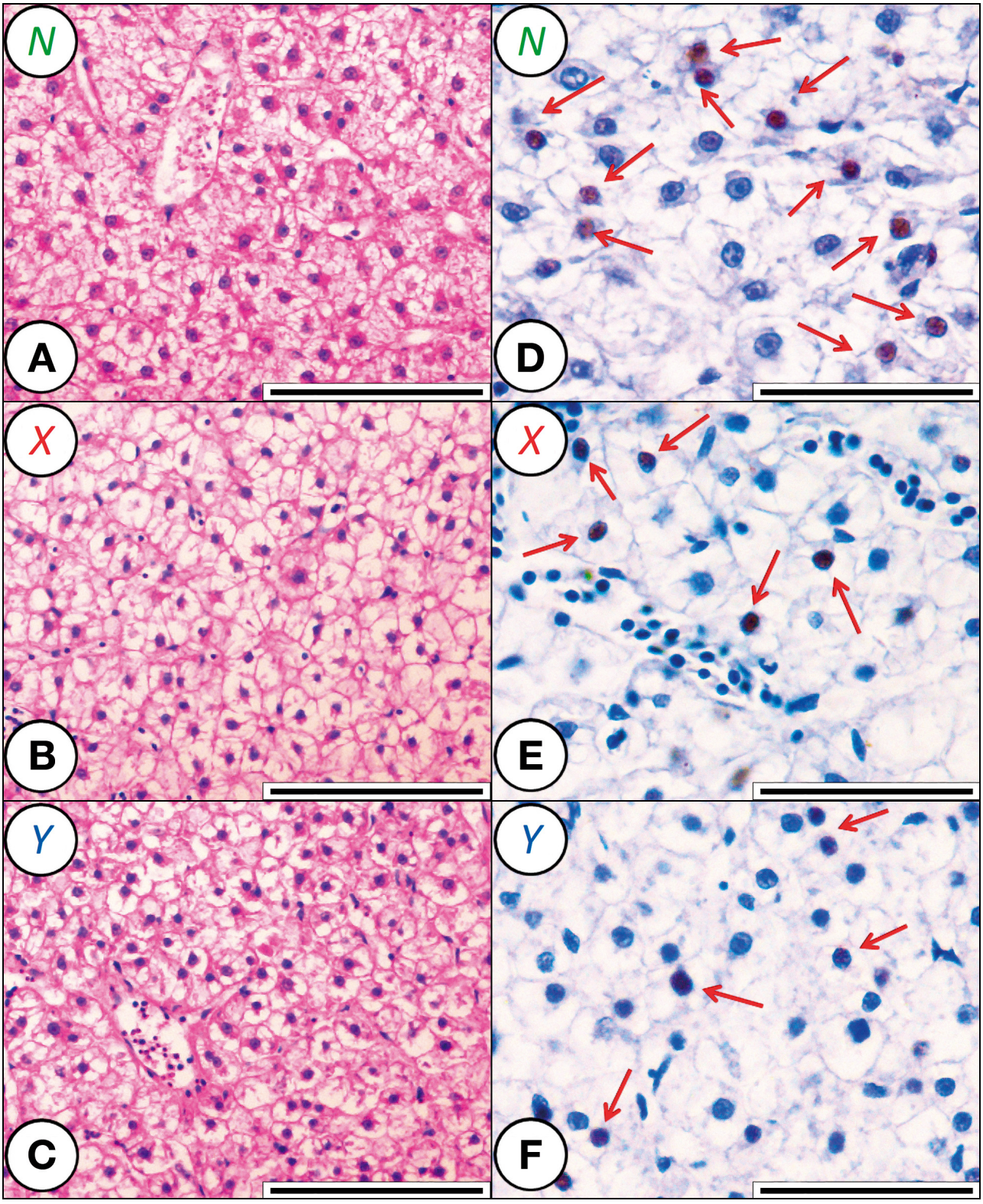

Fig. 4. Sections of juvenile crucian carp liver parenchyma, after 6 wk of experimental treatment with natural food (*N*) or commercial diets (*X* and *Y*) (see Table 1). Arrows indicate brown-stained proliferating cell nuclear antigen (PCNA)-positive hepatocyte nuclei. (A,B,C) H&E stain; scale = 100 µm. (D,E,F) Immunohistochemical stain with anti-PCNA monoclonal antibody, counterstained with hematoxylin; scale = 50 µm

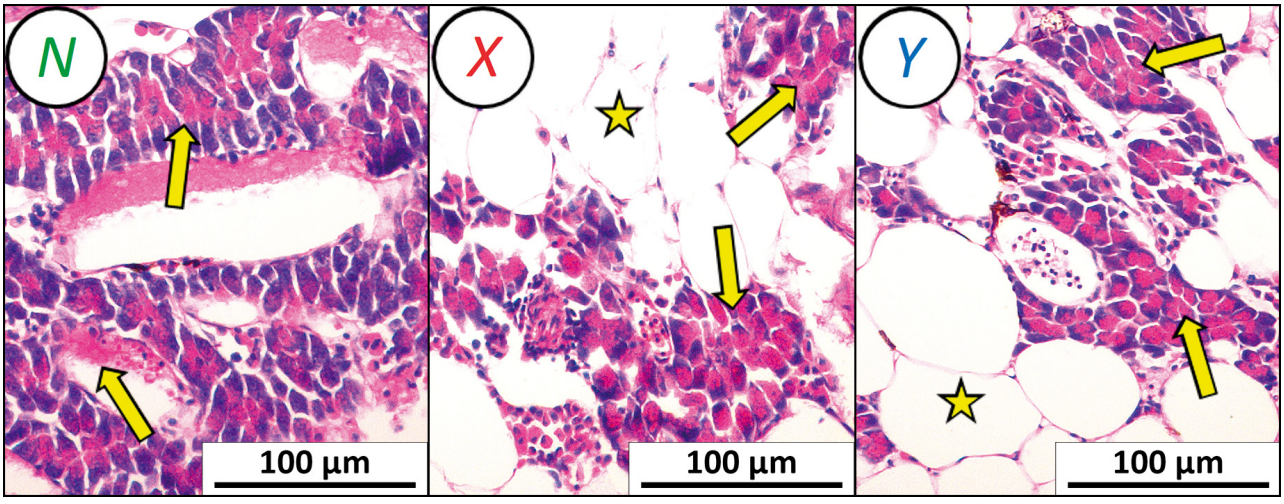

Fig. 5. Sections of the exocrine pancreas of juvenile crucian carp after 6 wk of experimental treatment with natural food (*N*) or commercial diets (*X* and *Y*) (see Table 1). Arrows: zymogen; stars: adipose tissue. H&E stain

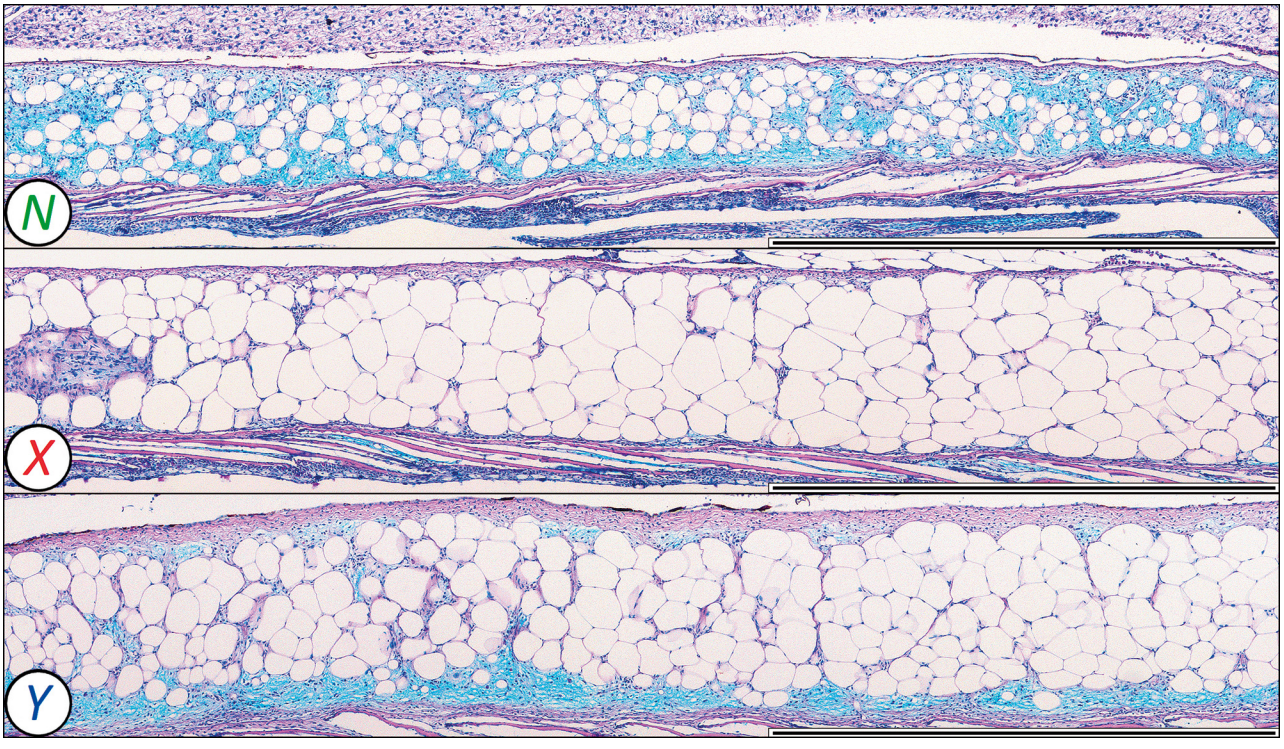

Fig. 6. Longitudinal sections of the skin fat on the bottom side of the abdominal cavity of juvenile crucian carp after 6 wk of experimental treatment with natural food (*N*) or commercial diets (*X* and *Y*) (see Table 1). AB-PAS stain; scale = 1 mm

the application of a preliminary, *Artemia*-based rearing period throughout (at least) the first month of life is obligatory to prevent high mortality and morbid growth (Demény et al. 2012, Łączyńska et al. 2016), but the development of the digestive tract has not been studied before in their early juvenile stage.

4.2. Histological analyses

Immunohistochemical detection of PCNA is a reliable indicator of cellular proliferation in fish (Ortego et al. 1994) and it has already been applied in several studies on *C. carassius* (Margotta et al. 2001, Alfei et al. 2004, Sollid et al. 2005). Lower proliferation in-

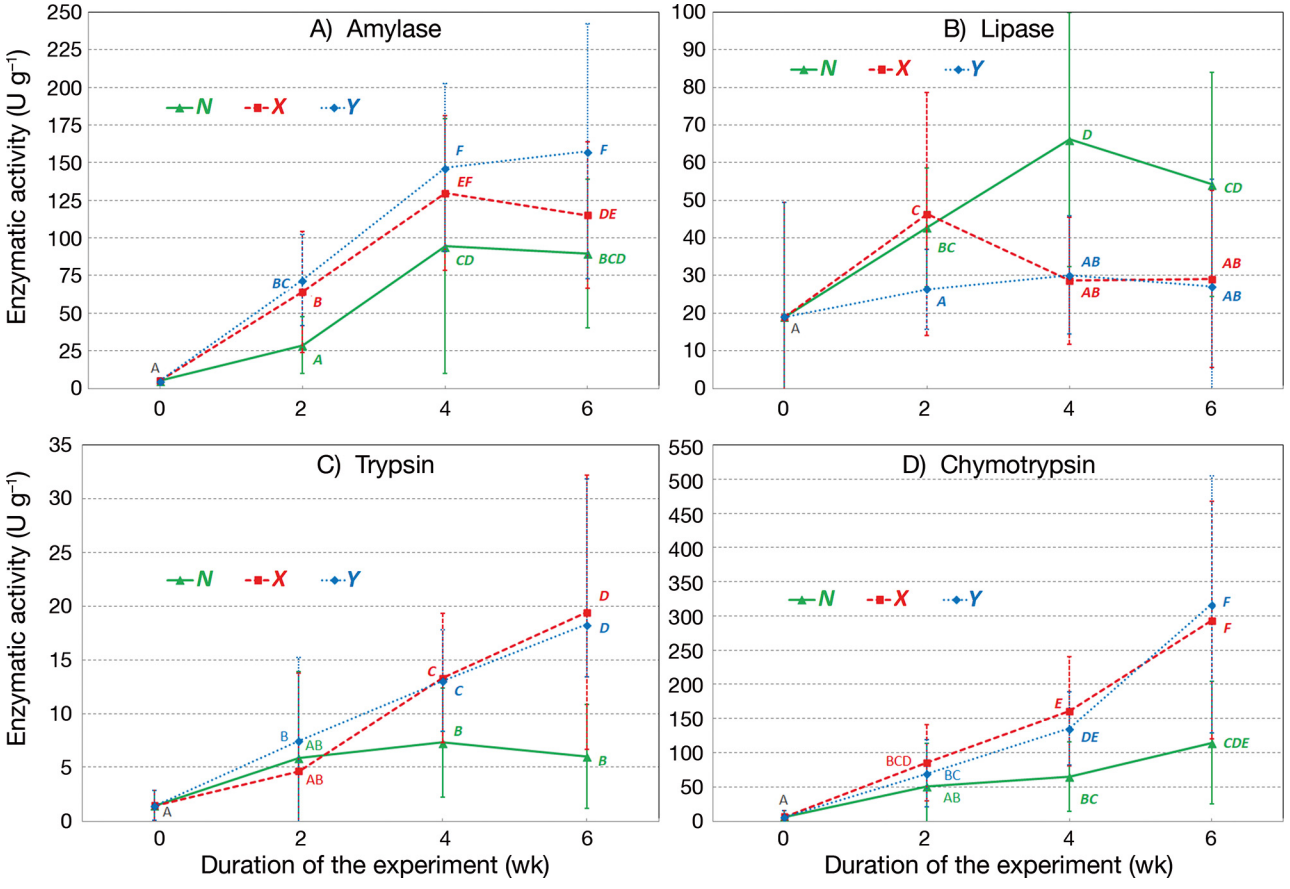

Fig. 7. Activities of (A) amylase, (B) lipase, (C) trypsin and (D) chymotrypsin throughout the feeding experiment with juvenile crucian carp (*N*: natural diet group; *X* and *Y*; commercial diet group; see Table 1); different letters reveal statistically significant differences among groups and/or sampling time points (2-way ANOVA, $p < 0.01$); **bold** and *italicized* letters indicate significant differences among the particular sampling time points

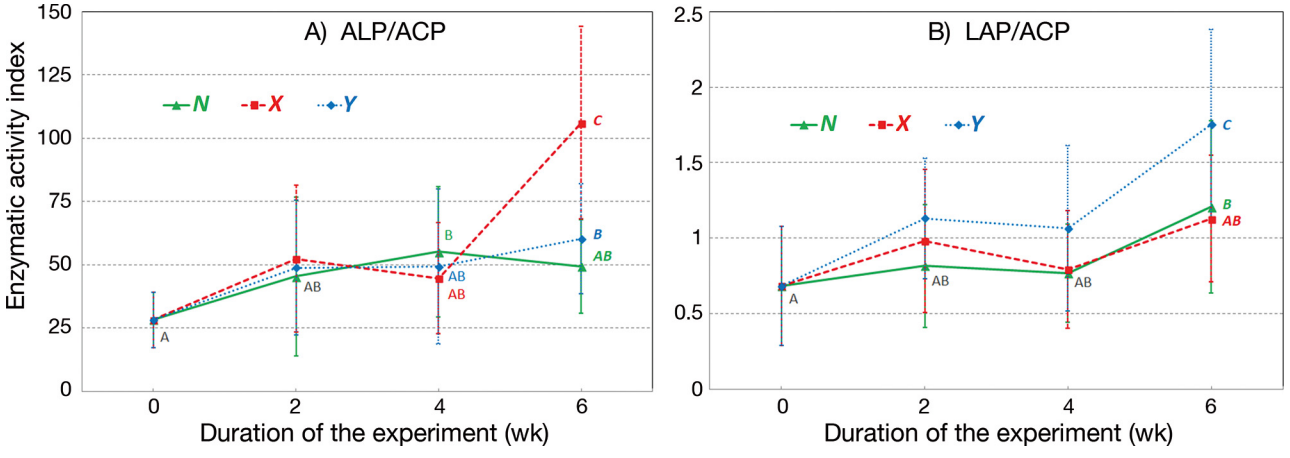

Fig. 8. Activity indexes of (A) alkaline phosphatase (ALP)/acid phosphatase (ACP) and (B) leucine aminopeptidase (LAP)/ACP throughout the feeding experiment with juvenile crucian carp (*N*: natural diet group; *X* and *Y*; commercial diet group; see Table 1); different letters reveal statistically significant differences among groups and/or sampling time points (2-way ANOVA, $p < 0.01$); **bold** and *italicized* letters indicate significant differences among the particular sampling time points

dexes are recognised as signs of impaired tissue development or toxicity, whether in the liver (Kraugerud et al. 2012, Ostaszewska et al. 2016) or muscles (Ostaszewska et al. 2008), but elevated cell division may also be linked to inflammation and damage repair processes in the intestine (Sanden et al. 2005) or liver (Machado et al. 2014).

Higher enterocyte proliferation and longer intestinal folds (as recorded here in fish from group *N*) are recognized as indicators of better nutritional condition in reared fish (Baeverfjord & Krogdahl 1996, Bakke-McKellep et al. 2007). Additional commentary about the mucosal folds is included in Section 4.3. Different proportions of acidic and neutral mucous cells recorded in fish fed commercial diets may indicate an abnormal secretion profile of the mucosa (Ostaszewska et al. 2010).

Overall hepatocyte area may reflect the amount of stored lipids and glycogen (Caballero et al. 1999) and such accumulation appears to be a reversible, non-pathological process (Castro et al. 2016). Meanwhile, the size of hepatocyte nuclei can be an indicator of their metabolic activity (Segner et al. 1988, Strüssmann & Takashima 1990); therefore, diminished nuclei (as in groups *X* and *Y*) may be markers of feeding inadequacies (Ostaszewska et al. 2005), although it should be noted that the nucleo-cellular index in group *Y* did not differ from group *N*. General observations of the hepatocytes, however, revealed that the nuclei in group *N* were usually round, while in groups *X* and *Y* other shapes occurred much more frequently. Furthermore, increased body fat levels, probably caused by impaired oxidative phosphorylation, are a common occurrence in phosphorus-deficient fish (Sugiura et al. 2004). Indeed, the cytoplasmic content of lipids in hepatocytes was higher in fish fed the 2 commercial diets than in fish from group *N*. Additionally, the lower hepatocyte proliferation in groups *X* and *Y* probably indicated that the development of the liver slowed down in these fish, especially when contrasted with the lack of differences in the numbers of apoptotic hepatocytes among the groups.

A similar principle regarding nuclear and cytoplasmic area could presumably also be applied to the exocrine pancreas cells, but only a handful of studies have shown the effects of malnutrition on the histological structure of this organ (Napora-Rutkowski et al. 2009). Furthermore, while extensive pancreatic steatosis (observed in *X* and *Y*) may lead to an impairment of proenzymatic production (Kamaszewski et al. 2010), the measurements did not show any differences regarding the proportion of cytoplasmic zymogen content, and the causes for this pathology are not known even in medical research (Smits & van Geenen 2011). Overall, it cannot be denied that the morphology of exocrine pancreas cells was visibly different between group *N* and groups *X* and *Y*.

Histomorphometrics revealed a distinct trend, as all of the parameters reached their highest values in group *N*, which may suggest a better overall condition of the digestive tracts of the fish in this group.

4.3. Enzymatic analyses

Amylase activity correlates with dietary carbohydrate content (Li et al. 2016), but high values of dietary lipids may impair the utilisation of these carbohydrates (Rueda-Jasso et al. 2004). In this study, amylase activity stabilised in all 3 groups after 4 wk, implying that the differences between groups were likely a result of an unspecified regulatory mechanism. Amylolytic activity in fish is still largely overlooked, but a number of studies have already shown the positive effects of carbohydrase supplementation (e.g. Castillo & Gatlin 2015).

Dietary lipids are extremely important in the early stages of fish growth (Izquierdo et al. 2000). Activity graphs of lipase were opposite to the dietary crude fat content, unsurprisingly, because it is the level and proportions of certain triglycerides that are recognised as modulators of lipolytic activity (Cahu & Zambonino-Infante 2001). Low lipase activity in fish fed dry food suggests that both diets comprise a fatty acid content which is unfavourable for juvenile crucian carp. Freshwater fish require more dietary *n*-6 than *n*-3 highly unsaturated fatty acids (HUFAs) (Kamler et al. 2008). Lastly, the much higher lipase activity in group *N* might relate directly to the higher hepatocyte volume in these fish due to an increased amount of processed lipids.

Lower trypsin and chymotrypsin activities in the fish fed natural food corresponded with the lesser total protein content of that diet, which is in accordance with various reports (Zambonino-Infante & Cahu 2007). However, the constant active increase of these enzymes throughout the experiment in fish fed both dry diets might have been an emergency response to borderline malnutrition, in order to maximise the utilisation of injested peptides (Cara et al. 2007). This is likely a result of an imbalanced amino acid supply, which is a common problem in fish fed artificial protein sources (Rønnestad et al. 2013).

Overall, the combined activity graphs of 4 pancreatic enzymes showed that the digestion pattern for natural food differed significantly from the 2 nearly

identical patterns of the commercial feeds. This shows a noteworthy flexibility of juvenile *C. carassius* to adjust to diverse dietary compositions by varying the blend of pancreatic secretions.

Enterocyte maturation is essential for the ongoing development of fish larvae because it leads to an increase of epithelial digestion and absorption (Cahu & Zambonino-Infante 1995). Over time, pancreatic peptidases lose significance in favour of intestinal BBEs, but simple activity measurements are insufficient for a thorough examination of this process. The calculation of an activity ratio of a selected BBE and an intracellular digestive enzyme provides a better representation of the progressive brush border development (Cahu & Zambonino-Infante 2001). A similar approach was used in this paper, but ACP was used in place of the Leu-Ala peptidase as the referential enzyme. Studies on fish enterocytes have revealed that ACP is usually associated with lysosomes and vesicular transport (Bakke-McKellep et al. 2000, Faccioli et al. 2016), which supports previous findings considering ACP participation in pinocytotic absorption and digestion (Govoni et al. 1986). Intestinal ACP activity stabilises after early fluctuations during larval metamorphosis (Zacarias-Soto et al. 2013); therefore, we suggest that ACP may be used as an intracellular reference for BBE analysis in fish which have already passed the larval transformation.

Intestinal ALP is a multifunctional enzyme because it modulates the surface pH of the mucosa, shapes the microbial community in the tract, regulates lipid absorption and participates in the anti-inflammatory response in the intestine (Lallès 2014). Increasing ALP activity during early larval growth marks the transition to an adult type of digestion due to the development of the brush border (Kolkovski et al. 2009). However, the distinct activity spike observed in group *X* after 6 wk does not necessarily imply that enterocyte maturation processes suddenly accelerated in that particular group. A study on the development of microbial communities in goldfish concluded that these bacterial populations establish around 2 mo post-hatching (Sugita et al. 1988), which occurred exactly between the 4 and 6 wk sampling points in our study. Feeding-induced changes in intestinal microbiota composition are not uncommon in fish breeding (Wang et al. 2017); therefore, it is possible that a microbiological distortion occurred in the *X* group which led to an anti-inflammatory response of the epithelium. In support of this theory, adverse microbial changes in the intestine may coincide with shortened intestinal villi, as was observed in group *X* (Heikkinen et al. 2006), while the dietary addition of probiotic bacteria may promote elongation of intestinal folds (Anguiano et al. 2013).

LAP is a highly specific BBE (Ma et al. 2005). Enterocyte maturation in fish larvae is commonly assessed with activity measurements of both ALP and LAP (Vizcaíno et al. 2014, Gu et al. 2016). However, the observed pattern was not similar to ALPs because the highest activity was recorded in group *Y*. This may be another example of a dietary-specific response due to the intake of an as yet undetermined nutritional component (Cahu & Zambonino-Infante 2001). Moreover, increased intestinal LAP may mirror liver damage (Feng et al. 2017). We assume that the higher LAP activity in group *Y* occurred in response to malnutrition as an attempt to increase mucosal digestion.

5. CONCLUSIONS

This study has shown that neither of the applied commercial diets appear to satisfy the nutritional demands of juvenile crucian carp, even though initial conclusions based on fish growth rates and survival might have been different. In a previous article (Kasprzak et al. 2019) it was revealed that crucian carp fed the 2 examined diets were characterised by a multitude of skeletal disorders; the current study showed that the alimentary tracts of these fish were also visibly affected by the employed dry feeds. Among the described observations, the activity patterns of digestive enzymes and an excessive accumulation of lipids are probably the most pronounced evidence of the inadequacy of these diets. While the general production parameters initially appeared to be very promising, it now seems reasonable to conclude that the chances of survival (and propagation) of these fish would be lower than those of fish fed natural food (upon release into their natural environment), especially after a rearing period which would last longer than the 6 wk shown in the present experiment. The described dietary effects would very likely continue to accumulate, to a point where they would significantly affect the overall condition of these fish, especially by weakening their ability to avoid predators or compete for food due to diminished mobility caused by deformities and malnutrition.

These results highlight that much more work still needs to be done regarding rearing protocols for juvenile crucian carp. It is obvious now that developing formulated diets specifically for individual species is necessary when accounting for the performance and welfare of the cultured fish, rather than being just an economical improvement of the pro-

duction process. This is also a tangible example of how deceiving initial growth data might be in fisheries, highlighting that additional scientific verification is valuable and that advanced analyses should always be performed in such studies.

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