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ORIGINAL PAPER

Effects of dietary protein levels on growth and physiology of domesticated European perch (*Perca fluviatilis*) reared in a recirculating aquaculture system

W. Wiechetek[1], R. Kasprzak[1,*], D. Adamek-Urbańska[1], P. Gomułka[2], M. Wójcik[3], J. Bochenek[3], H. Bober[2], M. Woźniak[4], J. Śliwiński[1], A. Szczepański[1], H. Szudrowicz[1], R. Wild[1], K. Kawalski[1], J. Martynow[1], P. Bujarski[1], J. Król[5] and M. Kamaszewski[1]

[1] Warsaw University of Life Sciences, Institute of Animal Science, Department of Ichthyology and Biotechnology in Aquaculture, 02-787 Warsaw, Poland
[2] University of Warmia and Mazury, Faculty of Animal Bioengineering, Department of Ichthyology and Aquaculture, 10-719 Olsztyn, Poland
[3] The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Department of Genetic Engineering, 05-110 Jabłonna, Poland
[4] University of Warmia and Mazury, Faculty of Geoengineering, Department of Tourism, Recreation and Ecology, 10-719 Olsztyn, Poland
[5] National Inland Fisheries Research Institute, Department of Salmonid, 10-719 Olsztyn, Poland

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* Corresponding author:
e-mail: robert_kasprzak@sggw.edu.pl

ABSTRACT. This study investigated the effects of dietary protein levels on the growth and physiology of domesticated European perch (*Perca fluviatilis*) reared in a recirculating aquaculture system (RAS). Perch fingerlings (initial body weight of 72.8 ± 1.9 g) were divided into three groups (P41, P46 and P51; 275 fish each), and fed isoenergetic diets with varying levels of fish meal inclusion: 44, 52 or 60%, corresponding to 41.3, 46, and 50.5% protein in the diet, respectively. The experiment lasted 12 weeks. The level of dietary protein significantly influenced the specific growth rate, feed conversion ratio, and protein efficiency ratio, with group P41 performing significantly worse than other groups . Moreover, fish from group P46 had lower muscle fat content (5%) than P41 (11.2%) and P51 (12.2%), as well as decreased blood plasma creatinine levels, and alanine aminotransferase and aspartate aminotransferase activity. Furthermore, histological analysis revealed alterations in hepatocyte parameters (reduced cytoplasmic and nuclear areas) in group P51. Fish from group P51 also had a significantly lower expression level of the interleukin 6 gene when compared to P46, both in the anterior intestine and liver. Group P46 showed a significantly lower expression level of the solute carrier family 15 member 1b gene in the liver. So, the recommended dietary protein proportion for maximising the growth and feed utilisation was determined at 46% (group P46). The current findings contribute to the optimisation of feed formulation for domesticated European perch and provide valuable insights for the sustainable development of RAS-based perch aquaculture.

Introduction

Aquaculture has recently emerged as the fastest-growing branch of the food industry. Owing to a dramatic increase in the production volume, the global consumption per capita of aquaculture products has more than doubled, increasing from 9.9 kg to 20.2 kg since the 1960s (FAO, 2022).

This growth has largely been facilitated by new technologies and various optimisations (Føre et al., 2017; Pedersen and Wik, 2020; Szczepanski et al., 2022; Yue and Shen, 2022). Consequently, aquaculture is progressively being relied upon as a solution to the pressing issue of growing global demand for food (Béné et al., 2015; 2016), not only as a mean to intensify, but also diversify food production. However, as in other sectors of the global economy, aquaculture development is constrained by a number of phenomena. These factors include problems of disease control (Granada et al., 2015) due to the expansion of new (mainly viral and bacterial) pathogens in fish farming facilities (Bigarré et al., 2017; Way et al., 2017; Adamek and Kasprzak, 2018), environmental alterations (Policar et al., 2019), accelerating climate change (FAO, 2022), or the need to reduce the use of products sourced from increasingly impoverished aquatic ecosystems, such as fish oil (FO) and fish meal (FM), for feed formulation (Vilhelmsson et al., 2004; Zaid et al., 2023).

To some extent, the development of the aquaculture industry has relied on domestication efforts centred around a limited number of commonly farmed species (FAO, 2019; Sicuro, 2021; Teletchea, 2021). Domestication itself is a fairly difficult process to define precisely. Broadly, a population's adaptation to man-made environments drives numerous genetic and behavioural alterations that are passed on in successive generations (Price, 1984; 1999). For fish, this is a lengthy and dynamic process that begins with the transfer of wild-caught individuals from natural conditions to controlled culture settings (Milla et al., 2021; Podgorniak et al., 2022). A fish population is considered domesticated only when it completes its entire life cycle in captivity, regardless of the ontogenic stage at which founding specimens were introduced (i.e., eggs, larvae or juveniles) (Teletchea, 2021). Teletchea and Fontaine (2012) proposed a five-level domestication scale for wild fish populations, starting with acclimation to an artificial environment (level 1) and ending with selective breeding programmes, targeting traits such as growth rate or meat quality (level 5).

In the late 1980s and early 1990s, European researchers began evaluating the aquaculture potential of the European perch (*Perca fluviatilis*) (Fontaine and Teletchea, 2019). After approximately 30 years of breeding of this species, it has been assumed that some stocks can be classified at level 4 of domestication. This claim was supported by the completion of a closed life cycle under intensive culture conditions, without selective breeding programmes (Palińska-Żarska et al., 2020). The European perch is also one of the few freshwater fish in Europe for which researchers have convenient access to both-domesticated strains and various wild populations. It could even be considered a model species for studies on fish domestication as its breeding in recirculating aquaculture systems (RAS) has been well-documented over several generations (Palińska-Żarska et al., 2021). In addition, recent consumer interest has increased its commercial production potential (Fontaine and Teletchea, 2019).

Similar to other sectors of intensive livestock farming industry, aquaculture relies heavily on feed expenditures to maintain production levels, and these expenses account for up to 50% of total fish production costs (Craig et al., 2017; Bochert, 2020). As global fish and seafood catch rates have stabilised and most major fishing areas have reached their maximum potential (FAO, 2022), ensuring an optimal supply of FM and FO is becoming increasingly challenging. Therefore, more cost-effective and readily available alternatives are being sought, including plant-based (Kamaszewski et al., 2010; Szczepański et al., 2022) and animal-derived (Kamaszewski and Ostaszewska, 2014) protein sources.

In the wild, the European perch is an opportunistic, schooling predator whose diverse diet ranges from zooplankton to fish (Thorpe, 1977). Researchers have studied the species' dietary preferences and acclimation to cultured environments, including various maintenance parameters (Strand et al., 2007) and the type (live/dry) of administered food (Kestemont et al., 1996). In 2015, Geay and Kestemont analysed previous studies, comparing basic production parameters, such as specific growth rate (SGR), feed conversion ratio (FCR) and feed efficiency (FE), as well as the protein/energy ratio. In conclusion, they proposed the following dietary requirements for the European perch: 43–50% protein, 13–18% fat and 10–15% carbohydrates. These guidelines enabled an optimisation of the nutritional composition of perch feed for successive life stages. However, until recently, a dedicated feed was not available on the market, leading perch farmers to frequently utilise pellets designed for salmonids (Bochert, 2020) or other marine fish species (Stejskal et al., 2010). In May 2020, the French company Le Gouessant Aquaculture introduced a new range of perch feeds, including one specifically for fingerlings weighing 10–15 g (55% protein, 10% fat) and another for juveniles weighing 15–400 g (49% protein, 12% fat). Nevertheless, the nutritional requirements of cultured perch remain inadequately defined, and producers often must rely on their own

experience and, to a large extent, on the uncertain results of ongoing experiments (Bochert, 2020).

Considering the steady increase in the production of European perch in global aquaculture (Tran et al., 2024), it becomes essential to optimise existing feeding strategies and tailor them to specific culture conditions. Therefore, the purpose of the present experiment was to evaluate formulated feeds with varying protein contents for adult perch maintained in RAS. We hypothesised that the proportion of protein in the feed that approaches or even falls below the typically recommended minimum levels could improve weight gain, feed utilisation and other parameters important in fish farming, while also exerting a beneficial effect on various physiological parameters of the digestive tract.

Material and methods

Experimental design

According to the opinion of the Local Ethics Committee for Animal Experiments in Olsztyn (LKE 34/2022, dated 08/11/2022), the experimental design was standard for nutritional studies ending with tissue collection for analysis, and therefore not requiring approval from the Local Ethics Committee.

Three isoenergetic feeds with different protein contents, with fish meal as the sole protein source (Table 1), were formulated at the Stanisław Sakowicz Institute of Inland Fisheries and the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, both located in Olsztyn. The feeds were then prepared at the Department of Ichthyology, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn. The proximate composition of the feeds was determined using standard methods (AOAC International, 2016). Dry matter (DM) content was determined by drying in an oven at 105 °C for 24 h. Total protein content was measured using Kjeldahl's method, and crude fat content by Soxhlet's method. Crude ash content was determined gravimetrically by measuring the mass loss after combusting the sample in a muffle furnace at 550 °C for 12 h, while crude fibre was estimated using the standard AOAC 973.18 method. Carbohydrate content (nitrogen free extract, NFE) was calculated by subtracting moisture, protein, fat, ash and fibre contents from 100%. Finally, gross energy (GE) contained in DM (MJ/kg) was calculated using the following formula: GE = 0.01 × (2.385 × protein + 3.891 × fat + 1.715 × NFE).

Table 1. Composition of isoenergetic experimental feeds differing in fish meal content as the main protein source, g/kg

Ingredients	Diets		
	P41	P46	P51
Fish meal[1]	440	520	600
Wheat flour[2]	340	260	180
Fish oil[3]	200	200	200
Premix[4]	20	20	20
Nutrients			
Dry matter	917	931	928
Crude protein*	413	460	505
Crude fat*	227	220	220
Ash*	84	103	119
Crude fibre*	7	5	3
Nitrogen-free extract*	269	212	153
Gross energy*, MJ/kg	23.3	23.2	23.2

P41, P46 and P51 isoenergetic diets with varying fish meal inclusion levels: 44%, 52% or 60%, respectively (41.3/46/50.5% protein in diet); [1] fish meal (70–72% protein; FF Skagen, Denmark); [2] wheat flour (Polskie Młyny, Poland); [3] fish oil produced (ROL-PASZ, Poland); [4] dolmix Pstrąg 1.5%, (DOLFOS, Poland); * calculated based on dry matter

For the feeding experiment, a total of 675 domesticated juvenile perches (average initial body weight, IBW = 72.8 ± 1.9 g) were obtained from the Żurawia Stocking Center (Biała Rawska, Poland). The fish were evenly distributed into 9 tanks (75 fish/tank; each tank with a volume of 0.3 m^3), all of which were part of a singular RAS. Water parameters were measured daily at the outlet of the main storage tank and were as follows: temperature = 20.6 ± 1.4 °C, dissolved O_2 = 10.4 ± 1.1 mg/dm^3, pH = 7.0 ± 0.10 (6.92–7.15), total ammonia = 0.005 ± 0.001 mg/dm^3, NO_3 = 0.20 ± 0.02 mg/dm^3, NO_2 = 0.002 ± 0,001 mg/dm^3 and PO_4 = 0.005 ± 0.001 mg/dm^3. Three experimental groups, replicated in three tanks, were fed one of the three aforementioned feeds differing in FM inclusion levels, designated as P41, P46 and P51 (i.e., 44, 52 and 60% FM, providing approx. 41.3, 46.0 and 50.5% protein in the feed, respectively). The daily feed dosage was set at 1% of the fish biomass, and the experiment lasted 12 weeks.

Sampling, body parameters and rearing indices

Upon completion of the experiment, all fish were placed in a buffered solution of 0.3‰ MS-222 (MilliporeSigma, Burlington, VT, USA) for euthanasia. They were subsequently subjected to morphometric measurements of final body weight (FBW), and final caudal length (FCL). Afterwards, the fish were eviscerated and randomly assigned for sample collection to assess muscle composition, blood

plasma biochemistry, histology and gene expression in the digestive tract.

Growth performance indices were determined using the following formulas:

- Fulton's condition factor (F) = 100 × FBW × FCL^{-3});
- Viscerosomatic index (VSI; %) = 100 × (digestive tract weight × FBW^{-1});
- Specific growth rate (SGR; %/days) = 100 × [(ln FBW − ln IBW) days of $study^{-1}$];
- Feed conversion ratio (FCR) = feed intake × $weight\ gain^{-1}$;
- Protein efficiency ratio (PER) = weight gain × crude protein fed^{-1}.

Muscle proximate composition

To analyse muscle composition, 15 fish from each tank were euthanised, filleted, skinned, and pooled (3 samples per tank; 9 per group) for laboratory assays. The proximate composition of the muscles (DM, crude protein, fat and ash) was determined using standard methods, as previously described for feed composition analysis.

Blood plasma parameters

Blood samples were collected from five fish per tank (n = 15 per group) via the caudal vein immediately after euthanasia using a syringe. Samples were centrifuged (StatSpin; Beckman Coulter, Brea, CA, USA) for 30 s at 15800 rpm, and the resulting plasma was analysed using a Catalyst Dx Chemistry Analyzer (Idexx Laboratories, Westbrook, ME, USA) with custom-panel test slides. The following biochemical assays were performed: total protein (TP; g/l), albumin (ALB; g/l), globulins (GLOB; g/l), glucose (GLU; mmol/l), cholesterol (CHOL; mmol/l), triglycerides (TRIG; mmol/l), total bilirubin (TBIL; µmol/l), creatinine (CREA; µmol/l), ammonia (NH_3; µmol/l), alanine aminotransferase (ALT; U/l), aspartate aminotransferase (AST; U/l) and alkaline phosphatase (ALP; U/l). Each plasma sample was thawed only once at room temperature, and all analyses were conducted in a single session to avoid multiple freeze/thaw cycles.

Histological analysis

Sections of the anterior and posterior intestine, and liver parenchyma (n = 8 each tissue per group), were fixed in Bouin's solution and processed using a standard paraffin-embedding histological protocol. Tissue sections (6 µm) were cut and stained with haematoxylin and eosin (HE), and Alcian blue with periodic acid Schiff's reagent (AB-PAS method) to identify glycogen in the liver and distinguish mucous cells with different secretion profiles (acidic, neutral, mixed) in the intestine.

Histomorphometric evaluation was performed on the stained slides using a Nikon Eclipse Ni-E microscope equipped with NIS Elements image analysis software (Nikon Corporation, Tokyo, Japan). The following parameters were analysed: intestinal fold height in the anterior (AFH) and posterior (PFH) sections, *lamina propria* width in the anterior (AWLP) and posterior (PWLP) sections, enterocyte height in the anterior (AEH) and posterior (PEH) sections, and enterocyte supranuclear height in the anterior (ASH) and posterior (PSH) sections. For each parameter, 20 measurements were taken from 8 individuals per group. In addition, hepatocyte total area (HA), cytoplasmic area (HCA) and nuclear area (HNA) were determined, with 100 measurements taken per individual (n = 8 per group). These data allowed for the calculation of the hepato-nuclear index (HNI) using the formula: HNI (%) = 100 × (HNA/HA).

Gene expression analysis

Total mRNA from the anterior and posterior intestine and liver (n = 10 each tissue per group), was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The yield of isolated RNA was estimated spectrophotometrically (NanoDrop Technologies, Wilmington, NC, USA), and its integrity was evaluated electrophoretically by separation on a 1% agarose gel containing ethidium bromide. First-strand cDNA synthesis was conducted with 1 µg of total RNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR, which included dsDNase (Thermo Fisher Scientific, Waltham, MA, USA). Specific primers for determining the expression of four housekeeping genes (actin, beta 2; glyceraldehyde-3-phosphate dehydrogenase; glucuronidase, beta; hypoxanthine phosphoribosyltransferase 1) and six target genes (interleukin 1, beta (*il1b*); interleukin 6 (*il6*); tumour necrosis factor b (*tnfb*); solute carrier family 15 member 1b (*slc15a1b*); solute carrier family 27 member 4 (*slc27a4*); peroxisome proliferator-activated receptor alpha a (*pparaa*)) were designed using Primer-Blast software (National Library of Medicine, Bethesda, MD, USA) and synthesised by Nextbio (Lublin, Poland). The full names and primer sequences of all genes under study were listed in Table 2. Real-time PCR reactions were performed using the HOT FIREPol EvaGreen qPCR Mix Plus (no ROX; Solis BioDyne, Tartu, Estonia) and HPLC-grade oligonucleotide primers. All reactions were performed in a total volume of 15 µl

Table 2. Oligonucleotide primer sequences for real-time PCR

Short name	Full name of gene	GenBank acc. no	Sequence 5'→3'	Amplicon size, bp
il1b	interleukin 1, beta	XM_039779729.1	TGACTTCGACCTGTCTCAAGC	94
			ATCCTGAACGTCCGTTGTGT	
il6	interleukin 6 (interferon, beta 2)	XM_039821186.1	GAGTACCCCGGCAACTCAAT	90
			GCCACGGTTTCTCATCTTTCG	
tnfb	tumour necrosis factor b (TNF superfamily, member 2)	XM_039804706.1	CACTGCTGAGAAAGATCCCCA	104
			CTTGCATACTCTCGCCGTCT	
slc15a1b	solute carrier family 15 member 1b	XM_039793389.1	GAGCACTGTAGGTCAAGCGA	111
			AGGTAATCCTCATTGGCCCTG	
slc27a4	solute carrier family 27 member 4	XM_039780588.1	TATCTTCGAGGGGACTGGGG	110
			CCACCACGTCACCATCCTTA	
pparaa	peroxisome proliferator-activated receptor alpha a	XM_039791719.1	GAGAGTTTCAGCCCCCTCAA	107
			CTTCTGAAGAAACCCTTGCAGC	
Housekeeping genes				
actb2	actin, beta 2	XM_039824642.1	CTGCGGAATCCATGAGACCA	188
			CTGGTGGGGCAATAATCTTGA	
gapdh	glyceraldehyde-3-phosphate dehydrogenase	XM_039805259.1	GGGACCCCGCTAACATCAAA	102
			CCTTCAAGTGAGCAGAAGCC	
gusb	glucuronidase, beta	XM_039777627.1	GGCATCGCAGATAGTCGCAG	96
			AGAGGCATGGTTCTTGTCCC	
hprt1	hypoxanthine phosphoribosyltransferase 1	XM_039813961.1	TTGACGGGCAAGAATGTCCT	157
			CTGGTCGGTAGCCAACACTT	

containing 3 µl of Master Mix, 10.05 µl of RNase free water, 2 × 0.225 µl of each primer (0.225 mM) and a 1.5 µl of cDNA template. Amplification was performed using a Rotor Gene 6000 thermocycler (Corbett Research, Mortlake, Australia) according to the following protocol: one cycle at 95 °C for 15 min (enzyme activation), followed by 35 cycles of: 95 °C for 10 s (denaturation), 59 °C for 20 s (annealing) and 72 °C for 10 s (elongation), and a final extension at 72 °C for 7 min (product stabilisation). The melting curve was generated between 70–95 °C at 0.5 °C intervals. Negative controls without cDNA template were included in each reaction, and specificity of PCR amplification was confirmed by direct sequencing of the products (Nextbio, Lublin, Poland). The average expression levels of the housekeeping genes were used to normalise the expression of genes of interest.

Statistical analysis

The numerical datasets were assessed for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. Depending on these results, statistical analyses were conducted using either the nonparametric Kruskal-Wallis test (for muscle composition, blood plasma indices and gene expression) or the parametric one-way ANOVA followed by Fisher's post hoc test (for remaining parameters). Statistically significant differences between groups were reported at $P < 0.05$. All calculations were conducted using STATISTICA 13 software (TIBCO Software, Palo Alto, CA, USA).

Results

Body parameters and rearing indices

Statistically significant differences occurred between the experimental groups in terms of individual body parameters, with each differing parameter having one group distinct from the other two, while no significant differences were observed for tank-based rearing indices (Table 3). Specifically, FBW in group P41 was lower compared to groups P46 and P51, while FCL was larger in group P51 than in the other two groups. The K factor was higher in P46 compared to P41 and P51, while the VSI was lower in P51 than in the remaining groups. Although rearing indices did not show statistically significant differences, clearly the worst values for all three parameters were obtained for group P41. In particular, SGR was approximately 25% lower than in P46 and P51, while FCR was almost twice as high as in those groups, resulting in an almost 60% lower PER.

Table 3. Body parameters and rearing indices of perch fed experimental diets

Parameters	Diets		
	P41	P46	P51
FBW, g	$103.62^{b} \pm 21.08$	$114.64^{a} \pm 28.53$	$113.70^{a} \pm 26.96$
FCL, cm	$16.93^{b} \pm 0.91$	$17.01^{b} \pm 1.03$	$17.59^{a} \pm 1.16$
K	$2.11^{b} \pm 0.24$	$2.28^{a} \pm 0.33$	$2.06^{b} \pm 0.21$
VSI, %	$15.08^{a} \pm 4.39$	$14.23^{a} \pm 2.89$	$12.78^{b} \pm 2.26$
SGR, %/day	0.47 ± 0.03	0.62 ± 0.01	0.63 ± 0.05
FCR	1.83 ± 0.31	0.97 ± 0.22	0.97 ± 0.10
PER	0.65 ± 0.16	1.55 ± 0.47	1.44 ± 0.40

P41, P46 and P51 isoenergetic diets with varying fish meal inclusion levels: 44%, 52% or 60%, respectively (41.3/46/50.5% protein in diet); FBW – final body weight, FCL – final caudal length, K – Fulton's condition coefficient, VSI – viscerosomatic index, SGR – specific growth rate, FCR – feed conversion ratio, PER – protein efficiency ratio; all results are presented as means ± SD; ^{ab} – means within a row with different superscripts are significantly different at $P < 0.05$

Proximate composition of muscles

There were no statistically significant differences between the groups in muscle composition parameters, except for the crude fat content in group P46, which was significantly lower when compared to group P51 (Table 4). Additionally, the crude protein content was slightly lower in P51 than in the other two groups.

Table 4. Proximate composition of muscles of perch fed experimental diets, g/kg wet weight

Parameters	Diets		
	P41	P46	P51
Dry matter	222.9 ± 3.0	221.4 ± 3.5	219.9 ± 2.5
Crude protein	202.5 ± 1.2	202.7 ± 4.9	189.5 ± 0.4
Crude fat	$11.2^{ab} \pm 0.8$	$5.0^{b} \pm 2.4$	$12.2^{a} \pm 1.1$
Ash	13.6 ± 1.0	12.5 ± 0.6	12.6 ± 0.4

P41, P46 and P51 isoenergetic diets with varying fish meal inclusion levels: 44%, 52% or 60%, respectively (41.3/46/50.5% protein in diet); all results are presented as means ± SD; ^{ab} – means within a row with different superscripts are significantly different at $P < 0.05$

Blood plasma parameters

No statistically significant differences were found between the groups in the twelve blood indices analysed (Table 5), which could partly result from relatively high standard deviations in many of the biochemical analyses. Nevertheless, some trends could still be observed in the data. Fish from group P41 had lower plasma TP, ALB and GLOB levels, but the highest GLU and CHOL values. Group P46 showed the highest average values of TP, ALB and GLOB and lowest of CHOL, TRIG and CREA, while for P51 the lowest GLU and TBIL levels were recorded. Additionally, the average activities of the three enzymes tested (ALT, AST and ALP) were highest in group P41 and lowest in P46 (Table 5).

Table 5. Blood plasma indices of perch fed experimental diets

Indices	Diets		
	P41	P46	P51
TP, g/l	46.27 ± 9.12	52.00 ± 6.44	49.30 ± 7.63
ALB, g/l	14.55 ± 2.38	16.20 ± 1.92	15.60 ± 3.24
GLOB, g/l	31.73 ± 6.87	35.80 ± 4.55	33.70 ± 4.81
GLU, mmol/l	10.37 ± 10.19	7.27 ± 6.48	6.56 ± 4.82
CHOL, mmol/l	5.41 ± 1.70	4.67 ± 0.93	5.04 ± 0.86
TRIG, mmol/l	3.13 ± 0.93	2.81 ± 0.52	3.18 ± 0.78
TBIL, µmol/l	5.91 ± 3.88	5.60 ± 3.36	4.80 ± 3.68
CREA, µmol/l	17.18 ± 15.85	11.80 ± 3.56	16.33 ±14.46
NH_3, µmol/l	155.67 ± 67.42	150.33 ± 63.03	156.11 ± 65.32
ALT, U/l	78.87 ± 58.52	46.00 ± 18.88	61.10 ± 55.42
AST, U/l	195.13 ± 129.06	113.00 ± 38.40	140.60 ± 99.19
ALP, U/l	70.00 ± 37.01	43.38 ± 16.92	54.20 ± 25.78

P41, P46 and P51 isoenergetic diets with varying fish meal inclusion levels: 44%, 52% or 60%, respectively (41.3/46/50.5% protein in diet); TP – total protein, ALB – albumin, GLOB – globulins, GLU – glucose, CHOL – cholesterol, TRIG – triglycerides, TBIL – total bilirubin, CREA – creatinine, NH_3 – ammonia, ALT – alanine aminotransferase, AST – aspartate aminotransferase, ALP – alkaline phosphatase; all results are presented as means ± SD; $P > 0.05$

Histological analysis

The overall structure of the gastrointestinal tract was generally consistent in all three fish groups, with structural differences observed mainly in the mucosa layer, both in the anterior and posterior sections (Figure 1). In the anterior intestine of fish from groups P41 and P46, mucous cells were predominantly of the acidic subtype, while in group P51, they showed a mixed profile. Although there were no significant differences in terms of intestinal fold height in either of intestinal sections (AFH and PFH), most of the enterocyte and *lamina propria* parameters differed statistically between the groups (Table 6). These changes were more pronounced in the anterior intestine, where enterocyte height (AEH) progressively decreased with higher dietary protein levels ($P < 0.05$), while supranuclear height (ASH) was the lowest in group P46 and highest in P51. Conversely, group P41 had significantly smaller enterocytes in the posterior intestine (PEH), while their supranuclear height (PSH) remained similar in all groups. Lastly, *lamina propria* width showed an opposite trend in the two intestinal sections, as AWLP increased significantly with higher dietary protein inclusion (the widest in P51), while the PWLP was significantly narrower in P51 compared to the other two groups (Figure 1; Table 6).

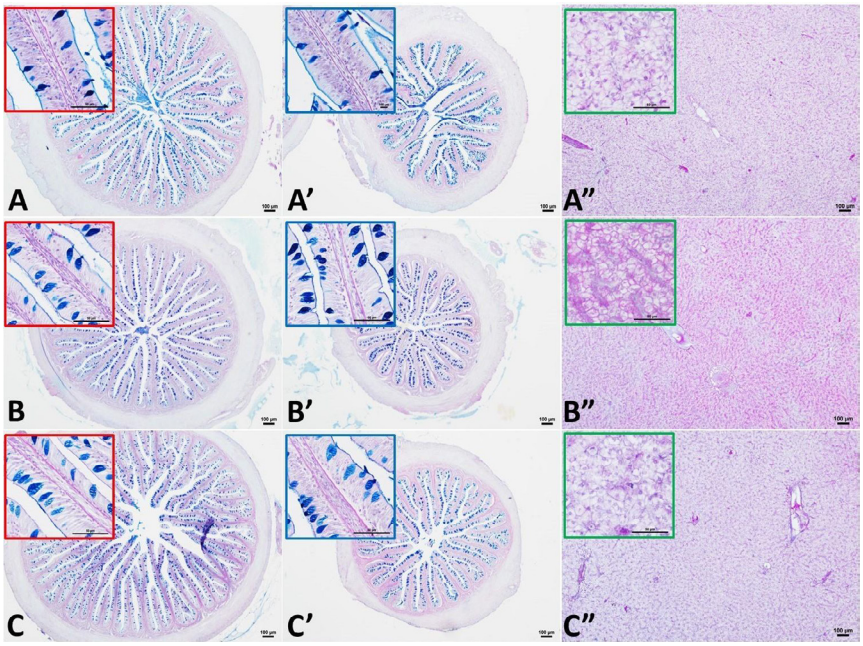

Figure 1. Histological structure of the digestive organs of perch: anterior intestine (A, B, C), posterior intestine (A', B', C') and liver parenchyma (A", B", C"). Fish were fed diets with different protein levels: P41 (A, A', A"), P46 (B, B', B") and P51 (C, C', C"). AB-PAS staining; scale bars: 100 µm (A–C') and 500 µm (A"–C"). The insets represent blow-ups of specific areas from the respective panels, magnified 10-fold

The histological structure of hepatocytes exhibited high variability depending on their localisation in relation to blood vessels. In groups P41 and P46, these cells were relatively large and were characterised by low glycogen deposition but significant lipid accumulation (Figure 1). In contrast, the total hepatocyte area (HA) and cytoplasmic area (HCA) were significantly smaller in group P51 (approx. 30% smaller than in the other two groups), as was the nuclear area (HNA) (Table 6). Thus, an increase in the proportion of fish meal in the perch diet was associated with a reduction in hepatocyte and cytoplasmic size, resulting in a corresponding increase in the HNI values.

Gene expression analysis

In most instances, the expression of the studied genes remained unaffected by varying dietary protein levels, as exemplified by *il1b* and *pparaa*, whose expression was comparable between all experimental groups in each of the three organs studied (Figure 2). Nevertheless, a few statistically significant differences were observed. Specifically, the expression of *il6* in the anterior intestine decreased with rising dietary protein levels and was significantly lower ($P < 0.05$) in group P51, while in the liver it was significantly lower ($P < 0.01$) in P51 when compared to P46. Conversely, *tnfb* transcription in the anterior intestine was significantly higher ($P < 0.01$) in P51 than in P46. Additionally, the expression of the *slc15a1b* gene varied only in the liver, where group P46 displayed significantly lower

Table 6. Histomorphometric parameters of digestive organs of perch fed experimental diets

	Diets		
	P41	P46	P51
Anterior intestine			
AFH, µm	818.16 ± 164.50	816.35 ± 173.43	815.96 ± 91.09
AEH, µm	30.32^{a} ± 3.75	28.12^{b} ± 2.87	26.53^{c} ± 2.58
ASH, µm	16.11^{b} ± 1.90	15.09^{c} ± 1.93	17.18^{a} ± 2.19
AWLP, µm	8.61^{c} ± 2.22	10.84^{b} ± 2.33	13.04^{a} ± 2.04
Posterior intestine			
PFH, µm	553.08 ± 74.02	547.98 ± 78.18	583.09 ± 124.20
PEH, µm	34.64^{b} ± 6.55	37.03^{a} ± 5.80	37.24^{a} ± 5.45
PSH, µm	17.08 ± 2.68	17.41 ± 3.10	17.70 ± 3.01
PWLP, µm	14.69^{a} ± 3.72	14.71^{a} ± 4.63	12.11^{b} ± 2.74
Liver parenchyma (hepatocytes)			
HA, µm^2	215.304^{a} ± 68.91	210.54^{a} ± 72.97	149.08^{b} ± 35.16
HCA, µm^2	196.95^{a} ± 67.08	190.24^{b} ± 70.84	131.39^{c} ± 34.00
HNA, µm^2	18.48^{b} ± 4.59	20.29^{a} ± 4.97	17.69^{c} ± 2.94
HNI, %	10.17^{c} ± 3.62	11.65^{b} ± 3.80	14.11^{a} ± 3.30

P41, P46 and P51 isoenergetic diets with varying fish meal inclusion levels: 44%, 52% or 60%, respectively (41.3/46/50.5% protein in diet); AFH – anterior intestine, fold height, AEH – anterior intestine, enterocyte height, ASH – anterior intestine, enterocyte supranuclear height, AWLP – anterior intestine, width of the lamina propria, PFH – posterior intestine, fold height, PEH – posterior intestine, enterocyte height, PSH – posterior intestine, enterocyte supranuclear height, PWLP – posterior intestine, width of the lamina propria, HA – total area of hepatocytes, HCA – cytoplasmic area of hepatocytes, HNA – nuclear area of hepatocytes, HNI – hepato-nuclear index; all results are presented as means ± SD; ^{abc} – means within a row with different superscripts are significantly different at $P < 0.05$

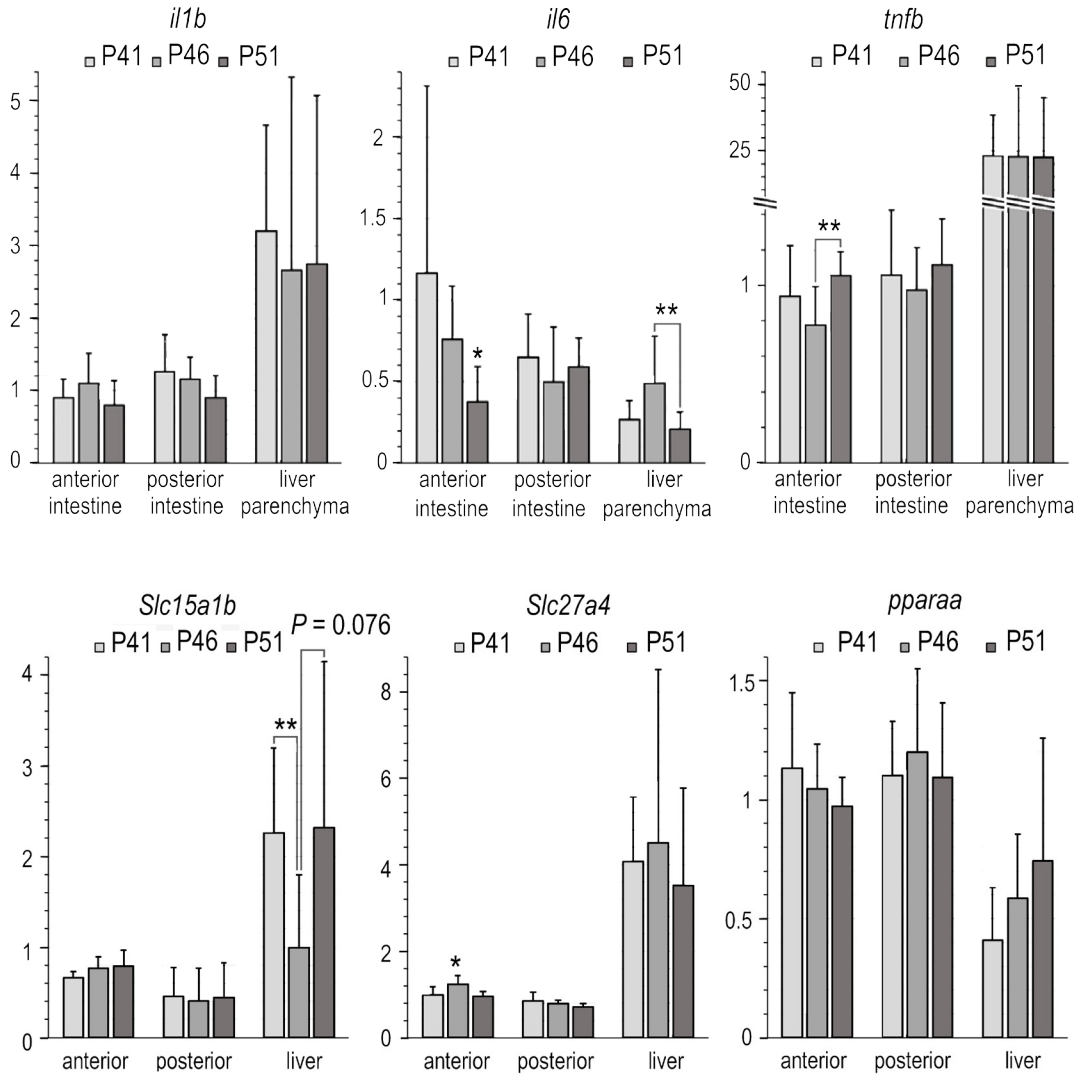

Figure 2. Normalised gene expression of *il1b*, *il6*, tnfb, *slc15a1b*, *slc27a4* and pparaa in three digestive organs of European perch fed diets with different protein levels (groups FM44, FM52 and FM60). Results are presented in arbitrary units, as the ratio of target gene expression to the mean expression of all four housekeeping genes.

il1b – interleukin 1, beta; il6 – interleukin 6 (interferon, beta 2), tnfb – tumour necrosis factor b (TNF superfamily, member 2), slc15a1b – solute carrier family 15 member 1b, slc27a4 – solute carrier family 27 member 4, pparaa – peroxisome proliferator-activated receptor alpha a

($P < 0.01$) relative mRNA levels than group P41 and almost significantly lower ($P = 0.076$) levels compared to P51. Lastly, the expression of *slc27a4* in the anterior intestine was significantly higher ($P < 0.05$) in group P46 than in the other groups.

Discussion

In our experiment, the highest body weight and best production indices for European perch were obtained with diets containing 46% and 50.5% protein. This appears to be consistent with the generally recommended protein content in the feed for *P. fluviatilis*, i.e., ranging between 43–50%, which is lower than the 50–54% threshold suggested for other percids (Geay and Kestemont, 2015). For instance, the closely related pikeperch (*Sander lucioperca*) reaches optimal production metrics on feeds containing 55–59% protein (Schulz et al., 2007; Kamaszewski and Ostaszewska, 2014). Furthermore, marine perciform species such as striped bass (*Morone saxatilis*) (Millikin, 1982) and Malabar grouper (*Epinephelus malabaricus*) (Teng et al., 1978) have also been shown to grow optimally on diets containing at least 50% protein. However, the scientific literature provides rather conflicting information about the effects of dietary protein content on European perch. For instance, Mathis et al. (2003) observed the most favourable growth at lower protein levels, up to 40%, while Bochert (2020) reported optimal growth at protein levels exceeding 45%. Similarly to the latter study, it was observed that

European perch fed a diet containing 45% protein showed a greater weight gain compared to an isoenergetic group fed 53% protein (Yang et al., 2002). Conversely, Fiogbé et al. (1996) found that a diet with 51% protein led to better growth than a 40% protein feed, though a further increase to 58% protein did not yield additional benefits. Thus, it should be noted that the availability of feed components and their chemical composition, in particular the amino acid composition, are important aspects of fish feed formulation, often more important than the gross protein content of the ration. Additionally, wild and domesticated fish may also have different nutritional demands, likely contributing to inconsistencies among the discussed studies.

The dietary protein level did not significantly affect whole-body protein content of the perch, although there was a slight decrease observed for feed with 50.5% protein. This outcome aligns with research on other perciforms, where optimally fed fish either showed no change in whole-body protein (Nyina-Wamwiza et al., 2005) or slight increases (Yang et al., 2002; Schulz et al., 2007), while excessive dietary protein in fact reduced whole-body protein levels (Alam et al., 2019). However, there was a significant difference in terms of whole-body lipid contents, as group P46 showed markedly lower whole-body fat levels (5%) than fish from groups P41 and P51 (11 and 12% fat, respectively). Similar findings have been reported under comparable experimental conditions (Yang et al., 2002; Alam et al., 2019). Nevertheless, it should be emphasised that the differences in whole-body fat content obtained in our study were significantly greater than previously documented.

In our study, although no significant differences were found for any of the evaluated serum parameters, there were indicators suggesting an improved condition of perch from group P46. Most notably, this group showed elevated levels of TP, ALB, and GLOB, markers that typically indicate good health status of the experimental fish (Çiçek and Özoğul, 2021; Zakaria et al., 2022). Moreover, raised serum CREA levels are a marker of kidney dysfunction and muscular deterioration, while increased activity of enzymes such as ALT, AST and ALP is associated with hepatic disorders (Alam et al., 2019; Bojarski and Witeska, 2020; Shahjahan et al., 2022). Therefore, the lower levels of those four parameters found in group P46 collectively suggest a more favourable health status of these fish, further highlighting the beneficial effects of the 46% protein feed diet.

Histomorphometric analyses of intestinal parameters are commonly applied in aquaculture research, with increased metrics such as fold or enterocyte height generally interpreted as positive, implying enhanced absorptive capacity of the mucosa. Conversely, an enlarged *lamina propria* is often an indicator of inflammation (Urán et al., 2008; Vatsos, 2021). For example, increased enterocyte supranuclear height was described in pikeperch fed a diet rich in linseed and peanut oils (Kowalska et al., 2010). In the present study, varying dietary protein content did not affect the height of intestinal folds, either anteriorly or posteriorly. However, enterocyte parameters showed varied trends of changes, as their height in the anterior intestine decreased with increasing dietary protein content, while the opposite relationship occurred in the posterior intestine (although groups P46 and P51 had similar averages). Interestingly, group P46 had the smallest supranuclear area in anterior enterocytes, making it challenging to form reliable conclusions from these contrasting results. Overall, intergroup differences in intestinal histology were less pronounced than in other measured parameters, such as serum enzyme activity or hepatocyte metrics (discussed below). In summary, it appears that the intestinal histology of the perch studied remained relatively unaffected by differences in dietary protein contents administered to each of the three feeding groups.

The size of fish hepatocytes and the proportion of cytoplasmic eosinophilic areas indicate either glycogen or lipid storage, indicating ongoing intestinal absorption and hepatic accumulation (Castro et al., 2015; Rašković et al., 2016). The hepatocyte nuclear area is an indirect marker of gene transcription rates, with reduced nuclear size likely implying nutritional deficiencies (Wold et al., 2009; Kasprzak et al., 2019; 2021). Lin et al. (2018) demonstrated that a diet rich in starch (>18%) increased hepatic glycogen content in largemouth bass (*Micropterus salmoides*). In our three formulated diets, the proportion of wheat flour decreased with increasing protein content. However, the livers of perch from groups P41 and P46, which were fed diets with a high proportion of wheat (34% and 26%, respectively), showed low glycogen deposition but visible fat accumulation, with relatively large hepatocytes compared to those in group P51. These observations may indicate a healthy nutritional status of the fish from these two experimental groups, rather than malnutrition (Ostaszewska et al., 2010). Moreover, the largest nuclei observed in hepatocytes of fish from group P46 points to enhanced hepatic protein

synthesis, contrasting with likely reduced synthesis in group P51 (Emiroğlu and Tarkan, 2012).

Alterations in gene expression patterns of inflammatory cytokines such as interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor b (TNF) may be related to the disruption of non-specific defence mechanisms, particularly the protective effect of intestinal mucus (Baloch et al., 2021). In this context, the lower expression of the *il6* gene in group P51 could be a sign of reduced levels of innate immunity, as IL-6 has been demonstrated to promote phagocyte proliferation in rainbow trout (Costa et al., 2011). Finally, the significantly lower expression of *tnfb* in the anterior intestine of fish from group P46 (compared to P51) seems to be a positive sign, as elevated TNF levels, with their strong pro-apoptotic and pro-inflammatory effects, are associated with various pathologies (Zou et al., 2016). On the other hand, Yousefi et al. (2024) reported that low expression levels of *il1b* and *tnfa* in the gut of rainbow trout coincided with improved fish growth and gastrointestinal homeostasis. In summary, the expression patterns of these three cytokines confirm that the 46% protein diet (group P46) had the most beneficial effect on the gastrointestinal immunity of the studied perch.

The solute carrier family 15 member 1b (PepT1b, encoded by the *slc15a1b* gene) is an amino acid membrane transporter that is a highly sensitive marker of intestinal physiology (Verri et al., 2017). In the present study, although intestinal *slc15a1b* expression was consistent between the experimental groups, there was a marked 2-fold reduction in the liver expression level in group P46. Unfortunately, there is limited literature to contextualise these findings Meanwhile, transcript abundance of the second soluble carrier gene studied, *slc27a4*, encoding the solute carrier family 27 member 4 (long-chain fatty acid transport protein 4, FATP4), differed only in the anterior intestine, with group P46 having a significantly higher expression levels than the other groups. A comparable pattern was observed in the intestine (but not the liver) of Japanese seabass (*Lateolabrax japonicus*) fed diets enriched with arachidonic acid (Xu et al., 2017). In zebrafish (*Danio rerio*), however, FATP4 expression showed high variability, both in the intestine and liver, when subjected to starvation, diets with varying lipid concentrations, and their different sources (Wang et al., 2019). These results suggest an improved lipid uptake across the cellular membrane of enterocytes in fish from group P46, likely due to the specific proportions of ingredients in this dietary formulation. Interestingly, relative transcription levels of both *slc15a1b* and *slc27a4* were significantly higher in the liver than in the intestinal segments, contrary to previous reports on teleost fish (Romano et al., 2014; Wang et al., 2017; Xu et al., 2017; Zhao et al., 2022). The last of the genes examined, *pparaa*, encodes the nuclear peroxisome proliferator-activated receptor alpha a (PPAR-α), a regulator of the metabolism of fatty acids and their derivatives (Burri et al., 2010). In the current study, hepatic expression of *pparaa* increased progressively with rising dietary protein levels. According to Jin et al. (2015), who analysed PPAR-γ transcription in the liver, this increase could indicate reduced insulin sensitivity, which might partly explain the appearance of significantly smaller hepatocytes in the livers of P51 fish. Furthermore, hepatic *pparaa* expression was similarly elevated in Atlantic salmon (*Salmo salar*) showing early or advanced symptoms of fatty liver disease (Rojas et al., 2024). Overall, considering all results of gene expression analyses, it can be concluded that varying dietary protein levels did not induce significant inflammatory responses in digestive organs of European perch, and may have even provided a minor improvement of innate immunity in group P46.

Conclusions

The present study demonstrated that, of the three dietary protein levels tested, the feed containing 46% protein was the optimal choice for domesticated juvenile perches maintained under recirculating aquaculture system conditions. This diet had a favourable effect on growth rate, as well as the morphology and physiology of internal organs, without causing any serious pathological or metabolic alterations. This conclusion marks an important step towards refining rearing protocols for *Perca fluviatilis*, a species anticipated to play an increasingly important role in European aquaculture in the coming years.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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