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RESEARCH ARTICLE

Impact of feeding juvenile tench (*Tinca tinca* (L.)) feeds supplemented with vegetable oils on hematological indexes and liver histology

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Abstract. The aim of the study was to identify the impact of feeding juvenile tench commercial feed supplemented with fish oil, linseed oil, arachide oil, and rapeseed oil on the hematology, cytology, and histology indexes of the liver. The fish were reared in a recirculating system and fed feed containing 470 g protein kg^{-1} feed and 120 g fat kg^{-1} feed (1000 g of base feed containing 70 g fat kg^{-1} feed was supplemented with 50 g of additional fat, i.e., fish oil (FO) or vegetable oils – linseed oil (LO), arachide oil (AO), or rapeseed oil (RO)). Statistically significant inter-group differences were noted for hematocrit (Ht), hemoglobin (Hb), red blood cell (RBC) counts, and other red blood cell indexes, including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) ($P < 0.05$). The lowest Ht and Hb values were noted in fish fed feed supplemented with fish oil (group FO), while the highest was noted in the tench from group AO. These differences were mainly caused by the occurrence of smaller erythrocytes in the peripheral blood of the fish from the FO group. No pathological changes were observed in the blood cells, which was in contrast to the parenchymal cells of the liver. Congestion, lipid vacuolization in hepatocytes, regressive changes in cytoplasm density, nuclear chromatin, and nucleus disintegration were observed in all fish. These changes were more pronounced in groups LO and AO. The fish from these groups were characterized by smaller hepatocytes and significantly higher nucleocytoplasmic ratios ($P < 0.05$).

Keywords: tench, vegetable oil, liver histology, hematological index

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Introduction

The fats in fish diets are not only a significant source of energy, but they also play significant roles in a range of important physiological functions. This refers in particular to the so-called essential fatty acids (EFA). These have an impact on, among other processes, embryogenesis, ovulation, immunity, stress reactions, and adaptation mechanisms in fish (Sargent et al. 2002, Bell and Sargent 2003). It is crucial to meet the species-specific requirements for polyunsaturated fatty acids (PUFA) from the n-3 and n-6 families. An excellent source of these oils are marine organisms (Tacon 2004); however, increasing demands for aquaculture feeds and stable levels of fish oil and fish meal production has necessitated searching for alternative sources of fats. In the case of fish oil, suitable alternatives are thought to be

vegetable oils (VO) (Turchini et al. 2009). Replacing fish oil either partially or totally with VO significantly lowers the production costs of both feed and fish, but it can impact fish health and fillet quality. The vast majority of scientific studies concerning the application of VO in fish nutrition focuses on basic culture indexes such the feed conversion ratio obtained by the fish, the growth rates of the fish, the slaughter yield, and fillet quality (see the review in Turchini et al. 2009). Less attention is focused on the impact that feeding fish feed supplemented with VO has on the state of their health. In effect, the impact of VO on fish health remains unclear.

Development in aquaculture is signaled by, among other aspects, the introduction of new species. Tench (*Tinca tinca* (L.)), is one such species, and although it has been cultured in polyculture with carp (*Cyprinus carpio* L.) for decades (e.g., Steffens 1995), interest in its culture in recirculating aquaculture systems (RAS) has been on the rise in recent years (Quirós et al. 2003, Kamler et al. 2006, Wang et al. 2006, Wolnicki et al. 2006, Zakęś et al. 2006). This species does not assimilate formulated feed very effectively, which can even result in significant losses during culture in RAS (Quirós et al. 2003, Rennert et al. 2003). Very few studies to date have focused on the impact VO-supplemented feed has on tench, and those that have analyzed growth rates and fillet quality (Turchini et al. 2007, Zakęś et al. 2010a). These studies indicated that supplementing feed with VO impacts the metabolism of fats, which was confirmed by changes in fatty acid profiles of the fillets and viscera. This is an indication that feeding this type of feed potentially impacts the liver (the organ responsible for fat metabolism) and on other indexes that determine fish health.

The aim of the current study was to determine the impact feeding tench formulated feed supplemented with fish, linseed, arachide, or rapeseed oils had on liver histology and hematological indexes.

Materials and methods

Fish and culture conditions

The study material was obtained from intensive culture in recirculating aquaculture systems (RAS) using formulated feed. The fish used in the feeding trials had a mean body weight (b.w.) of 57.0 g, a mean standard length (SL) of 12.6 cm, and a mean total length (TL) of 15.2 cm. These specimens were cultured in tanks with a volume of 0.6 m^3, and each tank was stocked with 61 fish (5.9 kg m^{-3}). The water temperature during the trial was 22.0 ± 0.2°C. Water oxygen concentration at tank inflows and outflows did not fall below 8.0 and 6.0 mg O_2 dm^{-3}. The concentration of total ammonia nitrogen (TAN = NH_4^+-N + NH_3-N) at the tank inflows and outflows did not exceed 0.036 and 0.48 mg TAN dm^{-3}, respectively. The water pH was within the range of 7.6-7.7. Water temperature and oxygen concentration was measured daily, and ammonia and pH were measured every 7 days. The photoperiod applied was 24L:0D (light intensity at the tank water surface was 10-20 lux).

Feed and feeding

The experimental feed was prepared by adding different vegetable oils (VO) or fish oil to the base feed (Aller Safir XS, Aller-Aqua, Golub-Dobrzyń, Poland). The composition of 1000 g dry matter (d.m.) of feed was 510 g protein, 69 g fat, and 83 g ash. This feed was supplemented with the following: rapeseed oil (ZT Kruszwica, Poland; group RO); linseed oil (SOCPOL, Marki, Poland; group LO); arachide oil (SOCPOL, Marki, Poland; group AO), or fish oil (Lysi HF, Reykjavik, Iceland; group FO). The portion of a given oil (50 g; 42% of the total fat content of the feed) was added to 1000 g of base feed, mixed, and sealed with a vacuum pump (AGA Labor, Lublin, Poland). The proximate compositions of the feeds were as follows: protein 468-474 g kg^{-1} d.m.; fat 117-121 g kg^{-1} d.m.; ash 79-82 g kg^{-1} d.m. (Table 1). The proximate composition of the feed and the fatty acid

Table 1
Proximate composition (dry matter (d.m.)) and fatty acid profiles of experimental feeds supplemented with fish oil (FO), linseed oil (LO), arachide oil (AO), and rapeseed oil (RO)

	Experimental feeds			
	FO	LO	AO	RO
Proximate composition				
Protein (P; g kg^{-1} d.m.)	472	470	468	474
Fat (L; g kg^{-1} d.m.)	119	121	120	117
Cabohydrates (C; g kg^{-1} d.m.)(1)	270	266	274	270
Ash (A; g kg^{-1} d.m.)	81	80	79	82
Gross energy (MJ kg^{-1})(2)	20.22	20.18	20.21	20.19
Fatty acid profiles (% all fatty acids)				
Σ SFA(3)	20.94	16.48	15.65	14.90
Σ USFA(4)	79.06	83.52	84.35	85.10
Σ MUFA(5)	44.89	33.80	54.17	51.72
Σ PUFA(6)	34.16	49.72	30.18	33.38
Σ n-3(7)	19.71	32.98	11.47	13.69
Σ n-6(8)	11.66	15.14	17.15	18.10
Σ n3/Σn6	1.69	2.18	0.67	0.76

(1)C = 1000 – (P + L + A);
(2)Calculated taking into consideration the following values: P – 24 kJ g^{-1}; L – 39 kJ g^{-1}; C – 17 kJ g^{-1} (Jobling 1994);
(3)ΣSFA – saturated fatty acids 14:0; 15:0; 16:0; 18:0; 20:0; 22:0;
(4)ΣUSFA – unsaturated fatty acids including monosaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA);
(5)ΣMUFA – monosaturated fatty acids 14:1; 16:1; 17:1; 18:1cis9; 18:1cis11; 20:1n-9; 20:1n-7; 22:1n-11; 22:1n-9;
(6)ΣPUFA – polyunsaturated fatty acids 16:4; 18:2n-6; 18:3n-3; 18:4; 20:2; 20:3n-6; 20:4n-6; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-6; 22:5n-3; 22:6n-3;
(7)Σn-3 – PUFA from the n-3 family: 18:3n-3; 20:3n-3; 20:4n-3; 20:5n-3; 22:5n-3; 22:6n-3;
(8)Σn-6 – PUFA from the n-6 family: 18:2n-6; 20:3n-6; 20:4n-6; 22:5n-6

profile were determined in previous publications (e.g., Zakęś et al. 2010a). The daily feed ration was 0.7% of the biomass stocked in the tanks, and the feed was delivered by automatic band feeders. Each feeding trial variant was performed in three replicates, and the length of the trial was 55 days.

Study procedure

The fish were weighed (b.w. ± 0.1 g), and measured (TL and SL ± 0.1 cm) on the initial and final days of the experiment. The fish were anesthetized in a solution of 2-phenoxyethanol (MERCK-Schuchardt, Germany) at a concentration of 0.4 cm^3 dm^{-3} (Myszkowski et al. 2003). On the initial and final days of the feeding trial, the liver was removed from seven fish from each feeding treatment. After the fish had been anesthetized in an anesthetic solution (1.5 cm^3 dm^{-3}), they were decapitated and their organs were excised. The tissues were fixed in Bouin's fluid, dehydrated in ethanol, cleared in xyline, embedded in paraffin blocks, sliced into 5 μm sections with a Leica RM 2255 rotary microtome (Leica, Bensheim, Germany), and stained with H&E. Histological analysis and measurements were performed with a light microscope (Nikon E600, Japan), MultiScanBase v. 8.08 (Computer Scanning System Ltd., Warsaw, Poland) and NIS – Elements F2.30 v. 2.21 (Nikon, Japan) software. The diameter of 50 hepatocytes (C) and their nuclei (N; ± 0.01 μm) were measured. These data were used to determine the nucleocytoplasmic ratios (INC = N × C^{-1}). Hepatocyte vacuolization was determined on a scale of 0 to 3; higher numbers indicate the lipid vacuole occupies an increasingly larger area of the cell cytoplasm (low – 0, moderate – 1, high – 2, and very high – 3 vacuolization). The degree of vacuolization was examined in cells located within a 2500 μm^2 (50 × 50 μm) surface area. Twenty such fields

Table 2
Hematological indexes of tench fed feeds supplemented with fish oil (FO), linseed oil (LO), arachide oil (AO), and rapeseed oil (RO) (mean (± SD); n = 10)

Index	Feeding group			
	FO	LO	AO	RO
Hematocrit – Ht ($l\ l^{-1}$)	0.23 (± 0.02)^{a}	0.28 (± 0.04)^{b}	0.28 (± 0.02)^{b}	0.26 (± 0.03)^{ab}
Hemoglobin – Hb ($g \times dl^{-1}$)	4.12 (± 0.30)^{a}	5.92 (± 0.60)^{b}	6.67 (± 1.39)^{b}	5.73 (± 0.71)^{b}
Red blood cells – RBC ($\times 10^{6}\ mm^{-3}$)	1.02 (± 0.03)^{b}	0.94 (± 0.04)^{a}	1.04 (± 0.07)^{b}	1.00 (± 0.05)^{ab}
Mean corpuscular volume – MCV (mm^{3})	227.14 (± 23.15)^{a}	297.87 (± 14.24)^{c}	278.23 (± 26.21)^{bc}	256.00 (± 19.75)^{ab}
Mean corpuscular hemoglobin in red blood cells – MCH (pg)	40.39 (± 3.23)^{a}	62.98 (± 6.89)^{b}	64.13 (± 11.38)^{b}	57.30 (± 5.43)^{b}
Mean corpuscular hemoglobin concentration in blood cells – MCHC ($g \times dl^{-1}$)	17.91 (± 1.03)^{a}	21.14 (± 2.57)^{b}	23.83 (± 2.02)^{b}	22.04 (± 1.15)^{b}
Leukocytes – WBC ($\times 10^{3}\ mm^{-3}$)	39.95 (± 3.01)	44.56 (± 1.95)	45.74 (± 1.01)	41. 83 (± 2.87)
Lymphocytes (%)	92.00 (± 1.47)^{a}	94.07 (± 1.79)^{ab}	95.15 (± 1.52)^{b}	93.58 (± 1.04)^{ab}
Monocytes (%)	3.74 (± 0.51)	3.39 (± 1.14)	3.58 (± 0.82)	4.13 (± 0.80)
Granulocytes (%)	4.26 (± 1.28)^{b}	2.54 (± 1.13)^{a}	1.27 (± 1.16)^{a}	2.28 (±0.76)^{a}

Values in the same row with different letter indexes differ statistically significantly ($P < 0.05$)

were analyzed for each specimen. These same surface areas were analyzed for the occurrence of hepatocyte pathology, with a special focus on necrosis on a scale from 0 (no necrosis) to 2 (necrosis affecting nearly 100% of the cells in the analyzed area), and on the occurrence of inclusions between cells on a scale of 0 (none) to 2 (numerous).

At the conclusion of the experiment, blood was drawn from 10 fish from each group for hematological analyses. The blood was drawn from the tail vein with a 2 ml heparinized syringe. Blood smears were prepared and hematocrit (Ht), hemoglobin (Hb), and red blood cell (RBC) and white blood cell (WBC) counts were determined (Svobodova et al. 1991). The values of indexes such as erythrocyte mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated taking into consideration the RBC count, and hemoglobin (Hb) and hematocrit (Ht) levels (Dacie and Lewis 2001).

Statistical analysis

The data was analyzed with single factor analysis of variance (ANOVA). When statistically significant differences were confirmed ($P \leq 0.05$), Tukey's test was applied. Prior to statistical analysis, all percentage data were transformed with the *arcsin* function. The Kruksal-Wallis test was used to analyze the histological indexes of hepatocyte vacuolization and necrosis and inclusions between cells.

Results

Feeding juvenile tench feeds supplemented with different vegetable oils has a significant impact on their growth. On the last day of the experiment the fish had attained body weights of 77.1 g (group FO), 77.6 g (group LO), 83.2 g (group AO), and 78.6 g (group RO) ($P > 0.05$).

Statistically significant differences among groups were determined in hematocrit (Ht) and hemoglobin (Hb) levels, RBC count, the leukogram, and the values of red blood cell indexes including mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCH), and mean corpuscular hemoglobin concentration (MCHC) (Table 2; $P < 0.05$). The lowest Ht and Hb values were noted in fish fed feed supplemented with fish oil (FO), while the highest were noted in the tench from group AO. Additionally, the tench from group FO had significantly

Table 3
Liver histological and cytological indexes of tench fed feeds supplemented with fish oil (FO), linseed oil (LO), arachide oil (AO), and rapeseed oil (RO) (mean (± SD); n = 7)

	Feeding group				
Analyzed parameter	Initial sample	FO	LO	AO	RO
Hepatocyte size (μm)	13.82 (± 1.18)	16.06^{b} (± 0.99)	14.69^{a} (± 0.27)	15.09^{ab} (± 0.91)	15.66^{ab} (± 0.66)
Hepatocyte nucleus size (μm)	4.19 (± 0.22)	4.42 (± 0.03)	4.28 (± 0.14)	4.33 (± 0.15)	4.32 (± 0.05)
Nucleocytoplasmic ratios	0.30 (± 0.02)	0.27^{a} (± 0.02)	0.29^{b} (± 0.01)	0.29^{b} (± 0.01)	0.27^{a} (± 0.01)
Inclusions between cells (0 = none to 2 = numerous)	0.5	0.5	0.8	0.8	0.6
Hepatocyte vacuolization (0 = low to 3 = very high)	0	0.1	1.2	1.6	0.3
Hepatocyte necrosis (0 = none to 2 = significant)	0	0.1	0.4	0.4	0.1

Values in the same row with different letter indexes differ statistically significantly ($P < 0.05$)

lower values of MCV, MCH, and MCHC ($P < 0.05$). The analysis of the histological preparations indicated the occurrence in the peripheral blood of these fish of smaller erythrocytes and a significantly higher number of granulocytes ($P < 0.05$). It must be underscored that no pathology was noted in the blood cells, in contrast to that in the hepatocytes. Congestion, hepatocyte lipid vacuolization, and regressive changes in cytoplasm density, nuclear chromatin, and nucleus disintegration (Fig. 1) were observed in all fish regardless of feeding treatment group. These pathological changes were the most pronounced in groups LO and AO, and the fish from these groups were also characterized by smaller hepatocytes and significantly higher values of nucleocytoplasmic ratios (Table 3; $P < 0.05$).

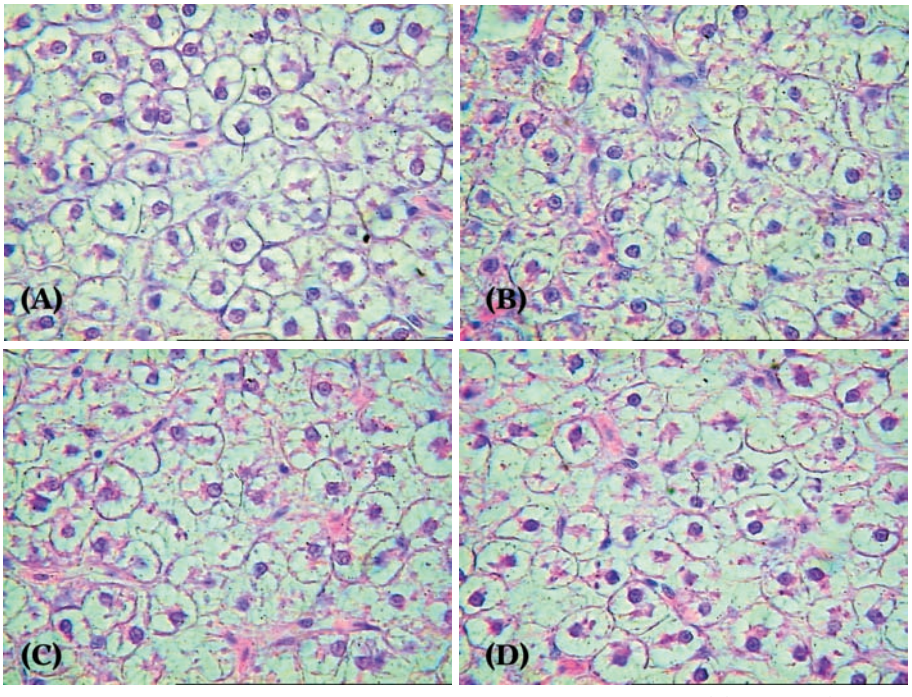

Figure 1. Cross-section of the liver of juvenile tench fed feeds supplemented with various fats: (A) – fish oil (group FO); (B) – linseed oil (group LO); (C) – arachide oil (group AO); (D) – rapeseed oil (group RO) (magnification 100×).

Discussion

Introducing new chemicals, diets, or technologies to aquaculture should be preceded by detailed scientific investigations focusing on fish health and welfare. This is why hematological studies are becoming increasingly important in aquaculture. Evaluating blood morphology permits evaluating fish health and diagnosing inflammation, infection, and many other disorders resulting from, among other causes, improper diet (Svobodova et al. 1991). Supplemental biochemical and/or macro- and microscopic examinations of tissues and organs (especially in the parenchyma) are extremely useful tools for determining fish health.

Generally, the tench peripheral blood parameters presented in the current paper are within normal ranges and are similar to those reported by other authors including Eddy (1973) and Shah (2010). Significantly, these fish came from different environments (RAS vs. natural waters), and they differed in age, size, and diet (formulated feed vs. natural feed). For example, Eddy (1973) obtained the following values for hematological indexes in tench with body weights ranging from 150 to 400 g: Ht 24.1%; Hb 6.89 g × dl^{-1}; RBC 1.05 × 10^6 mm^{-3}; MCV 244.9 mm^3; MCH 74.7 pg; MCHC 33.1 g × dl^{-1} . In turn, Shah (2010) reported the following values for tench caught in Lake Mogan in Turkey (fish body weight approximately 200 g): Ht 24.66%; Hb 6.89 g × dl^{-1}; RBC 1.48 × 10^6 mm^{-3}; MCV 186.79 mm^3; MCH 45.97 pg; MCHC 24.8 g × dl^{-1}.

Statistically significant differences were noted during the current study in the values of the hematological indexes of tench fed diets supplemented with FO or VO. The lowest values of Ht, Hb, MCV, MCH, and MCHC were noted in the fish from group FO. Similar observations were reported for African catfish (*Clarias gariepinus* (Burchell)) fed feed supplemented with cod liver oil (fat content of 90 g kg^{-1} feed) and/or palm oil (PO), the share of which was 0, 33.3, 66.6, or 100% of the total fat content (Ochang et al. 2007a), and also for Nile tilapia (*Oreochromis niloticus* (L.)) (Ochang et al. 2007b, Ochang 2011), which was cultured with feed with a fat content of 60 g kg^{-1} d.m., and supplemented with PO or soy oil (SO) at 33.3, 66.6, or 100% of the total fat content (Ochang et al. 2007b, Ochang 2011). The low values of Ht and Hb and the red blood cell indexes in the fish from group FO might indicate that tench, similarly to African catfish and Nile tilapia, cannot effectively utilize n-3 PUFA. Additionally, the high levels of these acids might be the cause of the low WBC count, which, in turn, determines the proper functioning of the immune system and increases susceptibility to disease, among other things. These hypotheses are confirmed by the results of experiments performed with African catfish and Nile tilapia in which fish survival increased with increased vegetable oil supplementation (Ochang et al. 2007a, b). Although diet was not noted to increase tench survival in the current study, the study by Shah (2010) confirms that low values of hematological indexes increased the susceptibility of this species to infection by *Saprolegnia* sp. The significantly increased levels of granulocytes in the FO group that was accompanied by slightly elevated numbers of rod-shaped white blood cells might indicate inflammation.

Fats are metabolized by the liver, and feeding fish feeds with different quantitative and/or qualitative lipid profiles can often impact the size of this organ or its histological structure. Feeding tench feeds supplemented with VO was reflected in the morphological structure of the liver and the degree of hepatocyte vacuolization. Although no statistically significant differences were noted, it should be emphasized that the highest degree of lipid vacuolization and the most advanced pathological changes, including parenchymal degeneration, necrosis, and congestion occurred in the tench from groups LO and AO. The changes noted in the fish fed feed supplemented with RO were less pronounced, and the size of the hepatocytes and their nuclei and the nucleocytoplasmic ratios were similar to those in group FO. In pikeperch (*Sander lucioperca* (L.)) fed feeds supplemented with RO, sunflower oil (SFO), or SO, the least pronounced changes in the hepatocytes were also noted in the RO group (Zakęś et al. 2010b). However, Caballero et al. (2002) reported that the

liver of rainbow trout (*Oncorhynchus mykiss* (Walb.)) fed feeds supplemented with SO exhibited a lesser degree of degeneration than did the fish fed feeds with RO or olive oil (OO). Parpoura and Alexis (2001) observed increased pathological changes in European seabass (*Dicentrarchus labrax* (L.)) fed feeds supplemented with either SO or OO. This indicates that the impact of feeding fish feeds supplemented with vegetable oils on liver histological structure and fat metabolism does not only depend on the type of VO, but also on the various characteristics of the fish species.

Liver pathology is usually noted in fish that are fed feeds with a high fat content (e.g., Kestemont et al. 2001, Kowalska et al. 2011a). Pathology in this organ is also linked to high contents of fat that is difficult to assimilate, including VO (Ostaszewska et al. 2006). Tench is a species that has difficulty in assimilating high-energy feeds (Wolnicki 2005). The feed used in the current experiment, with 120 g fat kg^{-1} feed, can be considered to have a high fat content in the case of this species. Undoubtedly, this could have impacted liver structure. Increased changes in liver structure depend, however, on the qualitative composition of the fat, which refers here to the quantity of VO supplementation. In the current study, the level of VO supplementation was about 42% of the overall fat content; however, Aderolu et al. (2011) reported that the optimal level of feed supplementation with AO or shea oil for African catfish was 15% of the total fat content. With European seabass the percentage of supplementation can be substantially higher. Figueiredo-Silva et al. (2005) reported that a share of SO that does not exceed 50% of the total fat content of feed does not have an impact on the histological structure of the liver or the degree of its vacuolization. Mourente et al. (2007) reported that the VO supplementation of European seabass feed can be as high as 60%, but they do recommend applying a mixture of RO, LO and AO. While some authors report it is possible to totally replace FO with VO, these studies are not always comprehensive and do not always include hematological, biochemical, and histological components (Ochang 2011).

When VO is added to feeds, it is important to note that the quantity and quality of polyunsaturated fatty acids change, including the ratio of acids from the n-3 and n-6 families (n-3/n-6 PUFA). Low levels of n-3 PUFA in marine fish feeds caused liver pathology (Tacon 1992); in the current study this referred to the feeds supplemented with AO and RO. The feed supplemented with LO had a high content of n-3 PUFA because of its high content of α-linolenic acid (18:3 n-3; Table 1). It was also demonstrated that supplementation with LO could disrupt the fat absorption process, as was indicated by the low level of pikeperch hepatocyte vacuolization, the occurrence of large nuclei, or their karyolysis (Kowalska et al. 2011b). No such similar changes were noted in the present study. Lipid vacuolization, and significantly smaller hepatocytes and higher nucleocytoplasmic ratios were noted in fish fed feed supplemented with LO as compared to that with FO supplementation. It should also be noted that greatest anomalies in liver tissue structure were noted in group AO, which is the group of fish with the highest values of the hematological indexes. Thus, it follows, that the key to normal tench function is not only the levels of n-3 PUFA, but also of n-6 PUFA.

Based on the results of the current study, none of the tested diets can be recommended unequivocally for tench culture. The results of the present study do indicate that it is warranted to continue feeding trials in an effort to lower fat content in feeds, and to determine the type of VO that should be used, and the amount of supplementation.

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