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Effects of dietary lipid levels on lipid accumulation and health status of adult *Onychostoma macrolepis*

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ABSTRACT

The effects of different dietary lipid levels on lipid accumulation, inflammatory response, serum bio-chemical index and histological features of intestine and hepatopancreas of *O. macrolepis* was experimentally evaluated in an eight-weeks study. Fish (initial weight 50.11 ± 2.86 g) were fed with five iso-nitrogenous diets (around 390 g/kg protein) varying with lipid level (5%, 7%, 9%, 11%, 13%, being LL5, LL7, LL9, LL11, and LL13 respectively) in triplicates. Results showed that the content of crude lipid in carcass and hepatopancreas were not affected by dietary lipid levels ($P > 0.05$). Serum ALT, TP, HDL-c and MDA, etc., were not significantly affected by diets ($P > 0.05$), while serum total antioxidant capacity in LL9 and LL11 groups were significantly higher than the other groups ($P < 0.05$). Histological features of hepatopancreas and intestine showed no significant difference among the five diets ($P > 0.05$), while the height of intestine villus showed the higher trend in LL9 compared with other groups. The relative expression of lipid metabolism related genes (*ppar α*, *cpt-1α*, *fas,* and *hsl*) and immune response related genes (*tlr 22*, *nrf 2*, *tnfα,* and *il-γ*) in the hepatopancreas of fish fed diets differing in their crude lipid levels were not significantly different ($P > 0.05$). The results suggested that a proper dietary lipid level of 9%–11% could maintain higher antioxidant and health status of adult *O.macrolepis*.

1. Introduction

Lipids is the main supplier of fatty acids, energy as well as the carrier of fat-soluble vitamins in the aquafeeds (NRC, 2011), so that high-fat diets have increasingly been used in aquatic feeds recently (Sagada et al., 2017; Wang et al., 2019). However, excessive dietary lipid often leads to severely lipid accumulation in the tissues of farming fish, causing metabolic disorder to fish and inhibiting the growth and feed utilization of fish (Ali & Jauncey, 2005; Chatzifotis et al., 2010; Yan et al., 2015). Furthermore, excessive dietary lipid levels were reported closely related to the oxidative defense and immune response of fish (Jin et al., 2013; Oliva-Teles, 2012; Zhou et al., 2020), including the induce of the production of reactive oxygen species, lipid peroxides and pro-inflammatory factors (Yin et al., 2021). Therefore, optimizing lipid levels in diet and the determination of lipid requirements of any new candidate fish species for aquaculture are very necessary, which not only boost growth performance but also reduce health hazards for the fish.

The *Onychostoma macrolepis* (*O. macrolepis,* Bleeker, 1871), still named *Gymnostomus macrolepis* (Yin et al. 2014) and belonged to the Cyprinidae family, is a freshwater benthopelagic cave fish species, distributed in the North of China, such as the Jialing, Huai, Wei, Hai, and yellow rivers (Yue et al., 2000). It is a wild indigenous fish in the upper stream of the Hanjiang River in the Qinling Bashan Mountains and commonly known locally as money fish for its scales are twinkling in the sunshine like the copper coins in ancient China or known as spring fish for its gush out of the spring hole accompanying with the spring water in each March and April. It still is a kind of popular fish that has high edible value due to its nutrient's variety that especially rich in highly unsaturated fatty acids of EPA (Eicosapentaenoic acid) and DHA (docosahexaenoic acid) (Chen et al., 2019; Li & Zheng, 2014; Xu et al., 2011) with the high fetching prices of being about 300 RMB/kg. However, fish stock has been severely lacking since the 1990s due to the overharvesting and habitat (caves) degradation (Chen, 2007; Zhao et al., 2011), which leads to that this species have been listed as a National Second-class Protected Animal by the Chinese authorities (List of Aquatic Wild Animal Protection in China).

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There are many ways to recovering the population of this cavefish, one is the artificial reproduction of *O. macrolepis*, where the reproductive biology and gonadial differentiation and development of the broodfish have been fully studied (Dong et al., 2016; Song et al., 2006; Zhang et al., 2015), the other is providing sufficient nutrients for the broodfish, for the favorable dietary nutrients are the key precondition in keeping the health status of the adult cavefish. Previous studies had showed the effect of dietary protein levels (Liu et al., 2016) and lipid levels (Zhou et al., 2022) on growth and gonad development of *O. macrolepis* broodfish, while up to date, no available data exists on the effect of dietary lipid on lipid accumulation and health status of this species. Therefore, the objective of the present study was to investigate the optimal dietary lipid levels for the cavefish by lipid accumulation, inflammatory response, serum biochemical index and histological features of intestine and hepatopancreas of *O. macrolepis*, aiming to provide basic reference data for the lipid nutrition in the adult fish.

2. Materials and methods

All experimental procedures were carried out under the Guidelines for Experimental Animals by the Animal Care and Use Committee of Northwest A&F University, China.

2.1. Feed, fish and feeding

The main sources of protein in this feed are fish meal, soybena meal, rapeseed cake meal, and cottonseed meal. Five isonitrogenous experimental diets (390 g/kg protein) with five increasing lipid levels (50, 70, 90, 110, and 130 g kg^{-1}, named LL5, LL7, LL9, LL11, and LL13 respectively) were formulated for *O. macrolepis* (initial weight 50.11 ± 2.86 g) by increasing soybean oil as the only source of dietary lipid. The ingredients composition of the diets were shown in Table 1.

The diet was prepared as followed. Firstly all ingredients were mealed, weighed and fully mixed, then the appropriate amount of distilled water was added and mixed again. Finally the mixture was mechanically extruded to obtain pellets with the diameter of 1–2 mm. The pellets were dried in a room temperateure for 24 h then stored at −20 °C until their use. The crude ash and crude moisture of the five diets were among 9.6%–10.2% and 10.3%–11.3% respectively.

The fish of 105 healthy adult *O. macrolepis*, reared in 15 tanks of 130 L volume evenly, five groups and triplicate per group, were fed continuously (8:30, 12:30, and 16:30 h/day) with five diets respectively for eight weeks. The fish were fed by hand to apparent satiation levels. The tanks, supplied by Ankang Fisheries Experimental and Demonstration Station of Northwest A&F University (Ankang, Shaanxi, China), equipped with aeration 24 h a day to maintain dissolved oxygen levels at 7.7 ± 0.2 mg/L (mean ± standard deviation). The water temperature and pH was 20.0 ± 1.5 °C and 7.8 ± 0.5 respectively. The photoperiod was outdoor natural light condition.

Table 1
Ingredients of experimental diets differ in their total crude lipids. (g kg^{-1}, air-dry weight).

Ingredients	Experimental diets				
	LL5	LL7	LL9	LL11	LL13
Fish meal	285	285	285	285	285
Soybean meal	145	141	140	140	140
Rapeseed cake	10	12	14	14	16
Cottonseed meal	195	214	230	247	263
Wheat flour	220	183	149	117	73
Rice bran	91	90	85	78	84
α-cellulose	10	10	10	10	10
Soybean oil	4	25	47	69	89
Bentonite	10	10	10	10	10
Ca(H2PO4)2	20	20	20	20	20
Premix of Fish	10	10	10	10	10
Total	1000	1000	1000	1000	1000
Proximate composition (g/kg)					
Crude protein	392	386	394	389	385
Crude fat	50	73	92	111	136
Ash	9.6	9.7	9.8	10.2	9.7
Moisture	11.3	11.3	10.7	10.3	10.3

2.2. Sampling procedures

At the end of the feeding trial, all fish were anesthetized with tricaine methane sulfonate (MS-222; 0.01 g/L, *Sigma-Aldrich,* USA), blood samples were taken from the caudal peduncle vein with heparinized syringes from three fish per tank (n = 9 per diet). The hepatopancreas and intestine of three fish from each tank were randomly sampled and fixed respectively in 4% buffered formalin for 48 h for subsequent histological observation. Three fish and its hepatopancreas per tank were sampled and stored at −80 °C respectively for further biochemical analyses of proximate composition and gene expression studies.

2.3. Proximate composition of diets and fish samples

Proximate composition of experimental diets, fish carcass, and tissues were analyzed according to the Association of Official Analytical Chemists (AOAC 2004). In particular, crude protein levels were determined by Kjeldahl's method, crude lipids were determined by the method of ethyl ether extraction, and moisture and ash contents were determined by sample drying in the oven at 105 °C for 24 h and by burning in a muffle furnace at 550 °C for 4 h, respectively.

2.4. Serum biochemical parameters

After blood extraction, serum was obtained by firstly storing blood at 4 °C for 8 h and then centrifuging at 2 000 r/min for 10 min (4 °C). In order to evaluate the effect of different dietary lipid levels on different serological biomarkers, the following biochemical parameters were determined using commercial reagent kits: alanine aminotransferase (ALT), total protein (TP), globulin (GLO), albumin (ALB), glucose (GLU), triacylglycerols (TAG), total cholesterol (T-chol), high-density lipoprotein (HDL-c) and low-density lipoprotein (LDL-c), total antioxidant capacity (T-AOC) and malondialdehyde (MDA) (Jian Cheng Biotechnology Company, Nanjing, China; Biosino Bio-Technology and Science Inc., Beijing, China).

2.5. Histological analysis of liver and intestines

Histological procedures for liver and intestine from different experimental groups were carried out according to the standard histological techniques (Humason, 1961). The tissues were dehydrated in ethanol, cleared in xylene, embedded in paraffin blocks, and then sliced with a rotating microtome into 5 μm sections and stained with H&E. All histological procedures were done at the Pathology Laboratory of the Yangling Demonstration Zone Hospital (Shaanxi, China). The analyses and histological measurements were performed and the morphology of hepatocytes and intestine were observed and taken pictures with a light microscope (Motic BA310, MOTIC CHINA GROUP CO., LTD manufacturer, XiaMen city, China). The villus height of the intestine was determined under a software of Emage J.

2.6. Gene expression by quantitative real-time PCR

The impact of dietary lipid level on lipidic metabolism and immune response in hepatopancreas was assessed using the following selected genes: i) fatty acid metabolism: peroxisome proliferator-activated receptor α (*pparα*), carnitine palmitoyl transferase 1 (*cpt1*), fatty acid synthase (*fas*), hormone-sensitive lipase (*hsl*); ii) immune response: toll-like receptor 22 (*tlr22*), nuclear factor related factors 2 (*nrf2*), tumor necrosis factor alpha (*tnfα*), interleukin gamma (*il-γ*).

Genes related to lipid metabolism and immune response were determined by quantitative real-time PCR, where total RNA was extracted firstly from the samples using RNAiso Plus (TaKaRa, Dalian, China), then RNA was purified and its quality and quantity were determined by spectrophotometry (NanoDrop 1000, Thermo Scientific Inc., Wilmington, USA). The methods of reverse-transcribed RNA (1 μg) and of quantitative real-time PCR were described in detain in previous informations in Zhou et al. (2022). The PCR primer sequences for each gene were synthesized by Sangon Biotech (Shanghai) Co., Ltd. and are shown in Table 2. Relative gene quantification was conducted according to the 2 (-Delta Delta C(T)) method (Livak & Schmittgen, 2001), using β-actin as a housekeeping gene.

2.7. Statistical analyses

Data are shown as means ± standard error. Data expressed as percentages were arcsine-square-root transformed before ANOVA analysis (data previously checked for normality and homoscedasticity). When significant differences were found ($P < 0.05$), the one-way ANOVA was followed by Duncan's post hoc test. All analyses were performed using SPSS 16 for Windows Software (SPSS, Chicago, IL, USA).

3. Results

3.1. Proximate composition of the carcass and hepatopancreas

The proximate compositions of the carcasses, including crude lipid (27.31%–31.30%), crud protein (44.89%–46.92%), etc., were not significantly different among these five groups ($P > 0.05$, Table 3). In addition, crude lipid levels of the hepatopancreas (7.46%–11.51%) were not significantly affected by the content of dietary lipids ($P > 0.05$, Table 3).

3.2. Serum biochemical parameters

No statistically significant differences were found for most serum biochemical indexes [ALT (108.3–143.3 U/L), TP (51.7–55.8 g/L), GB (35.7–38.5 g/L), AB (15.9–17.3 g/L), GLU (4.5–5.5 mmol/L), T-chol (13.0–14.7 mmol/L), HDL-c (2.9–3.3 mmol/L) and LDL-c (4.9–5.7 mmol/L] among dietary groups (Table 4; $P > 0.05$). Only serum TAG levels were significantly affected by dietary lipid levels, where TAG in fish from the LL11 group (8.5 ± 0.5 mmol/L) was significantly lower than the fish from the LL5 group (10.3 ± 1.0 mmol/L) ($P < 0.05$), whereas TAG levels in the rest of dietary groups showed intermediate levels with average values ranging from 9.0 to 9.5 mmol/L ($P > 0.05$).

Regarding the total antioxidant serum capacity (T-AOC), the highest average values (11.9 U/mL) were found in fish fed the LL9 and LL11 diets, which were significantly higher than that fish fed the LL5 diet (5.7 ± 0.1 U/mL). In contrast, the rest of dietary treatments showed intermediate T-AOC levels (9.1–9.7 U/mL) ($P < 0.05$). At the highest T-AOC levels, the dietary lipid level is about 9.15 g/kg. Serum MDA levels ranged from 25.9 to 39.4 mmol/L among dietary groups, even though no statistically significant differences were found among them due to high interindividual variability (Table 4; $P > 0.05$).

Table 3
The effect of dietary lipid levels on proximate composition of the carcass and hepatopancreas in *Onychostoma macrolepis* (air-dry matters, g/kg).

Items	Experimental diets				
	LL5	LL7	LL9	LL11	LL13
Carcass					
Crude lipid	273.1 ± 28.3	313.0 ± 2.8	278.1 ± 19.0	297.5 ± 14.7	309.5 ± 50.9
Crude protein	463.8 ± 36.0	468.1 ± 0.9	469.2 ± 18.8	468.5 ± 18.6	448.9 ± 45.3
Ash	99.0 ± 6.5	92.3 ± 7.5	93.5 ± 4.0	92.5 ± 4.1	89.3 ± 9.3
Moisture	49.9 ± 11.4	42.7 ± 6.9	45.3 ± 9.4	41.4 ± 5.4	49.7 ± 5.0
Hepatopancreas					
Crude lipid	115.1 ± 17.0	78.5 ± 29.5	74.6 ± 7.7	133.2 ± 50.5	112.2 ± 15.9
Moisture	60.7 ± 10.3	83.9 ± 15.0	93.4 ± 14.9	78.8 ± 12.9	93.8 ± 12.8

3.3. Histological organization of the hepatopancreas and intestine

The histological organization of the hepatopancreas was regular in all examined *O. macrolepis* specimens regardless of the dietary group considered. In particular, the general histological organization of the hepatic parenchyma consisted of polyhedral hepatocytes with central nuclei arranged in tightly packed anastomosed laminae around veins. The level of lipid inclusions within hepatocytes was similar among groups regardless of dietary lipid levels (Fig. 1a).

Regarding the histological organization of the intestine, it consists of

Table 2
Real-time PCR primer sequences.

Gene	Sequence (5′-3′)		Amplicon size (bp)	PCR efficiency (%)	Annealing temperature (°C)
Lipid metabolism					
pparα	*F*	*CGGAGCACGCTCAGTTAGTG*	66	103	53
	R	*GCAGTAAGGGATGCAGCGAA*			
cpt-1a	*F*	*GGCGATCCAGCATGGCAGAG*	86	99	56
	R	*ATGACTGAGCTGAAGGTCAATGCC*			
fas	*F*	*ATCCACAGAGCCACCATCCTACC*	145	115	56
	R	*CAAGTCCAGCATCCTCCAAGACAC*			
hsl	*F*	*GCTTCACCATCCAGACTGCTCAC*	109	102	55
	R	*CTGGCTGCACTGACTGACAACTC*			
Immune response					
tlr 22	*F*	*TCACACACCTGACTGATGGC*	81	99	50
	R	*ACACCTTCAGAGGGCCAGAG*			
nrf 2	*F*	*GACTCCGCTTCTGTTGGCTCTG*	168	92	57
	R	*TTCATACAGCGTTGCCTCCACAG*			
tnf α	*F*	*CGGATGCGGTGGAAGATTGGAAG*	97	98	55
	R	*AGGTAGATGGTGCTGTACGTGGTC*			
il-γ	*F*	*TTACAGCGTCCTGACCTGGT*	*100*	*97*	*60*
	R	*GCGTTTGGTGCCCAGTTTGA*			
β-actin	*F*	*GCCGGATTCGCTGGAGATGATG*	112	96	57
	R	*CACCAACGTAGCTGTCCTTCTGTC*			

Gene abbreviations: *pparα*: peroxisome proliferator-activated receptors α; *cpt-1α:* carnitine palmitoyltransferase-1a; *fas:* fatty acid synthetase; *hsl:* hormone-sensitive lipase; *tlr22:* toll-like receptor 22; *nrf2:* nuclear factor related factors 2; *tnfα:* tumor necrosis factor alpha; *il-γ:* interleukin gamma.

Table 4
Serum biochemical in *Onychostoma macrolepis* fed diets differing in their lipid levels.

Items	Experimental diets				
	LL5	LL7	LL9	LL11	LL13
ALT (U/L)	142.7 ± 36.9	108.3 ± 23.7	119.4 ± 18.4	136.9 ± 26.4	143.3 ± 49.1
TP (g/L)	52.5 ± 4.8	53.0 ± 2.7	55.8 ± 3.63	51.7 ± 2.1	52.8 ± 4.8
GB (g/L)	36.3 ± 3.9	37.1 ± 2.3	38.5 ± 2.4	35.7 ± 1.9	35.9 ± 3.4
AB (g/L)	16.3 ± 1.0	15.9 ± 0.4	17.3 ± 1.2	16.1 ± 0.4	16.9 ± 1.6
GLU (mmol/L)	4.5 ± 0.5	5.2 ± 0.7	5.5 ± 0.6	5.2 ± 0.2	5.1 ± 0.7
TAG (mmol/L)	10.3 ± 1.0^{a}	9.2 ± 0.6^{ab}	9.5 ± 0.7^{ab}	8.5 ± 0.5^{b}	9.0 ± 1.2^{ab}
T-chol (mmol/L)	14.7 ± 1.7	14.2 ± 0.6	14.6 ± 1.0	13.1 ± 0.7	13.0 ± 1.7
HDL-c (mmol/L)	2.9 ± 0.4	2.9 ± 0.1	3.3 ± 0.3	2.9 ± 0.1	3.1 ± 0.5
LDL-c (mmol/L)	5.7 ± 0.8	5.7 ± 0.7	5.5 ± 0.6	5.5 ± 0.4	4.9 ± 0.7
T-AOC (U/mL)	5.7 ± 0.1^{b}	9.1 ± 0.3^{ab}	11.9 ± 2.8^{a}	11.9 ± 1.4^{a}	9.7 ± 0.3^{ab}
MDA (mmol/L)	25.9 ± 0.1	39.2 ± 6.4	38.3 ± 2.1	35.7 ± 5.3	39.4 ± 8.6

Abbreviations: ALT, alanine aminotransferase; TP, total protein; GB, Globulin; AB, Albumin; GLU, glucose; TAG, triacylglyceride; T-chol, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein; T-AOC, total antioxidant capacity; MDA, malondialdehyde. Data were obtained from six different specimens per diet; different superscripts in the same row denote significant differences ($P < 0.05$).

a four-layered wall containing the mucosa, submucosa, muscular layer, and outer layer. The intestinal mucosa with prominent villi was organized in three layers: a simple columnar epithelium with prominent microvilli and scattered goblet cells, a *lamina propria* and muscular mucosae (Fig. 1b). No fat deposits were found within enterocytes regardless of the level of dietary lipids. Although the tallest villi were found in fish from the 9L group (581 ± 102 μm) and the shortest villi were found in fish fed the 5 L diet (445 ± 59 μm), no significant differences among dietary groups were observed regarding the size of villus height. The rest of the dietary groups showed intermediate values (450–578 μm) between those mentioned above dietary (Fig. 1c; $P > 0.05$).

3.4. Gene expression

The relative expression of genes involved in lipid metabolism and immune response in the hepatopancreas of *O. macrolepis* fed diets with different lipid levels are illustrated in Fig. 2. No statisitically significant differences in the relative expression of gene markers involved in lipid metabolism (*pparα*, *cpt-1α*, *fas,* and *hsl*) were found in the hepatopancreas of fish fed diets containing different lipid levels ($P > 0.05$; Fig. 2a). Similarly, different dietary crude lipid levels did not modulate gene expression patterns in selected immune markers (*tlr 22*, *nrf 2*, *tnfα* and *il-γ*) ($P > 0.05$; Fig. 2b).

4. Discussion

4.1. Effect of lipid level on lipid metabolism in adult O. macrolepis

In teleosts, lipid stores can generally be found in the muscle, subcutaneous tissue, liver, head, viscera, and other organs (Sheridan, 1988). Still, the overall proximate lipid content of the body and preferential sites of lipid deposition varies significantly among species (as reviewed by Tocher, 2003). Usually, lipid content of whole-body and hepatopancreas or liver increased in fish as dietary lipid levels increased (González-Félix et al., 2015; Gou et al., 2019; Huang et al., 2016; Jin et al., 2013; Lee & Putnam, 1973; Liao et al., 2017; Rahman & Lee, 2017; Wang et al., 2015). Meanwhile superabundant dietary lipid may lead to excessive lipid deposition in the liver and larger lipid vacuoles, and the more pronounced histopathology in the hepatocytes of fish (Kestemont et al., 2001; Kowalska et al., 2011; Santander-Avanceña et al., 2021) and the organ degeneration that leads to necrosis and structural distortions in cell membranes (Kowalska et al., 2011) receiving feed with higher lipid content had been observed. In contrast, a few previous result showed that dietary lipid levels did not affect the fat content in the liver and muscle of senegalese sole juveniles (Bonacic et al., 2016). Similar with senegalese sole juveniles, the present study showed that the fat accumulation in the carcass and hepatopancreas in *O.macrolepis* were found not affected by dietary lipid levels. By HE staining and observation, the histological organization of the hepatopancreas, including hepatocyte membrane, nucleus, and lipid-like vacuolation (if there is), were similar among five dietary groups (being in the same health status; Fig. 1a), probably indicating the superior lipid metabolism ability of hepatopancreas in *O. macrolepis* facing with these five dietary lipid levels.

Lipid deposition in hepatic or other organs and tissues was connected with the lipogenesis and uptake of mobilized fatty acids from the adipose tissue (Vyas et al., 2012), which inspired the present authors to explore the mechanisms and the gene expression of fatty acid metabolism proteins, relating with fatty acid synthesizing and lipid catabolic enzymes, were determined respectively.

The *ppar α* is a critical nuclear receptor that stimulates the expression of genes related to fatty acid transport and β-oxidation (Haemmerle et al., 2011; Pineda et al., 2003). C*pt 1* is an essential gene in transporting long-chain fatty acids across the membranes of mitochondrial for β-oxidation (Bremer, 1981). H*sl* is the major lipid catabolic enzymes (Smirnova et al., 2006) and the lipolytic proteins. Previous research in grass carp (*Ctenopharyngodon idella*) showed that the mRNA levels of *ppar* α were improved by higher dietary lipid levels (HLL) in hepatopancreas, and the mRNA levels of *cpt 1* were improved by HLL in muscle (Zhou et al., 2017). Meanwhile, a similar trend of *ppar* α and *cpt* 1 mRNA expression improved by increasing lipid levels was found in the hepatopancreas of juvenile *Onychostoma macrolepis* (Gou et al., 2019), in the liver of Japanese seabass (Xie et al., 2021) and juvenile largemouth bass (Zhou et al., 2020). At the same time, the contrary findings were that increasing dietary (soybean) lipid levels down-regulated the mRNA levels of the lipolytic genes (such as *pparα, cpt1,* and *hsl*) in the liver of juvenile pond loach (*Misgurnus anguillicaudatus*) (Li et al., 2018). Meanwhile, *pparα* and *cpt* 1 mRNA expressions suppressed by a high-fat diet were reported on *M. amblycephala* (Lu et al., 2013) and in hybrid yellow catfish (Fei et al., 2022). No effect of dietary lipid levels on mRNA expression of *ppar α* was found in rainbow trout (Liu et al., 2021).

Fas plays a vital role in *de novo* lipogenesis by catalysing the reactions associated with converting acetyl-CoA and malonyl-CoA to palmitate (Smith et al., 2003). In juvenile pond loach (*Misgurnus anguillicaudatus*), increasing dietary soybean lipid levels up-regulated the messenger (m) RNA levels of lipogenic genes (such as *fas*) in the liver (Li et al., 2018), while in juvenile tongue sole high dietary lipid level depressed the expressions of fatty acid synthase (FAS) mRNA expression significantly (Yuan et al., 2017).

Lipid levels affecting lipogenitic, or lipolytic genes in fish differently probably reflected the complexity of lipid metabolism, which might contribute to an explanation of fat deposition in the liver or other tissues of the fish fed diet with increasing graded levels of lipid. In the present study, *ppar α*, *cpt 1, hsl,* and *fas* gene expression levels in the hepatopancreas of *O.macrolepis* were not significantly different in fish fed the diets with increased lipid level, being in line with the present result that fat content in carcass and hepatopancreas were not significantly different in different dietary lipids levels. The expression of genes related with lipid metabolism was somehow in line with previous findings in other fish, like in rainbow trout whose mRNA expression of

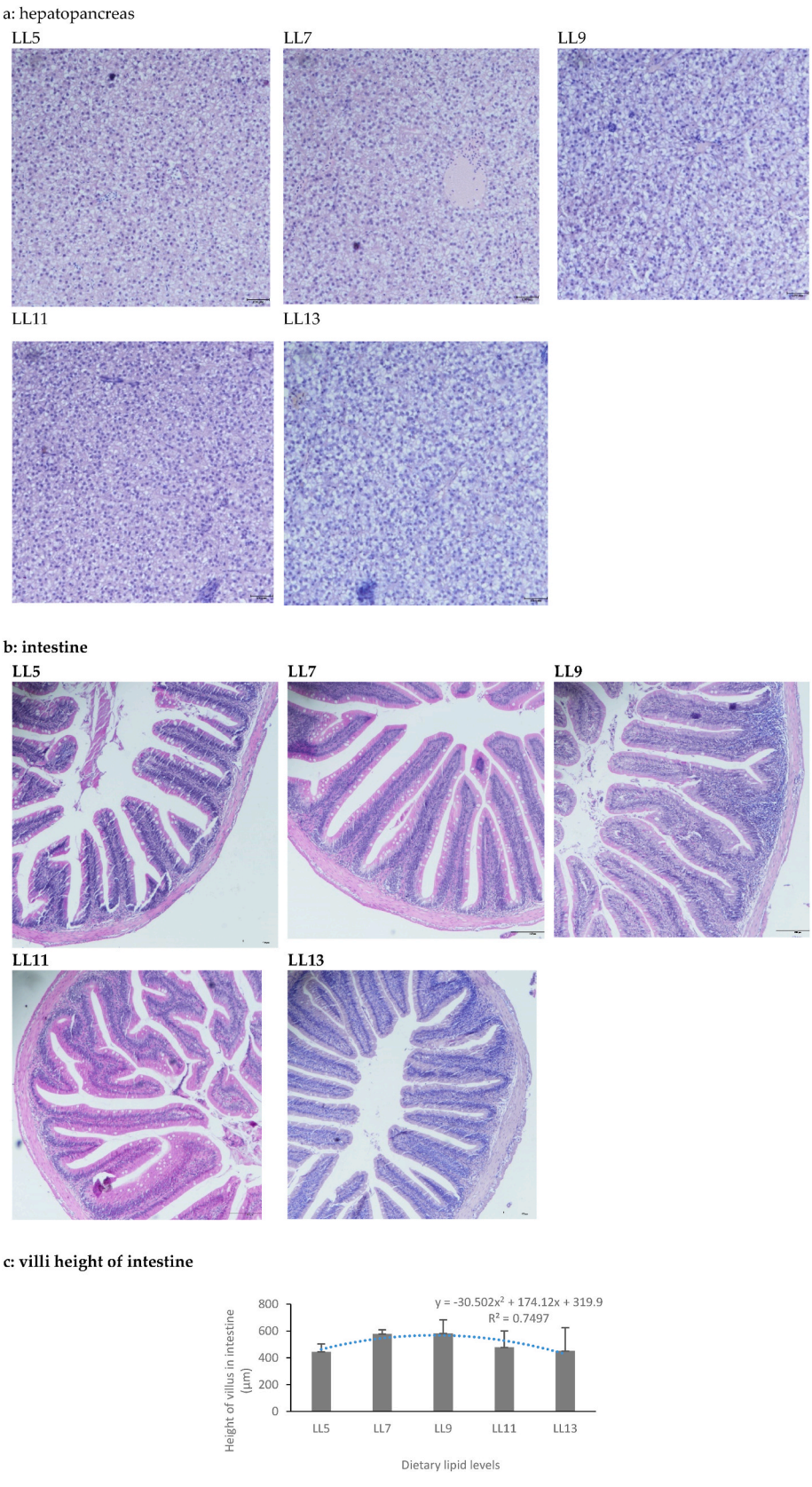

Fig. 1. Histological section and HE staining of hepatopancreas (a), intestine (b), and villus height of intestine (c) from adult *O. macrolepis* fed with five graded lipid levels. (40 × magnification; bar = 100 μm).

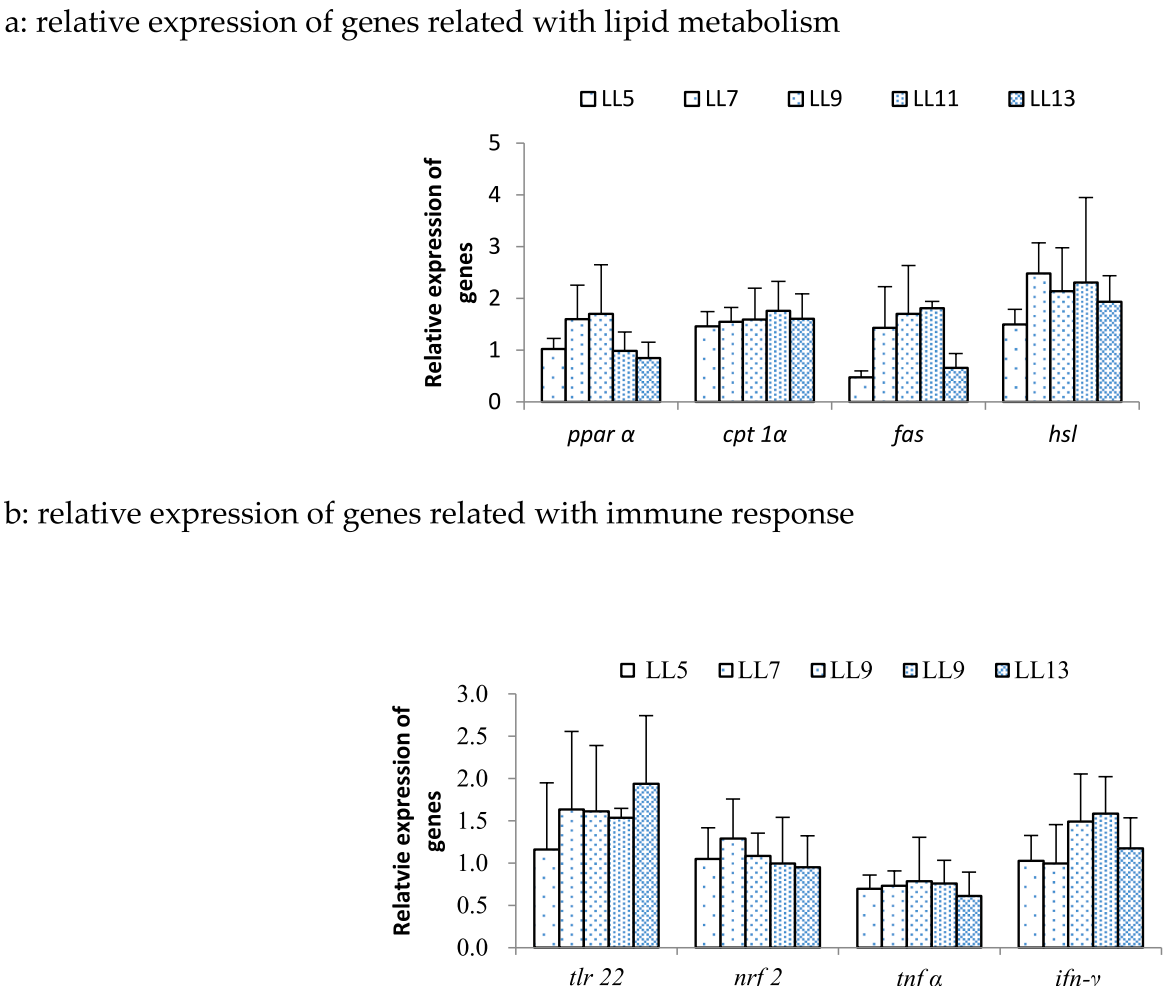

Fig. 2. Relative expression of genes from the hepatopancreas involved in lipid metabolism *(a; pparα, cpt-1α, fas,* and *hsl;a)* and immune response (b; *tlr 22, nrf 2, tnfα* and *il-γ;b*) in *Onychostoma macrolepis* fed diets differing in their crude lipid levels.

ppar α was not found affected by different dietary lipid levels (Liu et al., 2021). Considering the fish used in the present study is adult or up growing, the present result of lipid metabolism probably is caused by the possible fact that higher dietary lipid was utilized to supply gonad development, such as increasing the number of ovum or sperm but not only to store lipid in the liver or carcass. It also implies that adult fish may cope with high dietary lipid mainly through gonad development, being accompanied by regulating lipogenesis-related gene expression of *ppar α*, *cpt 1*, *fas,* and *hsl.*

4.2. Effect of lipid level on health status and immunity of O. macrolepis

Dietary nutrition, like lipid levels, obviously affects the health status of fish (Jin et al., 2013; Lim et al., 2009; Sheikhzadeh et al., 2012; Tocher et al., 2002; Yildirim-Aksoy et al., 2009). Biochemical indexes in blood or serum could show the status of nutrition and health of the fish (Patriche et al., 2011). Serum ALT is one of the important indexes of showing the function of the liver of the fish (Chen et al., 2003), where in health status the level of ALT is very low while it would increase highly as the liver or the hepatocytes are in damaged or unhealthy situation. In previous research, the trends of serum biochemical parameters showed varied in different fish as dietary lipid levels increased. For example, in bluefin tuna juveniles (*T. orientalis*), the hepatic ALT activity decreased as dietary lipid levels increased from 92 to 270 g kg^{-1} (Biswas et al., 2009). In rohu (*Labeo rohita* H.) fingerlings, liver ALT activities decreased with the increased inclusion of lipids in the diets (Siddiqua & Khan, 2022). Meanwhile, in tilapia (*Oreochromis aureus x Tilapia nilotica*), serum ALT activity was observed to decrease with the increasing dietary lipid level (Ma et al., 2016). In Tongue sole (*Cynoglossus semilaevis*), the hepatic ALT activities increased as dietary lipid levels increased (Liu et al., 2013). In the present study, serum ALT of *O. macrolepis* was not significantly different among five diets, being similar to the previous result that in grass carp and in the greenfin horse-faced filefish juvenile, serum ALT was not affected by lipid levels (Xu et al., 2021; Zhou et al., 2017). This discrepancy might be caused mainly by differences in fish species, sizes, dietary lipid sources and levels, feeding duration, and environmental factors used in these studies. The present result probably suggested that the dietary lipids of 60 g/kg and 130 g/kg are at in an appropriate levels without adverse effects on the liver function of the adult *O. macrolepis*.

Serum TP is the index of the function of the liver or hepatopancreas, showing the protein synthesize rate (Bernet et al., 2001), and the increase in plasma TP was postulated to due to the elevated lipoprotein levels required for the transport of excess lipid (Lim et al., 2009). Serum lipid levels, such as serum total glycerol (TG), total cholesterol HDL-c and LDL-c was correlated with lipid transportation between tissues or organs and lipid metabolism in the body (Gao et al., 2005; Zhao, 2006). As HDL contains apolipoprotein A-I (apoA-I), it is generally considered a good protein for its protection against the development of atherosclerosis induced by cholesterol in mammals (Gordon & Rifkind, 1989; Rubin et al., 1991). The previous results in hybrid snakehead fingerling showed no difference in plasma TG and TP content between lipid level groups (Zhang et al., 2017), and the contents of TC, TG, HDL-c, and LDL-c in serum of tilapia were not affected as dietary lipid level

increased (Ma et al., 2016). In line with these previous results, the present result showed no significant different effect in these serum biochemical parameters among diets respectively, which probably indicated the same efficient capacity of *O. macrolepis* in using lipid among these dietary lipid levels.

The health status of the intestine, decided by intestine morphology, is vital to absorb nutrients. The morphology of the intestinal villi, such as villus amount and villus height, directly affects the surface area of the villus, which in turn influences the ability to absorb nutrients (Petit et al., 2007). Previous research showed that the increase of exogenous lipid content enlarged the area of the intestine exposed to nutrients and enhanced the absorption capacity of the intestine for nutrients (Caspary, 1992), and high-lipid diets could increase the villus height of rats (Sagher et al., 1991) and rainbow trout (Liu et al., 2021). In pikeperch, more giant and more numerous lipid vacuoles and lipid droplets in the enterocyte supranuclear vacuoles were confirmed in higher dietary lipid groups (Kowalska et al., 2011). The present study showed that dietary lipid level of 9%–11% had the highest villus height compared to the other dietary lipid levels (Fig. 1c), probably suggesting that 9%–11% is the appropriate dietary lipid level for the intestine health of *O. macrolepis*.

Dietary nutrients could stimulate the immune system of fish and help to fend off diseases and keep the healthy status of fish (Viswanath, 2012), which had been identified by scientific evidence gathered over the past thirty years. The non-specific immune system is more important for disease resistance than the specific immune system (Anderson, 1992), and the antioxidant defenses, being a kind of non-specific immune system and functioning as removal of reactive oxygen species (ROS), somehow depend on nutritional factors (Sheikhzadeh et al., 2012). Lipid, as a principal source of energy and essential fatty acids (NRC, 2011), is a macronutrient and a crucial dietary regulation factor for immune system of the fish. In the present study, serum MDA contents showed the increasing trend as dietary lipid increased (Table 4), probably indicating the increased susceptibility of *O. macrolepis* to fatty acid peroxidation. Similar results were also observed in grass carp (Jin et al., 2013), greenfin horse-faced filefish juvenile (Xu et al., 2021), japanese seabass (Xie et al., 2021), juvenile Nile tilapia (Wangkahart et al., 2022), hybrid yellow catfish (Fei et al., 2022), juvenile largemouth bass (Zhou et al., 2020) and European grayling (*Thymallus thymallus*) (Rahimnejad et al., 2021), that lipid peroxidation and oxidative stress were revealed increased in a fish fed high-fat diet. Accompany with the increased MDA, and serum T-AOC activities increased firstly as dietary lipid levels increased from 5% to 9%, then decreased as dietary lipid levels continuously increased to 13% (Table 4), probably suggesting that dietary lipid as an immunostimulant (Lin & Shiau, 2003), fish may have limited ability to alleviate oxidative stress induced by high-fat diets and that dietary lipid level of 9%–11% would be available for adult *O. macrolepis* to keep high antioxidant ability.

The innate immunity in fish, as in all vertebrates, being the first line of defense and providing crucial signals for the activation of adaptive immune responses (Akira, 2003), could be regulated by dietary lipids. In humans, excess intake of a high-fat diet was reported to stimulate specific ligand of toll-like receptors (TLRs) of the immune system (Arya & Bhandari, 2020). In many fish, such as Japanese seabass, black seabream, blunt snout bream, and tilapia, the mRNA expression of inflammation-related genes (such as *interleukin 1β*, and *tumor necrosis factor α*) was reported up-regulated with the increasing dietary lipid levels (Xie et al., 2021; Dai et al., 2019; Jia et al., 2020; Jin et al., 2020). Reports in young grass carp showed that dietary low or excess levels of lipids impaired immune function by increasing inflammatory responses partly related to NF-kB and TOR-related signaling molecules, being both pro-inflammatory cytokines (such as IL-1b, IL-8, and TNF-a) and anti-inflammatory cytokines (such as TGF-b1 and IL-10) (Ni et al., 2016). In abalone, certain high dietary lipid levels significantly up-regulated the mRNA levels of inflammation-related genes (such as *tnfα*, *nf- κb*, *irak4*, *tlr2*,*tlr4,* and *perilipin- 2*) (Guo et al., 2021). These results indicated that arising of inflammation response is related to inappropriate dietary lipid levels, and the optimal lipids content could inhibit inflammation in the fish. In the current study, the TLR2 signaling pathway and cytokines, including *tlr22*, *nrf2*, *tnfα* and *il-γ* in hepatopancreas tissue, were evaluated and the relative expression level of thes genes, were not significantly affected by dietary lipid levels. Similar results were also reported in yellow catfish (Fei et al., 2022). The present result probably indicated that the dietary lipid level of 5%~13% would not do harm to the immune responses of the adult *O. macrolepis*.

5. Conclusions

In summary, the present data revealed that the lipid metabolism and fat accumulation were not affected by dietary lipid level in *O.macrolepis*, while 9%–11% dietary lipid levels had higher health status with considerable higher serum T-AOC and height of intestine villus.

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Institutional review board statement

This study was conducted following the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 53/2013) and authorized by the Ethical Committee of IRTA (Spain) and by the Ethical committee of NWAFU (China) for the use of laboratory animals.

Data availability statement

The data that support the findings are available upon direct request to the corresponding author.

CRediT authorship contribution statement

Jishu Zhou: Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Peng Feng:** Validation, Writing – review & editing. **Yang Li:** Methodology, Writing – review & editing. **Hong Ji:** Conceptualization, Writing – review & editing, Funding acquisition. **Enric Gisbert:** Writing – review & editing, Visualization.

Declaration of competing interest

None.

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