Original Research Article

A comparative study on the tolerance of tilapia (*Oreochromis niloticus*) to high carbohydrate and high lipid diets

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ABSTRACT

A 12-wk trial was conducted to compare the tolerance of tilapia to high carbohydrate and high lipid diets. Three isonitrogenous and isoenergetic diets, whose carbohydrate and lipid levels were the following: 35.0% and 8% (control), 44.2% and 4% (D1, high carbohydrate), and 25.8% and 12% (D2, high lipid), respectively. Three hundred tilapias (27 ± 0.11 g) were fed the diets for 10 wk (4 replicates per group); 72 fish from the D1 group were continually fed the D1 (D1D1) and 72 fish from the D2 were continually fed the D2 (D2D2) diet for 2 wk (3 replicates each group) to evaluate the tilapia's capacity to tolerate high carbohydrate and high lipid diets, respectively. Another 36 fish from D1 group were continually fed D2 (D1D2) for comparison with D1D1 and D2D2 groups. In phase 1, hepatosomatic index, liver triglycerides (TG), glucose tolerance (GT) and crude protein in the whole body in D1 group were higher than those in D2 group ($P < 0.05$). During phase 2, D1D1 group had lower feed intake and weight gain, as well as lower serum total protein and albumin than that of D2D2 group ($P < 0.05$), while its liver glycogen was significantly higher than that in D1D2 and D2D2 groups ($P < 0.05$). Moreover, serum glucose and GT were higher in D1D1 and D1D2 groups than those in D2D2 group ($P < 0.05$). By contrast, D2D2 group had significantly higher intraperitoneal fat, subcutaneous adipose tissue (SCAT) and liver TG than those in D1D1 group ($P < 0.05$). The mRNA expression of brain *npy*, hepatic *nrf2*, *gst1* and hepatic transcriptomic data showed that immune-related genes (*gama*, *mrc2*, *mhc2* and *cd163*), were downregulated in D1D1 group compared to D2D2 and D1D2 groups. Taken together: 1) tilapia have higher tolerance to a high lipid diet than high carbohydrate diet; 2) despite retention of glucose tolerance, the continuous feeding of D1 diet impaired tilapia's appetite, weight gain rate and host immune response; 3) specific distribution of fat in intraperitoneal regions, SCAT and liver may be a risk-avoidance strategy in tilapia in response to a continuous D2 diet.

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1. Introduction

Globally, fish and seafood products constitute the third highest source of dietary protein consumed by humans after cereals and milk, representing 6.4% of total protein supply (19.8% of total animal protein supply) (Tacon and Metian, 2018). Capture fisheries have not grown in recent years with populations instead relying on the growing aquaculture industry. The growing aquaculture sector has seen an increased need for ingredients such as fishmeal or other protein sources, which has resulted in overfishing and/or

pressure on protein sources (Naylor et al., 2021). It has been reported that an appropriate amount of digestible carbohydrates and lipids in diets formulated for farmed aquatic species is important to spare the use of protein as an energy source (Zhou et al., 2016). Therefore, different carbohydrate and lipid levels have been studied in many fish species for their "protein sparing effect", which minimizes protein content in aquafeed formula (Abdel-Ghany et al., 2021; He et al., 2015; Hillestad and Johnsen, 1994; Honorato et al., 2010; Liu et al., 2022; Yan et al., 2015).

Until now, dietary carbohydrate and lipid levels have been investigated in species including, but not limited to, Australia redclaw crayfish, blunt snout bream, juvenile black seabream and juvenile rockfish (Lee and Kim, 2009; Li et al., 2013; Taj et al., 2020; Zhu et al., 2013). The ability of fish to use dietary carbohydrates and lipids as an energy source varies among species, such that the tolerated dietary carbohydrate levels in omnivorous fish, such as juvenile Australia redclaw crayfish (21.98% to 29.01% carbohydrate, 8.07% to 10.09% lipid) and fingerling blunt snout bream (23.6% to 32.9% carbohydrate, 5.8% to 9.6% lipid), are higher than that of carnivorous fish, such as juvenile black seabream (10.05% to 18.00% carbohydrate, 7.95% to 13.14% lipid) and juvenile rockfish (13.2% to 19.8% carbohydrate, 12.7% to 16.2% lipid). This suggests that omnivorous species have a higher capacity to utilize dietary carbohydrates compared to carnivorous fish, while the capacity for dietary lipid utilization was the opposite (Lee and Kim, 2009; Li et al., 2013; Taj et al., 2020; Zhu et al., 2013). All of these expected results have been explained by the poor use of dietary carbohydrates in carnivorous fish species, which possess poor regulation of lipogenesis, gluconeogenesis and amino acid catabolism in the liver (Rocha et al., 2016). Nevertheless, tilapia, an omnivorous fish, can tolerate high carbohydrate levels of 38% to 46% (7.0% to 7.38% lipid) (Wang et al., 2005), as well as lipid levels of 11% to 14% (28.82% to 35.19% carbohydrate) (Ali and Al-Asgah, 2001), which differ from classical fish nutrition theory, which postulates that fish have limited ability to utilize dietary carbohydrates and high fat diets are likely to impair lipid homeostasis, resulting in metabolic disorders. For example, the tolerated level of glucose in blunt snout bream is below 45% and lipids are tolerated at a level of less than 10.5% in golden pompano (Ren et al., 2021b; Xu et al., 2021). Tilapia generally require 30% to 36% starch and 7% to 10% lipid for optimum growth, thus 38% to 46% starch or 11% to 14% lipid in the diet has been commonly called a "high-starch" or "high-lipid diet" (Abdel-Ghany et al., 2021; Wang et al., 2005; Wei et al., 2012; Xie et al., 2017). Therefore, it is important and interesting to verify whether higher levels of macronutrient (high carbohydrate or high lipid) are sustainable and their possible difference impacts on tilapia feeding.

Tilapia (*Oreochromis niloticus*) is an important aquaculture species worldwide and a good fish model for metabolic studies due to its rapid growth, high disease and stress resistance (Deng et al., 2010), and availability of a complete reference genome (Guyon et al., 2012). With these aforementioned considerations, 10 wk of isonitrogenous and isoenergetic diets were fed to tilapia divided into 3 groups: control (moderate carbohydrate and lipid), D1 (high carbohydrate) and D2 (high lipid). The fish were fed a further 2 wk of the D1 (D1D1, high carbohydrate in phase 1 and high carbohydrate in phase 2) or D2 (D2D2, high lipid in phase 1 and high lipid in phase 2) diet to investigate and compare the tolerance of tilapia to high carbohydrate and high lipid diets. A hepatic transcriptomic study was also performed to identify the regulatory pathway of nutrients in tilapia. Based on this study, we may be able to provide an overview of tilapia with respect to tolerating high carbohydrate and high lipid diets, respectively.

Table 1
Formulation and proximate composition of the basal diet (g/kg, as-fed basis).

Item	Carbohydrate to lipid ratio		
	35:8	44.2:4	25.8:12
	Control	D1 (high carbohydrate)	D2 (high lipid)
Ingredients			
Corn starch	35.00	44.20	25.60
Casein	30.00	30.00	30.00
Gelatin	7.50	7.50	7.50
Soybean oil	4.51	2.25	6.76
Linseed oil	3.49	1.75	5.24
Cellulose	12.27	7.07	17.67
Monocalcium phosphate	2.00	2.00	2.00
Choline chloride	0.25	0.25	0.25
Vitamin premix[1]	2.00	2.00	2.00
Mineral premix[2]	2.00	2.00	2.00
Cr_2O_3	0.03	0.03	0.03
Ester vitamin C	0.20	0.20	0.20
Taurine	0.75	0.75	0.75
Total quantity	100.00	100.00	100.00
Dry matter	90.00	89.62	90.43
Crude protein	32.62	33.62	33.23
Crude lipid	7.89	4.13	11.90
Crude ash	1.68	1.81	1.71

[1] Vitamin premix: vitamin A, 3,500,000 IU/kg; vitamin D, 1,000,000 IU/kg; vitamin E, 20 g/kg; vitamin K_3, 2.5 g/kg; ascorbic acid, 63 g/kg; thiamin-HCl, 4 g/kg; riboflavin, 15 g/kg; pyridoxine-HCl, 6 g/kg; cyanocobalamin, 0.025 g/kg; D-calcium pantothenate, 20 g/kg; niacin, 25 g/kg; folic acid, 2.5 g/kg; D-biotin, 0.05 g/kg; inositol 70 g/kg and the carrier rice chaff. The vitamin premixes were obtained from Guangzhou Hinter Bio-technology Co., Ltd., China.
[2] Mineral premix (g/kg): $Ca(H_2PO_4)_2$, 123.2; Zeolite powder, 474.22; NaH_2PO_4, 42.03; K_2SO_4,163.83; $FeSO_4$, 10.8; ferric citrate, 38.4; $MgSO_4$, 44; $ZnSO_4$, 1.2; $MnSO_4$, 0.4; sodium selenite, 0.6; $CuSO_4$, 0.22; $CoSO_4$, 0.52; $CaIO_3$, 0.02; NaCl, 32.4; KCl, 66; microcrystalline cellulose, 157.2. The Mineral premixes were obtained from Guangzhou Hinter Bio-technology Co., Ltd., China.

2. Materials and methods

2.1. Animals ethics statement

All experiments were conducted under the Guidance of the Care and Use of Laboratory Animals in China (GB/T 35892-20118).

2.2. Diets, animals and experimental design

Casein and gelatin, soybean oil and fish oil were used as dietary protein and lipid sources and the diets were made as previously described (Ning et al., 2016). The formulation and proximate composition of the 3 diets are presented in Table 1. More than 500
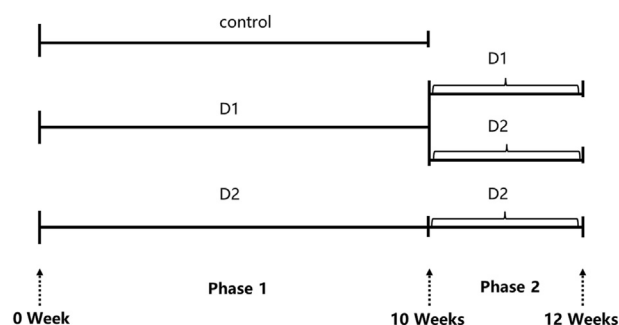

Fig. 1. Experimental protocol of the 2-phase feeding trial. D1 = high carbohydrate; D2 = high lipid; control = moderate carbohydrate and lipid.

Table 2
Biological status of juvenile tilapia fed test diets for 2 phases.

Phase	Groups	VSI, %[1]	HSI, %[2]	IPF,%[3]	CF[4],%[4]	SR[5], %[5]
Phase 1	Control	10.88 ± 0.49	2.29 ± 0.09[ab]	2.12 ± 0.33	3.6 ± 0.13	98.00 ± 3.46
	D1	9.76 ± 0.33	2.50 ± 0.24[a]	1.41 ± 0.15	3.50 ± 0.19	100.00 ± 0.00
	D2	10.66 ± 0.37	1.90 ± 0.13[b]	2.00 ± 0.26	3.67 ± 0.06	99.00 ± 1.73
Phase 2	D1D1	11.11 ± 0.93	3.16 ± 0.25	0.63 ± 0.15[b]	3.60 ± 0.13	100.00 ± 0.00
	D1D2	10.80 ± 0.43	2.53 ± 0.18	2.02 ± 0.21[a]	3.59 ± 0.05	100.00 ± 0.00
	D2D2	12.30 ± 0.27	2.48 ± 0.26	2.75 ± 0.55[a]	3.65 ± 0.13	100.00 ± 0.00

VSI = viscerosomatic index; HSI = hepatosomatic index; IPF = intraperitoneal fat index; CF = condition factor; SR = survival rate; Control = moderate carbohydrate and lipid; D1 = high carbohydrate; D2 = high lipid; D1D1 = high carbohydrate in phase 1 and high carbohydrate in phase 2; D1D2 = high carbohydrate in phase 1 and high lipid in phase 2; D2D2 = high lipid in phase 1 and high lipid in phase 2.
[ab] Values with the same column with different letters are significantly different ($P < 0.05$).
[1] Viscerosomatic index (VSI, %) = 100 × (visceral weight, g)/(whole body weight, g).
[2] Hepatosomatic index (HSI, %) = 100 × (liver weight, g)/(whole body weight, g).
[3] Intraperitoneal fat index (IPF, %) = 100 × (intraperitoneal fat weight, g)/(whole body weight, g).
[4] Condition factor (CF, %) = 100 × (fish weight, g)/(body length, cm)^3.
[5] Survival rate (SR, %) = 100 × (final fish number)/(initial fish number).

juvenile tilapias were obtained from a local fish hatchery. Before the feeding trial, all fish were acclimated in three 500-L tanks for 2 wk. During the acclimation period, fish were fed with a commercial diet. The schedule of the formal 2-phase feeding trial is shown in Fig. 1. The first feeding phase started after acclimation. Three hundred healthy juvenile tilapia of similar weights (27.00 ± 0.11 g) were selected and randomly distributed into 12 tanks (500 L, 25 fish per tank, 4 replicated tanks per group) which were connected as a recirculating system containing freshwater at

the feeding facility of South China Agricultural University. All fish were divided into 3 groups and fed with 3 isonitrogenous and isoenergetic diets (33.0% protein, 16.9 MJ/kg gross energy): a control group (carbohydrate and lipid, 35.0% and 8%), the D1 group (carbohydrate and lipid, 44.2% and 4%), and D2 group (carbohydrate and lipid, 25.8% and 12%), respectively. At the end of the first feeding phase trial, fish growth among the 3 groups was comparable. Seventy-two and 36 tilapias from the D1 and D2 groups, respectively, with similar body weights (91.58 ± 0.96 g) were
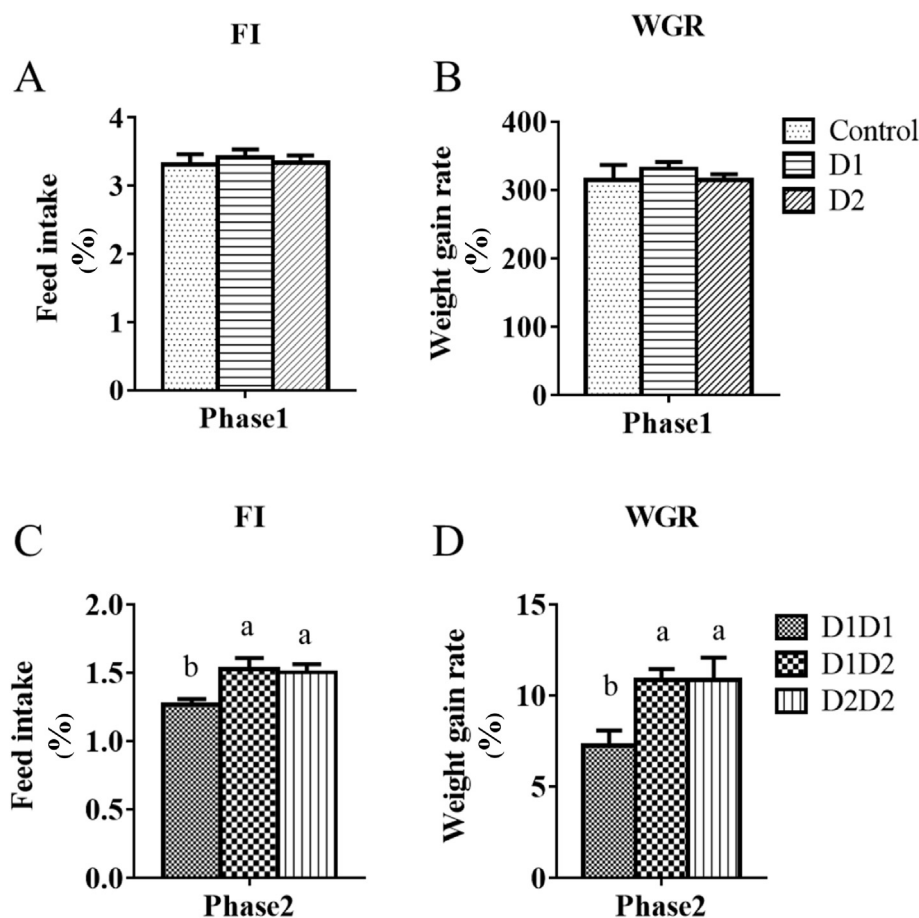

Fig. 2. Effects of treatments on feed intake and growth during 2 phases. (A and B) Feed intake and weight gain rate during phase 1. (C and D) Feed intake and weight gain rate during phase 2. Values are means ± SEM ($n = 4$ for A to B, $n = 3$ for C to D). Bars with different letters are statistically different ($P < 0.05$). D1 = high carbohydrate; D2 = high lipid; D1D1 = high carbohydrate in phase 1 and high carbohydrate in phase 2; D2D2 = high lipid in phase 1 and high lipid in phase 2.

Table 3
Whole body composition of juvenile tilapia fed test diets for 2 phases (%, as-is basis).

Phase	Groups	Crude protein	Crude lipid	Crude ash	Moisture
Phase 1	Control	15.53 ± 0.17^{ab}	11.62 ± 0.35	3.63 ± 0.13	68.8 ± 1.13
	D1	15.91 ± 0.14^{a}	10.56 ± 0.27	3.37 ± 0.19	69.82 ± 0.83
	D2	15.36 ± 0.13^{b}	10.90 ± 0.23	3.40 ± 0.15	68.44 ± 0.39
Phase 2	D1D1	15.13 ± 0.38	9.99 ± 0.29	3.48 ± 0.19	70.02 ± 2.42
	D1D2	15.84 ± 0.41	9.37 ± 0.53	3.26 ± 0.05	70.07 ± 2.52
	D2D2	15.05 ± 0.38	10.51 ± 0.55	3.20 ± 0.06	69.54 ± 1.70

Control = moderate carbohydrate and lipid; D1 = high carbohydrate; D2 = high lipid; D1D1 = high carbohydrate in phase 1 and high carbohydrate in phase 2; D1D2 = high carbohydrate in phase 1 and high lipid in phase 2; D2D2 = high lipid in phase 1 and high lipid in phase 2.
[ab]Values with the same column with different letters are significantly different ($P < 0.05$).

selected for the second phase of the feeding trial. Fish selected from the D1 group were then randomly divided to 2 subgroups and fed with the D1 or D2 diet (2 subgroups × 3 tanks, 12 fish/tank) and fish from the D2 group were further fed the D2 diet (1 subgroup × 3 tanks, 12 fish/tank) for 2 wk. During the 10-wk and 2-wk trial, fish were fed at 09:00 and 18:00 with an equal portion of diet at a feeding rate of 3% to 4% BW per day. Fish per tank were counted and weighed every 2 wk. Water temperature was maintained at $29 \pm 1\ °C$ with a 12-h light–dark cycle for 12 wk.

2.3. Growth performance

At the end of the 2 phases, fish were weighed and feed intake (FI), initial body weight (IBW), final body weight (FBW), weight gain rate (WGR), survival rate (SR), condition factor (CF), viscerosomatic index (VSI) and hepatosomatic index (HSI) were calculated using the following equations:

FI (%/day) = 100 × (total dry feed intake)/[(IBW + FBW)/2]/feeding days,

WGR (%) = (FBW − IBW) × 100/IBW,

CF (%) = (fish weight, g) × 100/(body length, cm)^3,

VSI (%) = (viscera weight, g) × 100/(whole body weight, g),

SR (%) = (final fish number) × 100/(initial fish number),

HSI (%) = (hepatic weight, g) × 100/(whole body weight, g).

2.4. Sampling and measurements of biochemical parameters

At the end of the 2 phases, all fish fasted overnight, and then 12 and 9 fish from phase 1 and 2, respectively, were euthanized with eugenol (1:10,000) and tissues and serum were collected (drawn from the caudal vein). Among them, 2 fish from each group of phases 1 and 2 were randomly selected for body parameter and biochemical measurements, and transcriptome and gene expression analysis. Another 4 and 3 fish from phases 1 and 2, respectively, were used for whole body composition measurements. Liver and muscle triglycerides (TG) and glycogen, serum amino acid (AA), free fatty acid (FA), pyruvate, glucose and lactate were assessed using commercial kits (Jiancheng Biotech Co., China). Remaining serum TG, total cholesterol (TC), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), globin, glucose and urea were measured using a COBAS c702 trans auto analytic machine (Royal, Netherlands).

2.5. Glucose tolerance test

At the end of the trials, 15 fish from each group were collected for a glucose tolerance test using an i.p. injection of D-glucose (500 mg/kg BW, 20% in 0.85% NaCl) (Sigma) after fasting overnight. Blood of 3 fish per group was drawn from the caudal vein at 0, 0.5, 1.0, 3 and 6 h after injection. Blood glucose was measured using glucometry (Baier Biotech Co.).

2.6. Quantitative real time PCR

RNA isolation, cDNA synthesis and quantitative PCR were performed as previously described (He et al., 2014). Primer details are provided in Supplemental Table S1. The genes for β-actin and elongation factor 1a (EF1α) of tilapia were used as housekeeping gene controls for normalization of gene expressions. The relative quantification of gene amplification by RT-PCR was performed using the value of the threshold cycle (Ct). The relative mRNA expression was estimated using the $2^{-\Delta\Delta Ct}$ method, $\Delta Ct = Ct_{target} - (Ct_{EF1\alpha} + Ct_{\beta\text{-actin}})/2$.

2.7. Transcriptomic analysis

The RNA for sequencing was equally pooled from 6 liver RNA samples after the feeding trials. Transcriptome sequencing was conducted using an Illumina HiSeq xten/Novaseq 6000 according to the manufacturer's instructions. The raw reads were filtered for quality control as described previously (Ning et al., 2016). To identify differential expression genes (DEGs) between 2 different samples, the expression level of each transcript was calculated according to the transcripts per million reads (TPM) method. RSEM (http://deweylab.biostat.wisc.edu/rsem/) (Dewey and Bo, 2011) was used to quantify gene abundances. Cluster analysis was conducted using distance algorithms (Spearman between samples and Pearson between genes). In addition, functional-enrichment analysis (KEGG) was performed to identify which DEGs were significantly enriched in metabolic pathways, set at a Bonferroni-corrected P-value of 0.05, compared with the whole-transcriptome background (Chen et al., 2011). KEGG pathway analysis was carried out by KOBAS (http://kobas.cbi.pku.edu.cn/home.do).

2.8. Histological study

Pieces of liver, muscle and subcutaneous adipose tissue (SCAT) (including muscle and skin) were fixed in 4% paraformaldehyde and embedded in paraffin as previously described (Betancor et al., 2015). Sections of 5 μm thick liver tissue (first phase) were stained with the hematoxylin and eosin mixture, Oil Red O and periodic acid-Schiff (PAS); other sections of 5 μm thick tissue (liver, SCAT and muscle, second phase) phase 2 were stained with PAS, and all of the above were examined under a light microscope (Abdel-Ghany et al., 2021).

2.9. Statistical analysis

Results are expressed as mean ± SEM. Differences among the 3 groups in the 2 phases were evaluated using one-way ANOVA followed by Tukey's multiple comparisons. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using SPSS 22 (SPSS, IL, USA).

3. Results

3.1. Growth performance

Growth performance indexes are shown in Table 2 and Fig. 2. During phase 1, there were no significantly differences in WGR, FI, SR, CF, VSI and IPF among groups after 10 wk of feeding ($P > 0.05$). HSI in D1 group was significantly higher than that in D2 group ($P < 0.05$). During phase 2, IPF, FI and WGR of D1D2 and D2D2

groups were significantly higher than those of D1D1 group ($P < 0.05$). No significant differences were observed in SR, HSI, VSI and CF among D1D1, D1D2 and D2D2 groups ($P > 0.05$).

3.2. Whole body composition

The effects of different diets on proximate composition of the whole body in tilapia after 2-phase feeding are presented in Table 3. ANOVA showed that crude protein levels were significantly affected
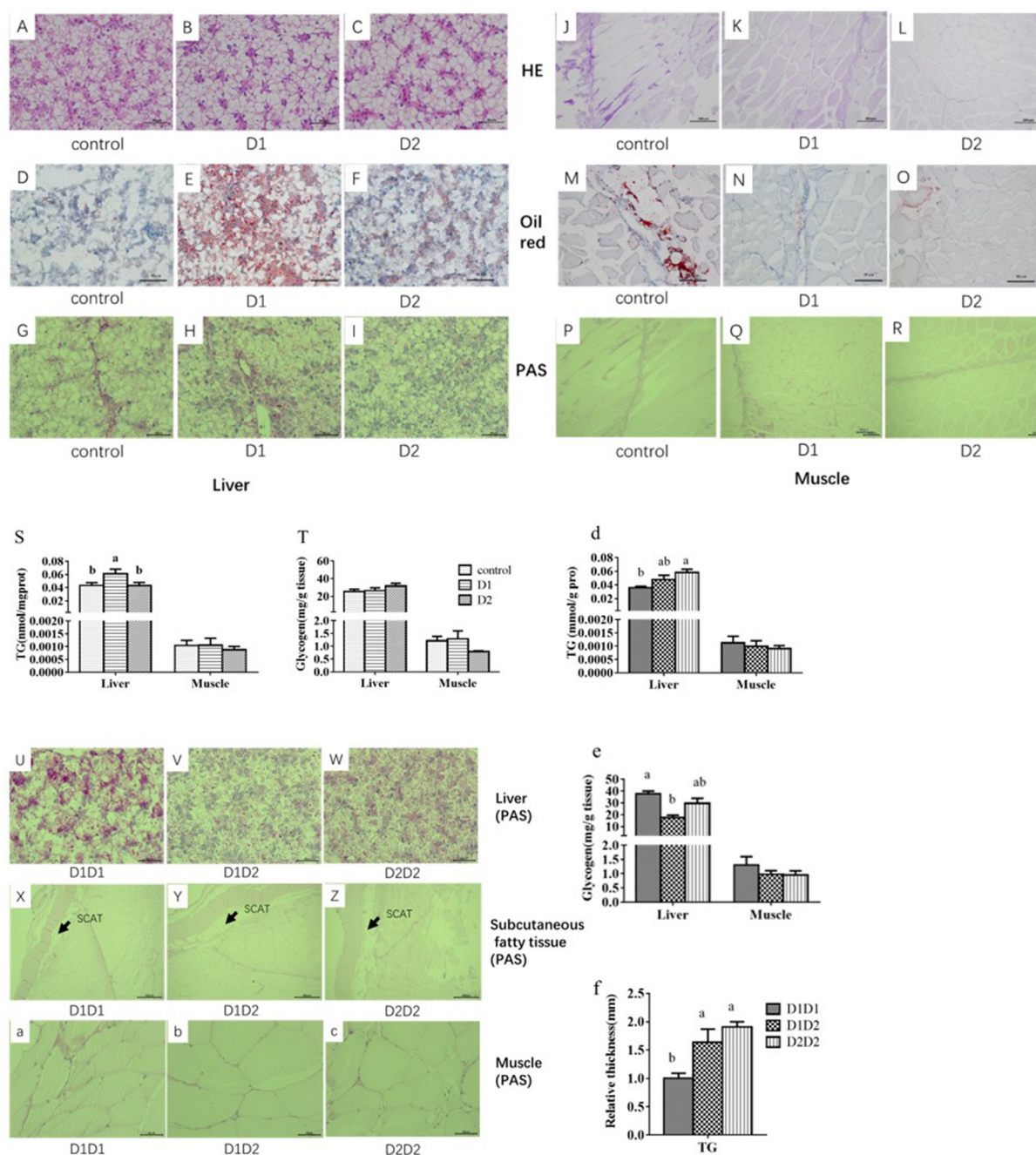

Fig. 3. Histological characteristics and lipid and glycogen quantitative analysis of liver, muscle and subcutaneous fatty tissue (SCAT). (A to I) Comparative photomicrographs (Hematoxylin & Eosin, Oil red O and PAS staining) of liver in phase 1. (J to R) Comparative photomicrographs (Hematoxylin & Eosin, Oil red O and PAS staining) of muscle in phase 1. (S to T) Triglycerides (TG) and glycogen contents of liver and muscle in phase 1. (U to Z) Comparative photomicrographs (PAS staining) of liver (U to W) and subcutaneous fatty tissue (SCAT) (X to Z) in phase 2. (a to c) Comparative photomicrographs (PAS staining) of muscle in phase 2; (d to e) TG and glycogen contents of liver and muscle in phase 2. (f) Quantitative analysis of thickness of SCAT. Values are means ± SEM ($n = 4$ for S to T, $n = 3$ for d to f). Bars with different letters are statistically different ($P < 0.05$).
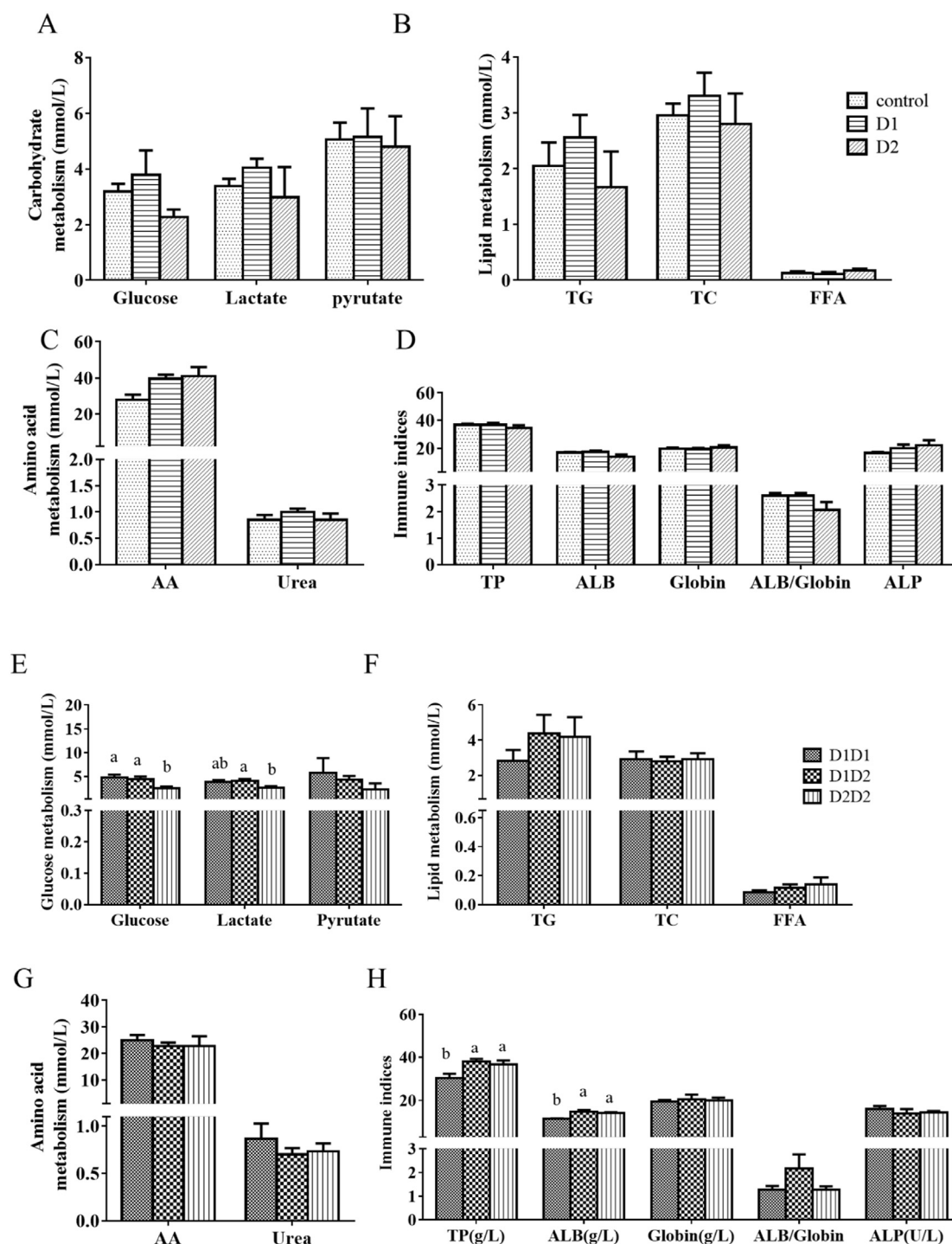
Fig. 4. Effects of treatments on serum biochemical indices during 2 phases. (A to C) Glucose, lipid and amino acid metabolism related triglycerides (TG), lactate, pyruvate, total glyceride, total cholesterol (TC), free fatty acid (FFA), amino acids (AA) and urea in phase 1. (D) Immune related total protein (TP), albumin (ALB), globin, albumin/globin ratio and alkaline phosphatase (ALP) in phase 1. (E to G) Glucose, lipid and amino acid metabolism related TG, lactate, pyruvate, TG, TC, FFA, AA and urea in phase 2. (H) Immune related TP, ALB, globin, albumin to globin ratio and ALP in phase 2. Values are means ± SEM ($n = 4$ for A to D, $n = 3$ for E to H). Bars with different letters are statistically different ($P < 0.05$).

by treatments during the first phase, where, the crude protein levels in fish fed D1 were significantly higher than those of D2 group ($P < 0.05$). Remaining indices showed no difference. In phase 2, moisture, crude protein, crude lipid and crude ash contents of whole-body composition of fish were not significantly different among D1D1, D1D2 and D2D2 groups ($P > 0.05$).

3.3. Tissue glycogen and lipid content

Systemic glycogen and lipid variations from treatments are presented in Fig. 3. In phase 1, liver TG in the control and D2 group was significantly lower than that in D1 group ($P < 0.05$). Liver histology (Fig. 3A to F) clearly indicated that more liver fat
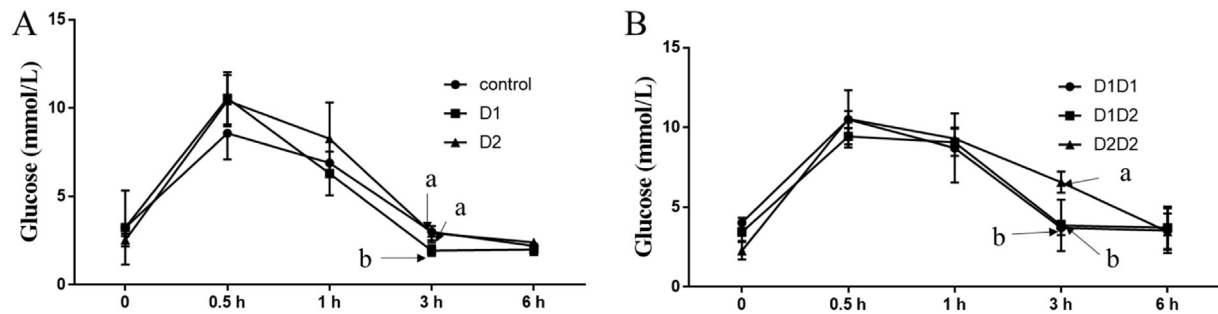
Fig. 5. Effects of the glucose tolerance test during the 2 phases. (A) serum glucose levels of fish subjected to glucose loading during phase 1. (B) Serum glucose levels of fish subjected to glucose loading during phase 2. Values are means ± SEM ($n = 4$ for A, $n = 3$ for B). Bars with different letters are statistically different ($P < 0.05$). D1D1 = high carbohydrate in phase 1 and high carbohydrate in phase 2; D2D2 = high lipid in phase 1 and high lipid in phase 2.

accumulated in D1 group than in the control and D2 group. Nevertheless, there were no significant differences in liver glycogen, muscle TG or muscle glycogen among the groups ($P > 0.05$). In phase 2, liver TG in D1D1 group was significantly lower than that in D2D2 group, and significantly higher than that in D1D2 group ($P < 0.05$) (Fig. 3). Liver histology (Fig. 3U to W) showed higher glycogen accumulation in D1D1 group than in D1D2 group. Fig. 3G and muscle histology (Fig. 3X to Z) showed thicker band gaps in D1D2 and D2D2 groups than in D1D1 group. There was no significant difference in muscle TG or glycogen among the groups ($P > 0.05$).

3.4. Serum biochemistry indices

Fig. 4 shows most indices were not affected by the treatments during the 2-phase trials. In phase 1, there was no significant difference in serum glucose, lactate, pyruvate, TG, TC, FA, AA, urea, TP, ALB, globin, ALB to globin ratio and ALP among control, D1 and D2 groups (Fig. 4A to D; $P > 0.05$). In phase 2, the serum levels of pyruvate, TG, TC, FA, AA, urea, globin, ALB to globin ratio and ALP showed no significant difference among the groups (Fig. 4E to H; $P > 0.05$). In contrast, serum glucose and lactate levels in D1D1 and D1D2 groups were significantly higher than those in D2D2 group
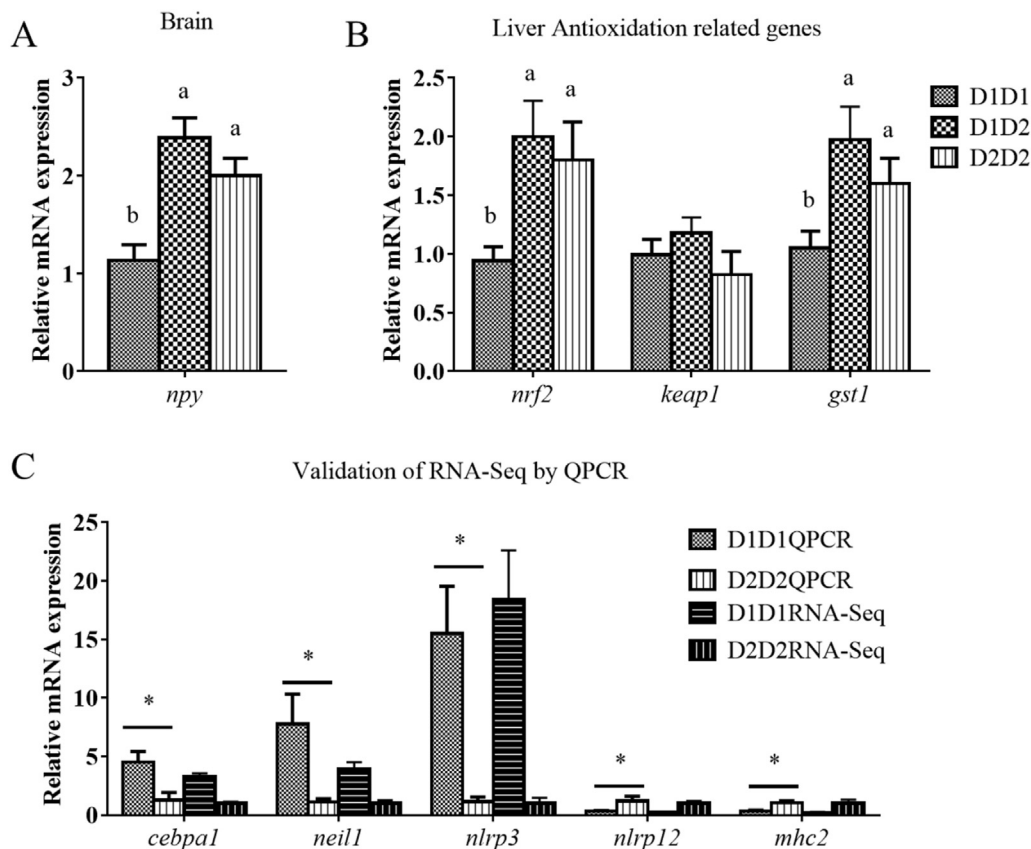

Fig. 6. Effects of a high carbohydrate or high lipid diet on gene expression during phase 2. (A) Appetite related gene: *npy*. (B) Antioxidation related genes: *nrf2*, *keap1* and *gst1*. (C) Validation of RNA-Seq by QPCR between D1D1 and D2D2. Values are means ± SEM ($n = 3$). Bars with different letters are statistically different ($P < 0.05$). Asterisk (*) indicates significant difference between D1D1 and D2D2 ($P < 0.05$). D1D1 = high carbohydrate in phase 1 and high carbohydrate in phase 2; D2D2 = high lipid in phase 1 and high lipid in phase 2. *npy* = neuropeptide Y; *nrf2* = nuclear factor erythroid 2-related factor 2; *keap1* = kelch-like ECH-associated protein; *gst1* = glutathione S-transferases 1; *cebpa1* = CCAAT/enhancer binding protein alpha 1; *neil1* = endonuclease VIII-like 1; *nlrp* = nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing; *mhc2* = HLA class II histocompatibility antigen.
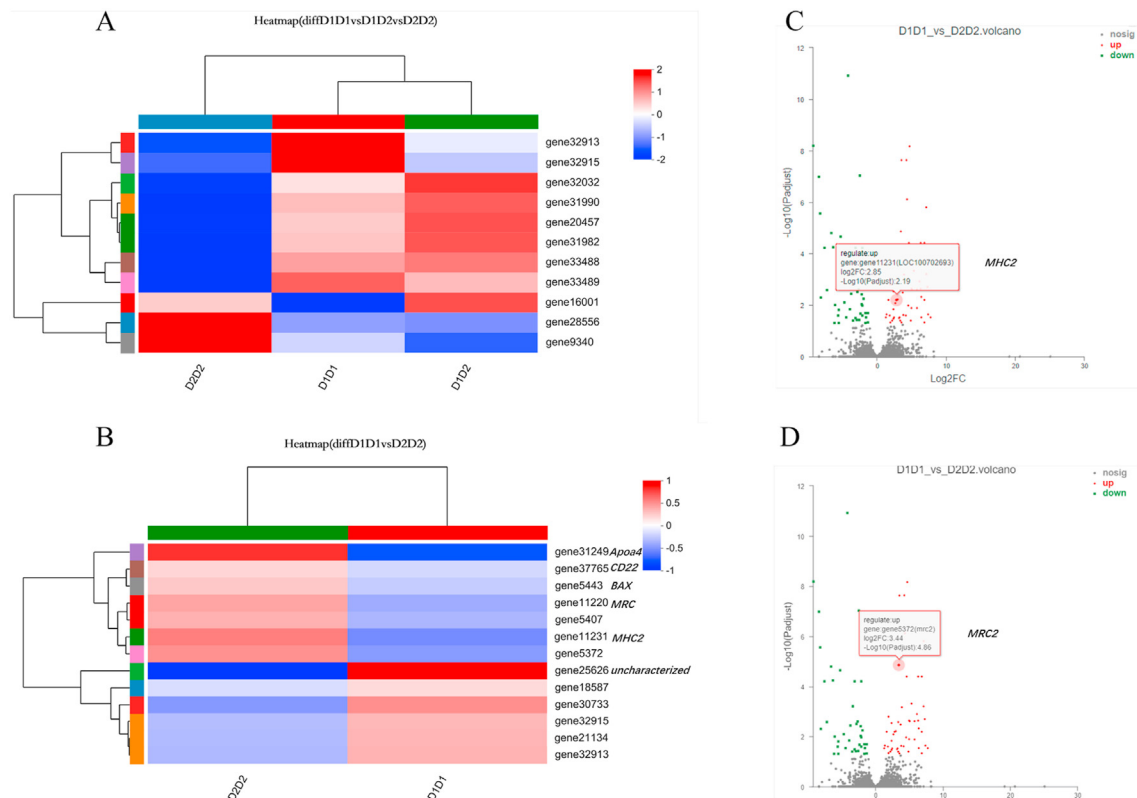
Fig. 7. Cluster and volcano analysis of differential expression genes (DEGs) among D1D1, D1D2 and D2D2. (A to B) Clustered heat map of hepatic transcriptome of tilapia among D1D1, D1D2 and D2D2, or between D1D1 and D2D2. Treatment groups showing similar expression patterns are clustered together. (C to D) Volcano map of DEGs between D1D1 and D2D2. Cluster analysis was conducted using a distance algorithm. Transcriptome enrichment in the heat map goes from low (blue) to high (red). *MHC2* = HLA class II histocompatibility antigen; *MRC* = mannose receptor C type 2; *Apoa4* = Apolipoprotein A-IV; *CD22* = cluster of differentiation-22; *BAX* = apoptosis regulator BAX-like.

($P < 0.05$), while serum ALB and TP levels in D1D1 group were significantly lower than those in D1D2 and D2D2 groups ($P < 0.05$). These results indicate that the D1 diet has negative effects on non-specific immunity indices of tilapia.

3.5. Glucose tolerance test results

During glucose tolerance tests, D1-fed fish showed faster glucose clearance (Fig. 5), indicating better insulin efficiency. In phase 1, glucose levels of D1 group were restored to normal levels after 3 h, which were significantly faster than control and D2 group ($P < 0.05$). In phase 2, glucose levels of D1D1 and D1D2 groups were restored to normal levels after 3 h, which were significantly faster than D2D2 group ($P < 0.05$), suggesting that D1 could strongly affect tilapia glucose utilization.

3.6. Molecular changes in brain and liver during the phase 2

Fig. 6 shows the gene expression levels in brain and liver during phase 2. The high carbohydrate diet decreased mRNA levels of brain nerve peptide y (Fig. 6A), hepatic nuclear respiratory factor 2 (*nrf2*) and *gst1* (Fig. 6B) after phase 2 feeding compared to D1D2 and D2D2 groups. By contrast, the high lipid diet (D2D2) significantly increased *mhc2* expression level compared to D1D1 (Fig. 6C).

3.7. Global regulatory effects of a high carbohydrate or high lipid diet on tilapia in a hepatic transcriptomic study

We conducted transcriptomic analyses using an Illumina Novaseq 6000 to identify differences in the liver related to

treatments. The number of high-quality reads (>Q30) were 49,042,077; 48,800,580; 48,607,682; 45,179,287 and 43,768,989 in the D1, D2, D1D1, D1D2 and D2D2 groups, respectively. Reads were mapped to the tilapia reference genome. Mapping rates of the D1, D2, D1D1, D1D2 and D2D2 groups were 95.23%, 95.24%, 94.80%, 95.04% and 94.83% respectively. A cluster analysis showed that the D1D1 and D1D2 groups were clustered together, and the D2D2 group was separate from the D1D1 and D1D2 groups (Fig. 7). Microarray analysis of the liver transcriptome returned 485, 251, 227, 413 and 327 DEGs when D1D2, D2, D2D2, D1D2 and D1D2 groups were compared with the D1, D1, D1D1, D1D1 and D2D2 groups, respectively. Fig. 8 shows the distributions of DEGs in 3 to 5 types of affected pathway groups among D1 and D2, D1 and D1D2, D1D1 and D2D2, D1D1 and D1D2, D2D2 and D1D2. The most predominantly affected pathway was "metabolism" in D1D1 group and "human disease" in D2D2 group when compared to that of D1D2 group, suggesting that D1D1 and D2D2 have a different pattern of physiological effects. Moreover, D1D1 group changed more metabolic pathways than D2D2 group when compared to that of D1D2 group. DEGs were also subjected to a more detailed analysis and we generated a schematic illustration of DEGs marched to their related pathways to more clearly demonstrate the magnitude of the effects of high carbohydrate and high lipid diets on major metabolic pathways (Fig. 9A and B). Those pathways from which indicated that systemic metabolic changes between high carbohydrate diet and high lipid diet in phase 2 are less than phase 1. Also, fewer genes were changed in almost all nutrient metabolic pathways compared to phase 1. Fig. 9C to D shows that D1D1 group had weaker glucose and protein metabolism than D2D2 group when compared to D1D2 group, as well as decreased gene
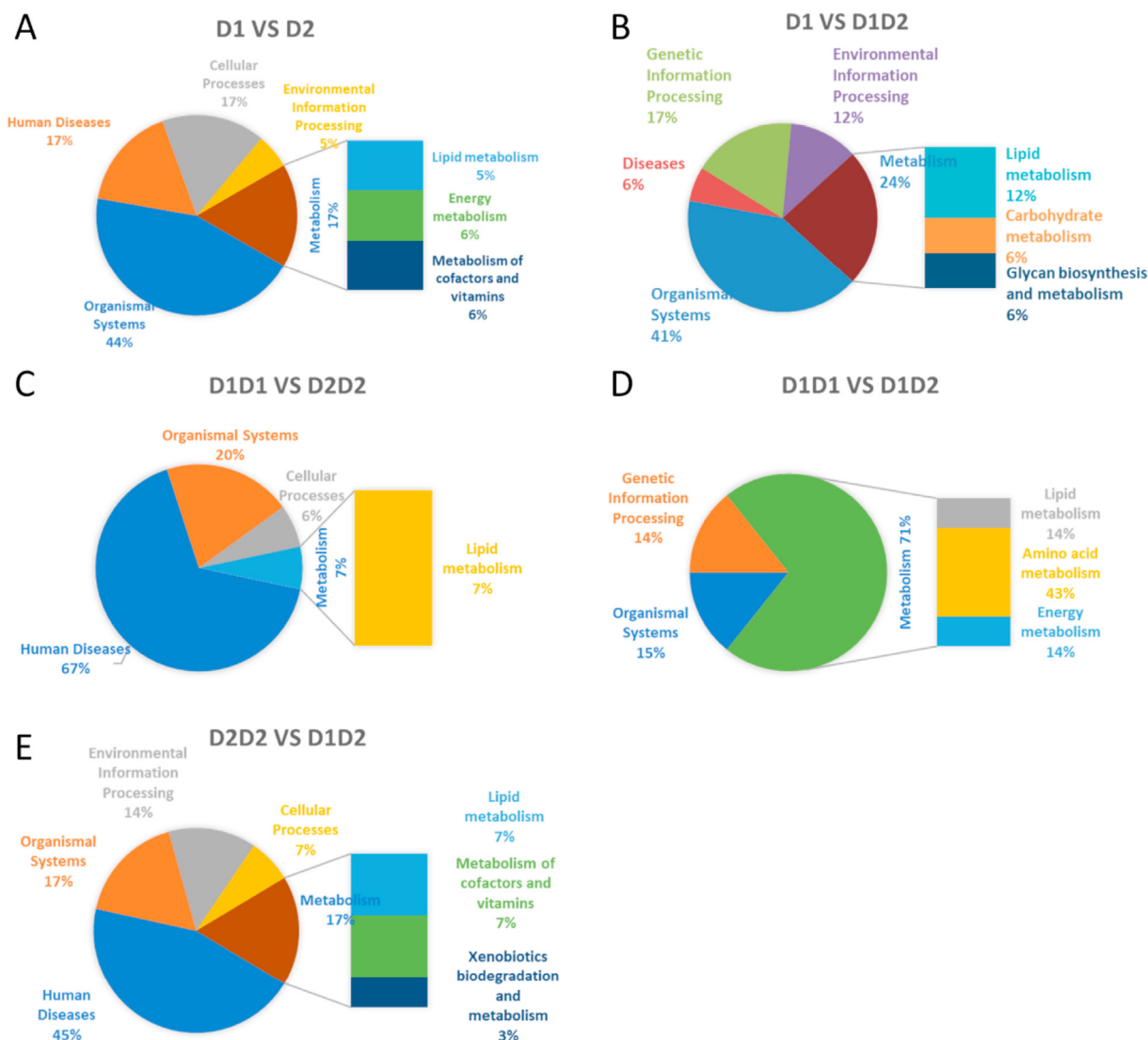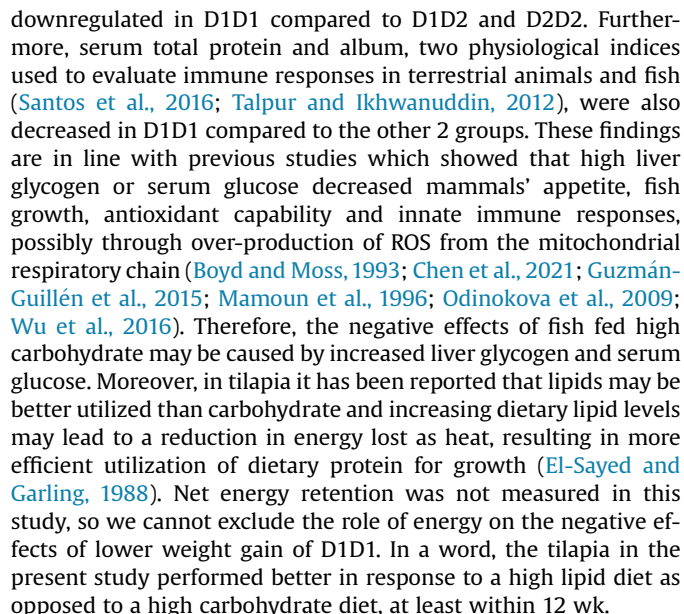
Fig. 8. Effects of high carbohydrate or high lipid diets on the differential metabolic pathways of tilapia between D1 and D2, D1 and D1D2, D1D1 and D2D2, D1D2 and D1D1, D1D2 and D2D2, respectively. (A to E) Distribution of categories of differentially expressed genes (236), (495), (51), (252) and (228) in liver when compared among D2, D1D2, D2D2, D1D1 and D2D2 to that of D1, D1, D1D1, D1D2 and D1D2, respectively (FDR < 0.05, $P < 0.05$). Non-annotated genes and features corresponding to the same gene are not represented. D1 = high carbohydrate; D2 = high lipid; D1D1 = high carbohydrate in phase 1 and high carbohydrate in phase 2; D2D2 = high lipid in phase 1 and high lipid in phase 2.

expression of immune related genes. Specifically, significant changes were detected in genes for key enzymes related to humoral immunity (*gama*, *mrc2*, *mhc2* and *cd163*), protein deposition (*tgm2* and tryptase) and inflammation (*nlrp3*) in the transcriptomic measurements between the D1D1 and D2D2 groups.

4. Discussion

Fish is a vital source of high-quality protein for people and over half of the fish consumed by people is produced by aquaculture (Anderson et al., 2019). Unlike mammals, whose main energy source is carbohydrate, fish preferentially use protein (Tacon and Cowey, 1985). Protein-rich ingredients have traditionally incurred considerable costs for aquaculture feed. There is currently a shortage which has increased their price over the years, which is detrimental to the sustainable development of aquaculture (Kok et al., 2020). Therefore, studies focused on the highest level of carbohydrate or lipid that fish can tolerate without growth retardation, which can potentially spare protein utilization, have been

investigated by a number of authors (Liu et al., 2022; Ren et al., 2021a; Taj et al., 2020; Yan et al., 2015). It was reported that tilapia could tolerate high carbohydrate levels of 38% to 46% (7.0% to 7.38% lipid) and high lipid levels of 11% to 14% (28.82% to 35.19% carbohydrate) without growth retardation (Ali and Al-Asgah, 2001; Wang et al., 2005). In partial agreement with those studies, tilapia in this study fed high carbohydrate (44.2%) not only had relatively high weight gain, but also gained the highest whole body crude protein among all the groups over phase 1. However, continually D1 feeding not only significantly decreased weight gain and feed intake, but also increased liver glycogen and/or serum glucose (D1D1) compared to D1D2 and D2D2 when fed for a further 2 wk (phase 2). It is a little bit surprising that tilapia is unable to tolerate high carbohydrate over a relatively longer-term period. When mRNA expression and schematic diagrams are taken into consideration, genes related to appetite (*npy*), antioxidation (*nrf2*, *gst1*) and immunity (*gzma*, *cd163*, *mrc2*, *mhc2*) (Catalan et al., 2012; Gazi and Martinez-Pomares, 2009; Semnani-Azad et al., 2021; Soengas et al., 2018; Zhao et al., 2015), were consistently and strongly

downregulated in D1D1 compared to D1D2 and D2D2. Furthermore, serum total protein and album, two physiological indices used to evaluate immune responses in terrestrial animals and fish (Santos et al., 2016; Talpur and Ikhwanuddin, 2012), were also decreased in D1D1 compared to the other 2 groups. These findings are in line with previous studies which showed that high liver glycogen or serum glucose decreased mammals' appetite, fish growth, antioxidant capability and innate immune responses, possibly through over-production of ROS from the mitochondrial respiratory chain (Boyd and Moss, 1993; Chen et al., 2021; Guzmán-Guillén et al., 2015; Mamoun et al., 1996; Odinokova et al., 2009; Wu et al., 2016). Therefore, the negative effects of fish fed high carbohydrate may be caused by increased liver glycogen and serum glucose. Moreover, in tilapia it has been reported that lipids may be better utilized than carbohydrate and increasing dietary lipid levels may lead to a reduction in energy lost as heat, resulting in more efficient utilization of dietary protein for growth (El-Sayed and Garling, 1988). Net energy retention was not measured in this study, so we cannot exclude the role of energy on the negative effects of lower weight gain of D1D1. In a word, the tilapia in the present study performed better in response to a high lipid diet as opposed to a high carbohydrate diet, at least within 12 wk.

Carbohydrates are the main ingredient of fish feed with a range of advantages like extensive availability and low price (Chen et al., 2021). Usually, carnivorous fish do not utilize dietary carbohydrates as well as omnivorous fish and high carbohydrate diets commonly induce excessive hepatic glycogen accumulation, which impairs hepatocyte function and further produces negative effects on the growth and health status of carnivorous fish (Liu et al., 2022). Interestingly, continually high carbohydrate diet feeding (D1D1) increased hepatic glycogen and serum glucose in omnivorous tilapia and decreased fish growth, feed intake and other indices in this study as outlined above. Thus, it is necessary to explore the hepatic glycogen and glucose metabolism of tilapia fed high dietary carbohydrate in this study. Glucose tolerance tests are traditionally

Fig. 9. Schematic diagrams of differentially expressed genes mainly related to carbohydrate, lipid, and protein metabolism, as well as immune response and other activities. (A) schematic diagram of differential expression genes (DEGs) between D2 vs D1; (B) schematic diagram of DEGs between D2D2 vs D1D1; (C) schematic diagram of DEGs between D1D2 vs D1D1; (D) schematic diagram of DEGs between D1D2 vs D2D2. Red indicates that the genes from later treatment are up-regulated compared to those in the earlier treatment. Green indicates down-regulation of genes in fish from the later treatment compared to those in the earlier treatment. A blank box indicates no obvious

induction during the 2 phases. Abbreviations: FFA = free fatty acid; LD = lipid droplet; LPL = low density lipoprotein lipase; FOXO3 = Forkhead box O3; Glut1 = glucose transporter1; PLIN2 = perilipin 2; CA = carbonic anhydrase; ACOT1 = acyl-CoA thioesterase 1; ApoA-I = apolipoprotein A to I; CPT1 = carnitine O-palmitoyltransferase1; HSP70 = heat shock protein 70; TNFaip3 = TNF alpha induced protein 3; HIF1A = hypoxia inducible factor 1 alpha subunit = ANGPT3 = angiopoietin-like 3; AMPKγ2 = AMP-activated protein kinase gamma 2; Bcl6 = BCL6 transcription repressor; SGK1 = SGK1 serum/glucocorticoid regulated kinase 1; CBS = cystathionine beta-synthase; PLCE1 = phospholipase C epsilon 1; EIF4g2 = eukaryotic translation initiation factor 4 gamma 2; EEF2k = eukaryotic elongation factor 2 kinase; PPARα = peroxisome proliferation receptor alpha; RXRα = retinol X receptor alpha; GS = glycogen synthase; PDP = pyruvate dehydrogenase phosphatase; TGM2 = transglutaminase; CEBPA = CCAAT/enhancer binding protein alpha; STAT3 = signal transducer and activator of transcription 3; CITED4 = cbp/p300-interacting trans-activator 3; MRC2 = mannose receptor C type 2; C1q = complement C1q-like protein 4; GZMA = granzyme A; MHC2 = HLA class II histocompatibility antigen; CD163 = scavenger receptor cysteine-rich type 1 protein M160-like; NLRP3 = protein NLRC3; TCA = tricarboxylic acid cycle; BAX = apoptosis regulator BAX-like; REDD1 = DNA damage-inducible transcript 4 protein; GCG = glucagon-2; GOT1 = glutamic-oxaloacetic transaminase 1; AMY = amylase; RPS27 = ribosomal protein S27; RPL29 = ribosomal protein L29; CEBPD = CCAAT/enhancer binding protein delta; ESR1 = estrogen receptor 1; LPGAT1 = lysophosphatidylglycerol acyltransferase 1; FABP = fatty acid binding protein; ANGPAT = angiopoietin; ATGL = Adipose triglyceride lipase; APO = apolipoprotein; VTG = vitellogenin; PEPCK = phosphoenolpyruvate carboxykinase; GPx = glutathione peroxidase; LYZ = lysozyme; COQ10 = coenzyme Q10; CCAR1 = cell division cycle and apoptosis regulator 1; GHSR1 = growth hormone secretagogue receptor; MAPKBP1 = mitogen-activated protein kinase binding protein 1; EPS8 = epidermal growth factor receptor pathway substrate 8; CASP7 = caspase 7; CYP46A1 = cytochrome P450 family 46 subfamily A member 1; DCR = decoy receptor; FADS2 = fatty acid desaturase 2; ELOVL6 = ELOVL fatty acid elongase 6; ADK = adenylate kinase; SOCS = suppressor of cytokine signaling.

performed to evaluate the efficiency of glucose use in fish (Polakof et al., 2012). Fish subjected to high-carbohydrate feeding have been found to improve glucose tolerance after glucose loading (Polakof et al., 2012; Xu et al., 2018). In agreement with those studies, the glucose tolerance of D1 fed fish in the present study was stronger than that of control and D2 groups after glucose loading during phase 1. Moreover, in phase 2, both groups, D1D1 and D1D2, from D1 had better glucose tolerance than the D2D2 group, regardless of whether further feeding of a D1 or D2 diet occurred. Metabolic pathway analysis showed differences between D1D2 and D1 were less than that of D1 and D2, possibly because D1D2 had a history of high carbohydrate diets. Moreover, schematic diagram analysis also showed that GLUT1 and GLUT12 were upregulated respectively in D1D1 and D1D2 when compared to that of D2D2. These results support a concept called "nutritional programming" which postulates that the effects of nutritional stimuli early in life may drive permanent changes in physiologic and metabolic pathways in later life (Kumkhong et al., 2021). It seems that tilapia from D1D1 retained high glucose catabolic capability after feeding for 12 wk. When we take the schematic diagram between D1D2 and D1D1 into consideration, amylase gene expression was significantly higher in D1D1 than D1D2, implying that D1D1 possessed better digestive capacity for carbohydrate since amylase catalyzes the hydrolysis of polysaccharides, such as starch and glycogen, into short-chain sugars for absorption (Champasri et al., 2021), possibly contributing to the differences in glycogen deposition between D1D1 and D1D2. On the other hand, glucose tolerance and GLUT1 expression were higher in D1D1 compared to D2D2, the latter of which plays an important role in the transport of glucose into cells for metabolism (Li et al., 2018), implying that high glucose metabolism cannot compensate for an overwhelming level of carbohydrate intake. Therefore, a continued high carbohydrate diet induced glycogen and glucose accumulation, possibly because the pressure of excessive carbohydrate input is not able to be relieved by the limited glucose catabolism of tilapia.

Generally speaking, animals including fish have developed adaptive strategies to address excess energy intake, such as storing more glycogen in the liver and muscle or more lipid in hepatic and intraperitoneal sites when carbohydrate or lipid content are abundant in their diets (He et al., 2015; Kamalam et al., 2017). A number of fish studies indicate that high carbohydrate diet supplementation can lead to higher glycogen accumulation, as well as

higher HSI and/or serum glucose (Chen et al., 2021; Lina et al., 2014; Ren et al., 2021a; Taj et al., 2020). High lipid diet supplementation is correlated with increasing whole body lipid and/or intraperitoneal fat deposition (Li et al., 2013; Lina et al., 2014; Ren et al., 2021a). In the present study, our results in both phases were mainly in line with those results of the aforementioned studies. Of note, our results provide additional data that a high lipid diet induced the deposition of not only hepatic fat and mesenteric fat, but also subcutaneous fat (SCAT) in tilapia after extended feeding for 2 wk (Fig. 2F), regardless of the 10-wk nutritional history. In mammals, increased amounts of SCAT, particularly in gluteofemoral regions, are associated with improved insulin sensitivity and lower risk of Type 2 diabetes relative to central obesity (Ibrahim, 2010). Also, SCAT in mammals has been reported to have higher absorption of FFAs from circulation (Ibrahim, 2010). Though those functions have not been fully determined in fish, a report on tilapia has demonstrated that SCAT has higher mitochondrial DNA, gene expression for fatty acid-oxidation, adipogenesis and brown adipose tissue characteristics (Wang et al., 2017). This could be as another factor as to why high lipid and mixed diets performed better than the high carbohydrate diet in this study on an energy equivalent basis in the 12-wk phase. On the other side, although muscle lipid levels were comparable among D1D1, D1D2 and D2D2, the intraperitoneal fat in D2D2 and D1D2 was significantly higher than in D1D1. We know that the liver, adipose tissue and muscle have the capability to store lipids in fish (He et al., 2015). It has been reported that excess lipid accumulation in hepatocytes makes them more susceptible to injury by oxidative stress and inflammatory cytokines (Lee et al., 2011). Therefore, we cannot exclude the role of intraperitoneal adipose tissue in improving hepatic fat deposition of tilapia fed a high lipid diet.

Until now, there has been limited evidence related to the sustainability and difference impacts of fish fed with high carbohydrate and high lipid diets on an energy equivalent basis. The predominant interest is in exploring the cost effectiveness of high carbohydrate and high lipid diets for different fish species. In the present study, both high carbohydrates and lipids were equally suitable for fish within the levels used in the first phase. However, metabolic homeostasis was affected by a high carbohydrate diet for a further 2 wk. By using D1D2 as comparison, we observed that D2D2 led to increased lipids in the liver, SCAT and adipose tissue. By contrast, D1D1 mainly distributed carbohydrates into the liver,
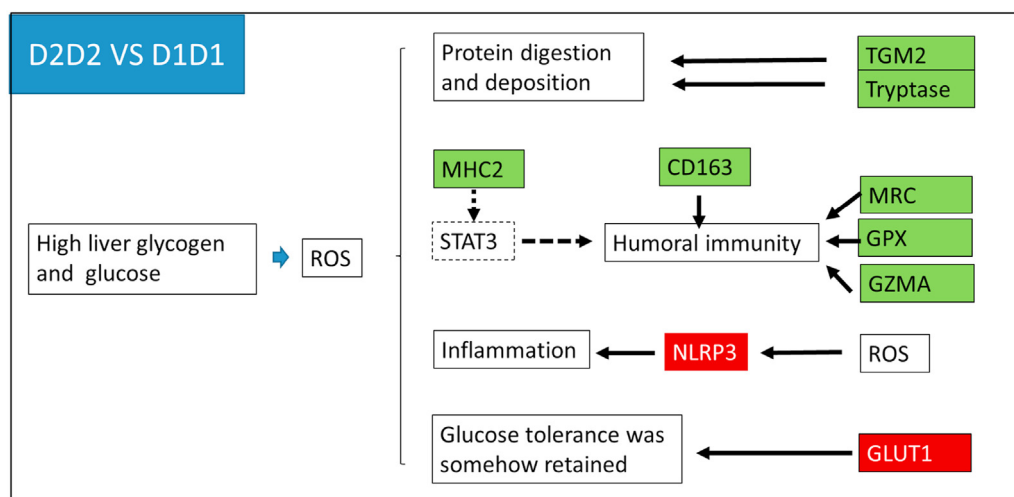

Fig. 10. Summary diagram of results between D1D1 and D2D2. D1D1 = high carbohydrate in phase 1 and high carbohydrate in phase 2; D2D2 = high lipid in phase 1 and high lipid in phase 2; TGM2 = transglutaminase; MHC2 = HLA class II histocompatibility antigen; CD163 = scavenger receptor cysteine-rich type 1 protein M160-like; MRC = mannose receptor C type 2; GPX = glutathione peroxidase; GZMA = granzyme A; NLRP3 = protein NLRC3; Glut1 = glucose transporter1.

impairing fish appetite, growth and immunity. For clarity, the results of this research have been presented in a summary diagram between D1D1 and D2D2 (Fig. 10).

5. Conclusion

The present results indicate that: 1) tilapia are better at tolerating a high lipid diet rather than a high carbohydrate diet after feeding at least for 12 wk; 2) a continuous 12 wk high carbohydrate diet could cause several negative effects, although glucose tolerance was retained; 3) tilapia were better at using a high lipid rather than high carbohydrate diet, possibly because of its delicate lipid distribution. In summary, our results elucidate the ability and systemic regulatory effects of tilapia in tolerating a high carbohydrate and high lipid diet and could be useful as a reference to study sustainable and cost-effectiveness diets in other fish species.

Author contributions

Lijun Ning and **Yuanyou Li:** Conceived and designed the experiments, wrote the paper. **Haotian Zhang** and **Xiaoying Chen:** Conducted the research. **Jia Zhen** and **Shen Chen:** Analyzed the data. **Junfeng Guang:** Contributed materials and analysis tools. **Chao Xu** and **Yuanyou Li:** Critically revised the manuscript. All authors read and approved the final version of the article.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.01.007.

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