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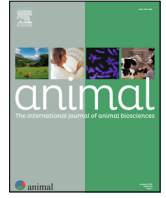
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Effects of refeeding with low- or high-carbohydrate diets on intermediary carbohydrate metabolism in juvenile and adult Nile tilapia



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ABSTRACT

Generally, energy expenditure and compensation according to food deprivation and refeeding often occur along the life cycle of farmed-raised fish. Fasting and refeeding are also hypothesised to modulate carbohydrate metabolism particularly for herbivorous and/or omnivorous. This study aims to investigate the effects of short-term fasting and subsequent refeeding with high or low-carbohydrate diets on the intermediary carbohydrate metabolism of juvenile and adult Nile tilapia (*Oreochromis niloticus*) which is known to be a good user of carbohydrate as an energy source. Fish were fasted for 4 days and subsequently refed with either a low carbohydrate and high protein (LC/HP) or high carbohydrate and low protein (HC/LP) diet for 4 days. Our results showed that 4 days of refeeding with either one of the diets could compensate for weight loss due to fasting. Thus, we investigated the effects of a 4-day-refeeding strategy and different carbohydrate-refeeding diets on plasma metabolites, nutrient composition, and glucose and its related metabolism in the liver and muscle of adult fish. Refeeding had similar effects in adults and juveniles and induced modulations to the intermediary metabolism: (1) refeeding with the HC/LP diet elevated plasma glucose levels; (2) refeeding with both diets increased triglyceride levels in the plasma, liver, and muscle, but the effect of the HC/LP diet was superior; (3) refeeding elevated plasma cholesterol levels in adults, irrespective of diet; (4) refeeding with both diets increased hepatic lipid levels in juveniles, with stronger effects observed in those fed the HC/LP diet, and refeeding with the HC/LP diet elevated hepatic lipid levels in adults; (5) refeeding with both diets increased the plasma protein content, but the effect of the LC/HP diet was superior; (6) refeeding with the LC/HP diet increased hepatic protein content in adults; and (7) refeeding with both diets increased hepatic glycogen levels, but the effect of the HC/LP diet was superior. Additionally, in juveniles and adults, refeeding with the HC/LP diet upregulated the expression of glycolytic genes in the liver and muscle, lipogenic genes in the liver, and glucose transport genes. Moreover, refeeding with the HC/LP diet downregulated the expression of gluconeogenic and amino acid catabolism genes in the liver and amino acid catabolism genes in the muscle. Collectively, the effect of short-term refeeding with a high carbohydrate diet on intermediary metabolism resembled that of long-term feeding, supporting the hypothesis that Nile tilapia, an omnivorous fish, is highly responsive to dietary carbohydrates.

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Implications

Since carbohydrates could be a primary source of energy during refeeding after fasting, understanding how fish instantaneously respond to different levels of dietary carbohydrate refeeding provides insights into the management of refeeding diets for Nile tilapia to compensate for energy expenditure. Refeeding had similar effects in adults and juveniles, and the effects of refeeding with high carbohydrate were superior in modulating intermediary car-

bohydrate metabolism. Refeeding with a high-carbohydrate diet induced glycolysis and lipogenesis but decreased gluconeogenesis and amino acid catabolism. This information could support the practical management of refeeding diets, leading to the mitigation of the negative effects of fasting.

Introduction

In aquaculture, feed accounts for a majority of production costs, and a rise in feed prices has a direct impact on production costs. Among feedstuffs, digestible carbohydrates (CHOs) are the cheapest to produce; therefore, intensive research and development

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have been conducted to drive the efficient use of CHO as an energy source. The optimum CHO levels and methods to increase dietary CHO levels to enable its protein-sparing effects during growth have been widely studied, particularly in herbivores and omnivores (Shiau and Peng, 1993; Singh et al., 2006; Li et al., 2013). Additionally, in carnivorous rainbow trout (*Oncorhynchus mykiss*), a glucose-intolerant phenotype is typically observed in juveniles fed a high CHO diet (more than 20%) (Polakof et al., 2012; Kamalam et al., 2017), however, female broodstock are adaptable to a 35% CHO diet without it having negative effects on their growth and reproductive cycle (Callet et al., 2021). These findings can not only be applied to other fish species but also provide basic knowledge of intermediary metabolism in fish which varies with feeding habits (Polakof et al., 2012). Moreover, energy expenditure and compensation according to food deprivation and refeeding which often occurs along life cycle of farmed-raised fish were also hypothesised to modulate CHO metabolism particularly for herbivorous and/or omnivorous fish. Indeed, intensive information of the effects of fasting and refeeding on CHO and its related metabolism is still limited although this nutritional status was demonstrated to influence several parameters including growth, liver size and composition, body composition, blood parameters and health status in various fish (Rios et al., 2006; Tian et al., 2013; Morshedi et al., 2017; Sakyi et al., 2020).

Nile tilapia is an economically important fish, and global tilapia aquaculture production reached 4.4 million tonnes in 2020 (FAO, 2022). The culture of Nile Tilapia is estimated to increase annually. Therefore, multidisciplinary nutritional research has been conducted not only to expand tilapia production but also to alleviate farming challenges. The tilapia is an omnivorous fish which is highly adaptable to high levels of dietary CHO as its main energy source. An investigation of glucose metabolism in Nile tilapia revealed that high dietary CHO induced glycolysis and lipogenesis and reduced gluconeogenesis and amino acid catabolism (Boonanuntanasarn et al., 2018a, 2018b). These experiments were conducted over several months and concluded that tilapia fed a high-CHO diet in the long term adequately metabolised glucose. In tropical areas, tilapia culture has been conducted in open freshwater environments in which fish often encounters fasting and refeeding states depending on seasonal variation, heavy rain, bacterial infection, and transportation. Since Nile tilapia is a good user of CHO as a primary energy source, food deprivation and subsequently refeeding might hypothesised to largely modulate glucose metabolism and CHO use. Information on how fasting and refeeding influence metabolic responses to different CHO diet is required which would be beneficial to improve aquaculture practices.

Previous study demonstrated that short-term fasting (4 days) and subsequent refeeding (4-days) of dietary high CHO diets led to hyperglycaemia and subsequently remodelling of the hepatic epigenetic landscape in rainbow trout, a model of glucose-intolerant fish (Marandel et al., 2015). This information suggested that fasting and subsequently instantaneous refeeding might also obviously result in metabolic responses of CHO which enable underlying CHO responses in tilapia, an omnivorous fish. Hence, the present study aims to investigate the short-term glucose metabolic response in Nile tilapia fed different CHO dietary levels after a short fasting period. Since glucose and its related metabolism depend on the physiological stage, this study provided a comparative investigation of CHO and its related metabolic responses at molecular level at two key life stages: juvenile and adult. In addition, the effects of different level of CHO refeeding on blood metabolites and muscle and hepatic composition were demonstrated, leading to the overall conclusion of intermediary carbohydrate metabolism.

Material and methods

Experimental design, diet, and fish culture

The experimental plan for fasting and subsequent refeeding, which was completely randomised with six replicates (tanks), is shown in Fig. 1. We conducted two separate experiments: one for juvenile and one for adult fish. For the first experiment, 60 juvenile fish (50–60 g) were randomly distributed in cement tanks (4 m³, water depth: 0.8 m) under continuous aeration. For the second experiment, 34 adult fish (450–550 g) were randomly distributed in cement tanks (water depth: 0.8 m) under continuous aeration. A flow-through water change system was implemented by replacing one-third of the water in each tank weekly with dechlorinated water.

The experimental fish were acclimated to the experimental conditions for 14 days (hereinafter referred to as the initial phase) and hand fed twice daily (0900 and 1600 h) with a commercial diet (36% CP + 4% crude fat) administered at 3% of their BW. Subsequently, the fish were fasted for 4 days. For a comparative investigation of the effects of instantaneous refeeding with different dietary CHO diets, two experimentally isoenergetic diets including high CHO and low protein (**HC/LP**) and low CHO and high protein (**LC/HP**) were formulated. The experimental diets and their proximate compositions, including moisture, CP, crude fat, crude fibre, and ash, which were analysed according to the standard method of the Association of Official Analytical Chemists (AOAC, 1990), are listed in Table 1. After fasting, the fish were divided into two groups for refeeding with two respective experimental diets for 4 days. During refeeding, tanks were divided into two partitions (replicates) for HC/LP and LC/HP refeeding. Fish (n = 6 partition replicates; juvenile; 22 fish/replication, adult: 13 fish/replication) were fed at 3% of their BW with either the HC/LP or LC/HP diet for 4 days. Throughout the experimental period, air and water temperature were determined daily and ranged from 29 to 31 °C and 27.0–28.0 °C, respectively. Dissolved oxygen and pH were measured daily using a dissolved oxygen and pH meter, and their values were within acceptable ranges of 4.30–5.58 mg/L and 7.49–8.71, respectively. During the experimental period, fish deaths were recorded to determine their survival rates, and no mortality was observed. Fish were weighed at the initial, 4-day fasting, and 4-day refeeding periods. Because the BW loss of fasted fish was recovered after the 4-day refeeding period, we collected samples prior to and after the refeeding period.

Fish sampling, blood collection and chemical composition analysis

Samples were collected after the 4-day fasting and 4-day refeeding periods (Fig. 1). After fasting for 4 days, fish (n = 6 tank replicates; juvenile; four fish/replication, adult: two fish/replication) were euthanised using 0.2% clove oil. For refeeding experiments, fish were sampled 5 h after the last meal based on the postprandial glycaemia curve of Nile tilapia determined in a previous study (Boonanuntanasarn et al., 2018a). Also, fish (n = 6 partition replicates; juvenile; four fish/replication, adult: two fish/replication) were euthanised using 0.2% clove oil. For each fish, blood samples were collected from the caudal vein using a hypodermic syringe and mixed with K₂EDTA (1.5 mg/mL blood) as an anticoagulant, after which the K₂EDTA-treated blood samples were centrifuged at 10 000 × g for 10 min at 4 °C to obtain plasma and stored at 80 °C for biochemistry analysis. After bleeding, liver and epaxial muscle samples were collected, snap-frozen in liquid nitrogen, and kept at –80 °C for analysis of CP, crude lipids, triglyc-

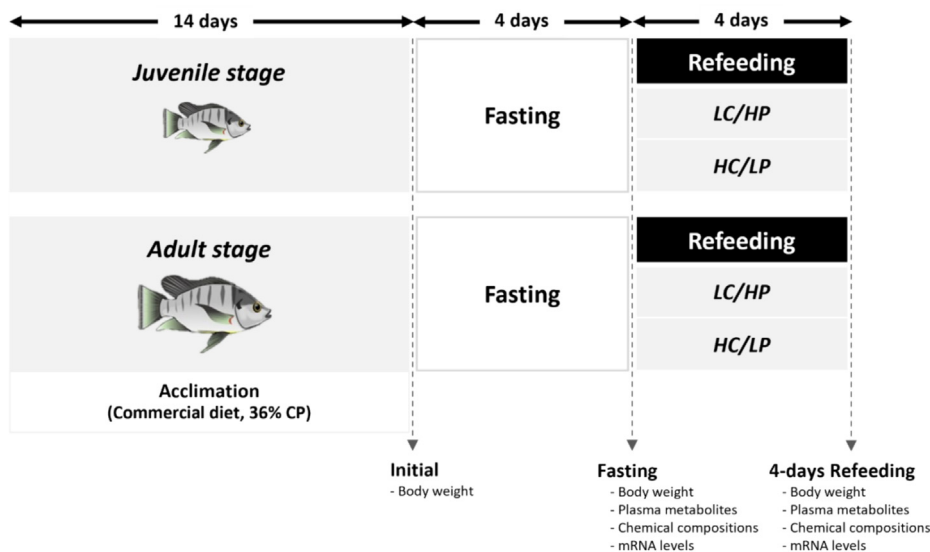


Fig. 1. Experimental plan for fasting and refeeding juvenile and adult Nile tilapia. Fish were fed a commercial diet during the acclimation period. During the 4-day refeeding periods, fish were re-fed with either a low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet.

Table 1

Ingredients and chemical composition (g/kg) of the commercial diets (during acclimation) and refeeding diets, namely high protein and low carbohydrate (LC/HP) and low protein and high carbohydrate (HC/LP) diets for the experimental Nile tilapia.

Ingredients	Acclimation diet	Challenge diets	
	Commercial diet	LC/HP	HC/LP
Fish meal	–	880	180
Rice flour	–	0	700
Fish oil	–	0	70
Soybean oil	–	20	0
Gelatin	–	80	0
Di-calcium phosphate	–	0	30
Fish premix ^a	–	20	20
Proximate composition (g/kg dry weight)			
DM	916.4	942.1	921.9
Protein	393.0	607.9	164.9
Fat	36.8	97.9	98.5
Fiber	77.7	5.2	4.6
Ash	137.0	241.6	92.9
NFE ^b	355.4	47.4	639.2
Gross energy (kJ/g)	13.13	14.40	15.6

Abbreviations: LC/HP = low carbohydrate and high protein; HC/LP = high carbohydrate and low protein.

^a Vitamin and trace mineral mix provided the following (IU/kg or g/kg diet): biotin, 0.25 g; folic acid, 0.003 g; inositol, 0.25 mg; niacin, 0.0215 g; pantothenic acid, 0.03 g; vitamin A, 5 000 IU; vitamin B1, 0.0025 g; vitamin B2, 0.0012 g; vitamin B6, 0.0075 g; vitamin B12, 0.00005 mg; vitamin C, 1 g; vitamin D3, 1 000 IU; vitamin E, 100 IU; vitamin K, 0.008 g; copper, 0.02 g; iron, 0.2 g; selenium, 0.3 mg; zinc, 0.32 g.

^b Nitrogen-free extract = DM – (CP + crude fat + crude fibre + ash).

eride (TAG), and glycogen content, as well as metabolic gene expression. Whole fish and livers were weighed, and the hepatosomatic index (HSI) was calculated.

Chemical composition, glycogen and triglyceride analysis

The chemical composition, including CP, crude fat, glycogen, and TAG, of the liver and muscle samples, was analysed after 4 days of fasting and 4 days of refeeding. The CP and crude fat content (one fish/replicate; n = 6 replicates) were analysed according to the standard method of the AOAC (1990). Glycogen content was analysed (one fish/replicate; n = 6 replicates) according to the

method described by Kirchner et al. (2003). In brief, samples (200 mg) were homogenised in 1 mL of 1 M HCl. An aliquot was transferred to a new tube, and 5 M KOH was added for neutralisation. The homogenised sample was centrifuged at 10 000 x g at 4 °C for 10 min, and the supernatant was used to measure free glucose content using a plasma glucose kit (Catalogue number: BLT00026, Erba Lachema s.r.o., Karásek Brno, Czech Republic), according to the manufacturer’s instructions. Another aliquot was boiled at 100 °C for 2.3 h to hydrolyse glycogen and was subsequently neutralised with 5 M KOH. After neutralisation, the sample was centrifuged at 10 000 x g at 4 °C for 10 min. Total glucose (free glucose and glucose obtained from glycogen hydrolysis) was determined using a glucose kit (Catalogue number: BLT00026, Erba). The glycogen content in the tissues was obtained by calculating the amount of glucose after subtracting the total glucose from the free glucose levels. The TAG content (one fish/replicate; n = 6 replicates) was analysed using a TG kit (Catalogue number: BLT00059; Erba), according to the manufacturer’s instructions. In brief, 100 mg of grounded tissue was homogenised with 1 mL of 5% IGEPAL in deionised water containing 0.1-mm glass beads using the Bioprep-24 homogeniser. Samples were heated in a water bath at 90 °C for 10 min and then cooled down to room temperature. After centrifugation at 10 000 x g at 4 °C for 10 min, the supernatant was transferred into a new tube and diluted with deionised water.

Blood chemistry analysis

Blood chemistry analysis was performed after 4 days of fasting and 4 days of refeeding. Determination of blood metabolites (two fish/replicate; n = 6 replicates), including glucose, TAG, cholesterol, and total protein, was performed as described in Kumkhong et al. (2020). Plasma glucose levels were quantitatively measured according to Trinder’s method (Trinder, 1969). Plasma TAG levels were determined using the glycerol-3-phosphate oxidase-sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (GPO-ESPAS) method as described by Bucolo and David (1973). Cholesterol levels were analysed using the cholesterol oxidase phenol + aminophenazone (CHOD-PAP) method according to Flegg (1973). Total plasma protein was analysed using the Biuret method (Gornall et al., 1949).

Total RNA extraction, cDNA synthesis, and quantitative reverse transcription PCR analysis of metabolic gene expression

Expression of genes related to CHO metabolism was examined in the liver and muscle samples after 4 days of fasting and 4 days of refeeding (at each sampling point: one fish/replicate; n = 6 replicates). Total RNA was extracted from the liver (50 mg) and muscle (100 mg) samples using the TRIzol reagent (Catalogue number: 15596026, Invitrogen, Carlsbad, CA, USA). The quantity of total RNA was measured using a NanoDrop spectrophotometer (Thermo Fisher, Madison, WI, USA), and the total RNA was verified using 1% agarose gel electrophoresis. cDNA synthesis was performed using 1 µg of total RNA and a SuperScript III RNaseH-Reverse transcriptase kit (Catalogue number: 18080093, Invitrogen) with random primers (Catalogue number: C1181, Promega, Charbonnières, France), following the manufacturer's protocol. The primer sequences used for real-time RT-qPCR are listed in Table S1. Reverse transcription (duplicate for each sample) was performed, and each PCR assay (duplicate for each PCR reaction) was performed to analyse the mRNA levels.

To measure the mRNA levels of glucose metabolism-related genes in the liver and muscle tissue, quantitative reverse transcription polymerase chain reaction (q RT-qPCR) was performed. Hepatic glycolysis was evaluated by determining the expression of glucokinase (*gck*), phosphofructokinase (*pfkfr*), and pyruvate kinase (*pkfr*). To evaluate hepatic gluconeogenesis, the expression of glucose-6-phosphatase (*g6pca1* and *g6pca2*), cytosolic phosphoenolpyruvate carboxykinase (*pkc1*), and phosphoenolpyruvate carboxykinase (*pkc2*) was determined. For lipogenesis in the liver, the expression of fatty acid synthase (*fasn*) and glucose-6-phosphate dehydrogenase (*g6pd*) was examined. The expression of several genes encoding enzymes involved in amino acid catabolism in the liver, including glutamate dehydrogenase (*gdh*), alanine aminotransferase (*alat*), and aspartate aminotransferase (*asat*), were analysed. In addition, the expression of CHO metabolism-related genes in the muscle was determined, including those related to glucose utilisation (glucose transporter, *glut4*), muscular glycolysis (hexokinase I/II, *hk1* and *hk2*; phosphofructokinase, *pfkma* and *pfkmb*; and pyruvate kinase, *pkma*), and amino acid catabolism (*alat*, *asat*, and *gdh*).

Real-time RT-qPCR to measure mRNA levels of glucose metabolism-related genes in the liver and muscle tissue was performed as described in Kumkhong et al. (2020). Reverse transcriptase- and cDNA-template-free samples were used as negative controls for real-time RT-qPCR. Relative quantification of gene transcription was carried out using the Roche Applied Science E-method according to Pfaffl (2001). Relative *ef1α* expression was used to normalise the measured mRNA levels. The expression of *ef1α* was not significantly different among the experimental groups (data not shown). Throughout the analysis, PCR efficiency values ranged between 1.8 and 2.0, as determined from the slope of a standard curve using serial dilutions of cDNA.

Statistical analysis

The statistical model utilised was $y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where y_{ij} was the response, μ was the general means, α_i was nutritive status (fasted and LC/HP and HC/LP refed) effects and ε_{ij} was the random error. All data were analysed using SPSS for Windows version 22 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was performed to analyse the differences among the 4-days fasting, 4-days HC/LP refeeding, and 4-days LC/HP refeeding groups. When significant differences were observed, Tukey's range test was performed to rank the treatment groups. The effects and differences were considered significant at $P < 0.05$.

Results

Effects of short-term fasting and refeeding with high- or low-CHO diet on BW and plasma metabolites

Juvenile and adult Nile tilapia were weighed after acclimatisation to the experimental conditions which was defined as the initial phase. The BWs of juvenile and adult Nile tilapia at the initial phase, after 4 days of fasting, and after 4 days of refeeding are shown in Fig. 2. In juveniles, compared with their weight in the initial phase, 4 days of fasting led to a decrease in BW ($P < 0.05$), and subsequent refeeding for 4 days, irrespective of diet, recovered their BW to that in the initial phase. In adult fish, compared with their weight in the initial phase, 4 days of fasting also resulted in a decrease in BW ($P < 0.05$) which was recovered after subsequent refeeding for 4 days, irrespective of diet. Indeed, continued refeeding through 7 days resulted in increased growth in juvenile (LC/HP only) and adult fish (Supplementary Figure S1). Because the BW loss of fasted fish was recovered after the 4-day refeeding period, we collected samples prior to and after the refeeding period.

The glucose, triglyceride, cholesterol, and total protein levels in juvenile and adult Nile tilapia were investigated (Fig. 3). Higher plasma glucose levels were observed in juvenile and adult fish fed the HC/LP diet ($P < 0.05$), but not in those fed the LC/HP diet, than those in the fasting phase. In addition, refeeding with either the HC/LP or LC/HP diet elevated plasma triglyceride levels; this effect was greater in fish fed the HC/LP diet ($P < 0.05$). In juveniles, there were no significant differences in cholesterol levels among experimental groups ($P > 0.05$). However, compared with plasma protein levels in the fasting phase, refeeding with either the HC/LP or LC/HP diet increased plasma protein levels, and the LC/HP diet had a greater effect than the HC/LP diet ($P < 0.05$). In adults,

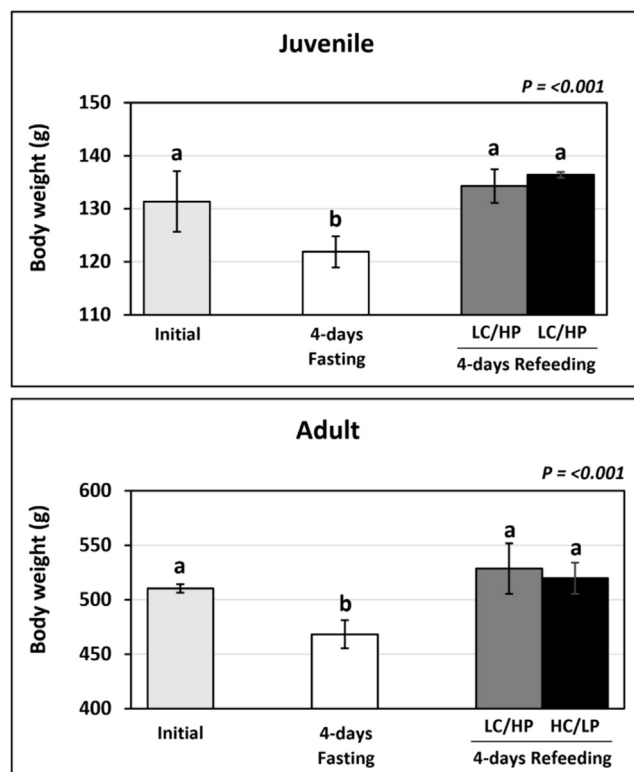


Fig. 2. BW (mean \pm SD, n = 6) of juvenile and adult Nile tilapia at initial, 4-day fasting, and 4-day refeeding periods. During the 4-day refeeding periods, fish were refed with either a low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet. Different lowercase letters indicate significant differences among nutritional status ($P < 0.05$).

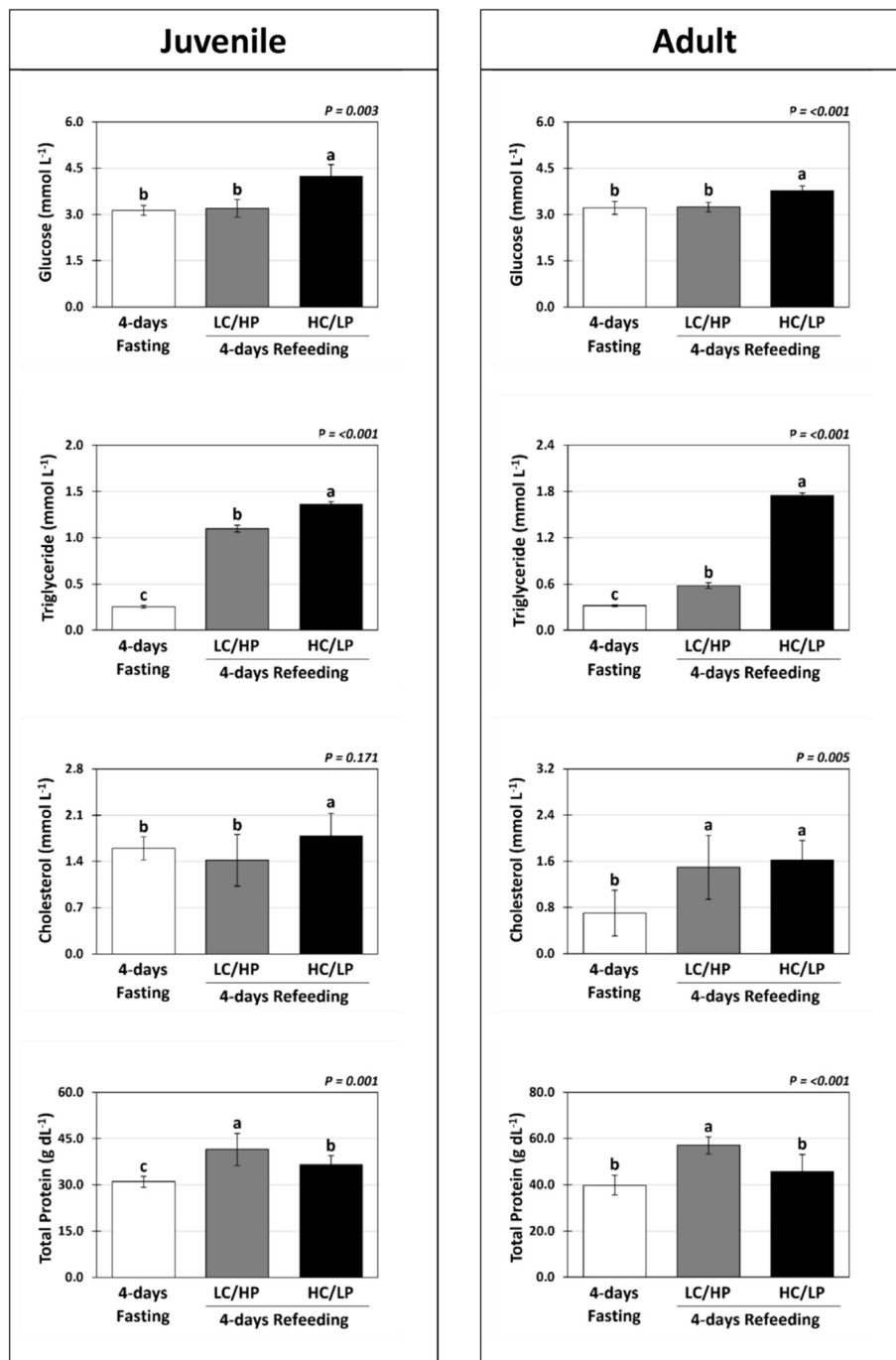


Fig. 3. Plasma metabolites of Nile tilapia that were fasted for 4 days (fasting) and refed for 4 days (4-day refeeding) with either a low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet. Data represent means \pm SD (n = 6 individuals per experimental group). Different lowercase letters indicate significant differences among nutritional status ($P < 0.05$).

both the HC/LP and LC/HP diets increased plasma cholesterol levels, while only the LC/HP diet increased plasma protein levels ($P < 0.05$).

Effects of short-term fasting and refeeding with low- or high-CHO diets on liver composition and mRNA levels of genes involved in glucose metabolism

The chemical composition of the liver samples of experimental Nile tilapia that were subjected to fasting and 4 days of refeeding are listed in Table 2. In juvenile fish, compared with those during fasting, refeeding with either HC/LP or LC/HP diets led to an

increased HSI and increased hepatic lipid and glycogen levels ($P < 0.05$); these effects were greater for the HC/LP diet ($P < 0.05$). Elevation of hepatic triglyceride levels was found only in juvenile fish fed the HC/LP diet ($P < 0.05$). In adult fish, compared with those during fasting, refeeding with either diets elevated hepatic glycogen and triglyceride levels; however, the effect of the HC/LP diet was superior ($P < 0.05$). In addition, higher hepatic lipid and HSI levels were found in adult fish fed the HC/LP diet but not in those fed the LC/HP diet ($P < 0.05$). Moreover, LC/HP refeeding resulted in significantly higher hepatic protein levels than those during fasting in adult fish ($P < 0.05$), whereas there were no significant differences in hepatic protein levels between fasting and

Table 2

Chemical composition of Nile tilapia that were fasted for 4 days (fasting) and refed for 4 days (refeeding) either the low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet.

Parameters	Juvenile stage				RSE	P-value ¹	Adult stage			
	Fasting	Refeeding		Fasting			Refeeding		RSE	P-value ¹
		LC/HP	HC/LP				LC/HP	HC/LP		
Liver composition										
Hepatosomatic index (%)	1.19 ^c	1.76 ^b	3.56 ^a	0.40	<0.001	1.92 ^b	1.98 ^b	2.95 ^a	0.39	0.013
Protein (g/kg)	72.32	75.08	74.15	1.92	0.087	73.14 ^b	75.57 ^a	74.61 ^{ab}	1.23	0.008
Lipid (g/kg)	30.63 ^c	34.74 ^b	41.65 ^a	1.76	<0.001	41.93 ^b	41.81 ^b	49.28 ^a	1.98	<0.001
Glycogen (mg/g tissue)	18.68 ^c	25.14 ^b	37.3 ^a	2.80	<0.001	25.44 ^c	40.76 ^b	50.17 ^a	2.62	<0.001
Triglyceride (mg/g tissue)	2.33 ^b	3.93 ^{ab}	5.88 ^a	0.20	0.001	4.02 ^c	5.64 ^b	8.41 ^a	0.40	<0.001
Muscle composition										
Protein (g/kg)	181.52	181.65	182.90	1.13	0.148	183.70	185.18	184.20	2.67	0.678
Lipid (g/kg)	8.45	8.61	8.38	1.03	0.912	8.04	8.55	8.84	0.76	0.171
Glycogen (mg/g tissue)	3.26	3.30	4.12	0.77	0.164	5.47	5.24	5.96	0.58	0.095
Triglyceride (mg/g tissue)	0.38 ^c	0.50 ^b	0.63 ^a	0.07	<0.001	2.01 ^b	2.14 ^b	2.98 ^a	0.20	0.001

Abbreviations: LC/HP = low carbohydrate and high protein; HC/LP = high carbohydrate and low protein; RSE = residual standard error.

¹ One-way ANOVA was used to determine the differences in nutritive composition in liver and muscle obtained from juvenile and adult fish. Means with different superscripts in each row differ significantly ($P < 0.05$).

refeeding periods with either the HC/LP or LC/HP diet in juvenile fish ($P > 0.05$).

The effects of fasting on the expression of genes related to glucose metabolism in the liver are shown in Table 3. In juveniles, compared with those during fasting, refeeding with the HC/LP diet upregulated *pklr* expression while refeeding with LC/HP downregulated *pfklr* ($P < 0.05$) expression. Between the refeeding diets, fish fed the HC/LP diet had higher mRNA levels of glycolytic genes (*gck* and *pfklr*) ($P < 0.05$). However, there were no significant differences in the expression of *pklr* between refeeding with HC/LP or LC/HP diets ($P > 0.05$). Compared with those during fasting, HC/LP refeeding resulted in significantly lower expression of gluconeogenesis genes (*g6pca1*, *g6pca2*, *pck1*, and *pck2*) and higher expression of lipogenic genes (*g6pd* and *fasn*) ($P < 0.05$). Note that refeeding with

a HC/LP diet induced a higher expression of *g6pd* than refeeding with a LC/HP diet ($P < 0.05$). Relative to fasting, refeeding modulated the expression of several genes involved in amino acid catabolism. Between the refeeding diets, the LC/HP diet induced the expression of *gdh*. There were no significant differences in *alat* and *asat* expression between the refeeding groups ($P > 0.05$).

In adult fish, compared with those during fasting, refeeding with the HC/LP diet resulted in higher expression levels of hepatic glycolytic genes (*gck*, *pklr*, and *pfklr*) ($P < 0.05$). Moreover, between the HC/LP and LC/HP diets, the HC/LP diet resulted in significantly higher *pklr* and *pfklr* expression than the LC/HP diet ($P < 0.05$). Compared with that in fish subjected to fasting, the expression of gluconeogenic genes (*g6pca1* and *pck1*) was downregulated in adult fish refed with the HC/LP diet ($P < 0.05$), while those refed

Table 3

mRNA levels of genes involved in intermediary glucose metabolism in liver tissue of Nile tilapia that were fasted for 4 days (fasting) and refed for 4 days (refeeding) either the low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet.

Parameters	Juvenile stage				RSE	P-value ¹	Adult stage			
	Fasting	Refeeding		Fasting			Refeeding		RSE	P-value ¹
		LC/HP	HC/LP				LC/HP	HC/LP		
Glycolysis										
<i>gck</i>	1.43 ^{ab}	0.27 ^b	1.91 ^a	1.06	0.013	0.74 ^b	1.35 ^{ab}	3.28 ^a	0.18	0.001
<i>pklr</i>	0.23 ^b	0.64 ^{ab}	1.51 ^a	0.30	0.001	0.47 ^b	0.42 ^b	4.17 ^a	1.67	0.003
<i>pfklr</i>	1.56 ^a	0.59 ^b	1.50 ^a	0.52	0.001	1.17 ^b	1.15 ^b	2.63 ^a	0.49	0.004
Gluconeogenesis										
<i>g6pca1</i>	2.10 ^a	1.49 ^{ab}	0.77 ^b	0.19	0.001	1.30 ^a	1.27 ^a	0.65 ^b	0.44	0.022
<i>g6pca2</i>	1.92 ^a	1.22 ^{ab}	0.44 ^b	0.21	0.001	1.30	1.88	0.88	0.48	0.099
<i>pck1</i>	6.46 ^a	0.98 ^{ab}	0.18 ^b	1.21	0.001	2.13 ^a	0.33 ^{ab}	0.01 ^b	0.38	0.001
<i>pck2</i>	2.19 ^a	0.94 ^{ab}	0.44 ^b	0.44	0.003	0.81 ^b	2.23 ^a	1.95 ^{ab}	0.97	0.044
Lipogenesis										
<i>g6pd</i>	0.34 ^b	0.60 ^b	2.22 ^a	0.65	0.002	0.91 ^b	0.26 ^b	2.63 ^a	1.12	0.001
<i>fasn</i>	0.002 ^b	0.56 ^{ab}	2.92 ^a	0.75	<0.001	0.17 ^b	0.14 ^b	3.25 ^a	1.38	0.001
Amino acid catabolism										
<i>alat</i>	2.02 ^a	0.65 ^b	0.66 ^b	0.39	0.003	1.62 ^a	0.96 ^{ab}	0.48 ^b	0.32	0.001
<i>asat</i>	3.10 ^a	1.47 ^{ab}	0.17 ^b	0.44	0.001	1.77 ^a	0.91 ^{ab}	0.15 ^b	0.27	0.001
<i>gdh</i>	0.87 ^b	2.03 ^a	0.73 ^b	0.71	0.003	1.08 ^{ab}	2.44 ^a	0.44 ^b	0.85	0.001

Abbreviations: LC/HP = low carbohydrate and high protein; HC/LP = high carbohydrate and low protein; *gck* = glucokinase; *pklr* = pyruvate kinase; *pfklr* = phosphofructokinase; *g6pca1* = glucose-6-phosphatase 1; *g6pca2* = glucose-6-phosphatase 2; *pck1* = cytosolic phosphoenolpyruvate carboxykinase 1; *pck2* = phosphoenolpyruvate carboxykinase 2; *g6pd* = glucose-6-phosphate dehydrogenase; *fasn* = fatty acid synthase; *alat* = alanine aminotransferase; *asat* = aspartate aminotransferase; *gdh* = glutamate dehydrogenase; RSE = residual standard error.

¹ One-way ANOVA was used to determine the differences in gene expression in liver obtained from juvenile and adult fish. Means with different superscripts in each row differ significantly ($P < 0.05$).

with the LC/HP diet had upregulated *pck2* expression ($P < 0.05$). However, there were no significant differences in *g6pca2* expression among the experimental groups ($P > 0.05$). Elevation of hepatic lipogenic genes (*g6pd* and *fasn*) was observed only in adult fish refed the HC/LP diet ($P < 0.05$). In addition, compared with that in fish subjected to fasting, there were no significant differences in the expression of amino acid catabolism genes (*alat*, *asat*, and *gdh*) in fish refed the LC/HP diet ($P > 0.05$), while fish refed the HC/LP diet had significantly lower *alat* and *asat* expression levels ($P < 0.05$). Fish fed the LC/HP diet had higher expression levels of *gdh* compared with those fed the HC/LP diet ($P < 0.05$).

Effects of short-term fasting and refeeding with low- and high-CHO diets on muscle composition and mRNA levels of glucose metabolism-related genes

The muscle composition of the fasted and refed juvenile and adult fish are shown in Table 4. In juvenile and adult fish, compared with those in the fasting group, there were no significant differences in muscular protein, lipid, and glycogen levels among the experimental groups ($P > 0.05$). Compared with fish in the fasting group, refeeding with either a HC/LP or LC/HP diet resulted in increased muscular triglyceride levels in juveniles; however, those fed the HC/LP diet had higher muscular triglyceride levels ($P < 0.05$). In adults, only those fed the HC/LP diet had increased muscular triglyceride levels compared with those in the fasting condition ($P < 0.05$).

The expression of glucose metabolism-related genes in the muscles of juvenile and adult Nile tilapia during fasting and subsequent refeeding conditions are shown in Table 4. Compared with that in fish under fasting conditions, *glut4* was upregulated in fish under refeeding conditions, and the HC/LP diet induced higher *glut4* expression than the LC/HP diet ($P < 0.05$). Compared with fasting, refeeding modulated the expression of several glycolytic genes in juveniles, such as the upregulation of *hk1*, *pfkma*, and *pkma* and downregulation of *hk2* and *pfkmb*. In contrast, refeeding upregulated the expression of glycolytic genes in adults, particularly that of *hk1*, *pfkmb*, and *pkma*. Between the two refeeding diets, higher expression levels of several glycolytic genes (juveniles, *hk1* and *pfkma*; adults, *hk1*, *hk2*, *pfkma*, *pfkmb*, and *pkma*) were observed in fish refed the HC/LP diet. Compared with those

during fasting, refeeding downregulated the expression of several genes related to muscular amino acid catabolism. Refeeding upregulated *alat* expression in juvenile fish fed the LC/HP diet. Comparison between the refeeding conditions at the molecular level revealed the elevation of muscular amino acid catabolism genes (*alat*, *asat* and *gdh*) in fish fed the LC/HP diet ($P < 0.05$).

Discussion

Tilapia is a good user of CHO as an energy source and thus responds sensitively to dietary CHO. Several studies have demonstrated the metabolic responses of Nile tilapia to a high-CHO diet throughout its life cycle, as well as the nutritional programming effects of CHO stimuli in early life (Boonanuntasarn et al., 2018a, 2018b; Kumkhong et al., 2020, 2021; Srisakultiew et al., 2022). Considering that CHO could be a primary source of energy during refeeding after fasting, understanding how fish instantaneously respond to different types of dietary CHO refeeding after fasting would be interesting. In rainbow trout, 4-day refeeding of high dietary CHO after fasting showed significant epigenetic remodelling in liver, demonstrating that this instantaneous refeeding period had an impact on metabolic disturbance (Marandel et al., 2015). This study provides information on how Nile tilapia adapted to short-term refeeding with different dietary CHO levels after fasting for plasma metabolites, nutrient composition in the liver and muscle, and CHO and its metabolic responses at the molecular level in different growth phases in Nile tilapia. Our results demonstrated differences in intermediary metabolic responses in Nile tilapia according to different CHO-refeeding diets, suggesting their susceptibility to the use of CHO as an energy source. These findings would be beneficial for further applications of refeeding diets to recover fish from short-term fasting, which often occurs during tilapia culture.

Previous studies have shown that food deprivation results in lower BW, and refeeding leads to compensatory growth which varies according to fish species, the duration of fasting, and the subsequent refeeding period, as well as the nutrient composition of the refeeding diet and/or refeeding regime (Oh et al., 2007; Cho and Heo, 2011; Urbinati et al., 2004). For Nile tilapia, the effects of fasting and subsequent refeeding have been demonstrated. Refeeding

Table 4

mRNA levels of genes involved in intermediary glucose metabolism in muscle tissue of Nile tilapia that were fasted for 4 days (fasting) and refed for 4 days (refeeding) with either the low carbohydrate and high protein (LC/HP) or high carbohydrate/low protein (HC/LP) diet (n = 6).

Parameters	Juvenile stage				Adult stage				RSE	P-value ¹
	Fasting	Refeeding		Fasting	Refeeding		RSE	P-value ¹		
		LC/HP	HC/LP		LC/HP	HC/LP				
Glucose transport										
<i>glut4</i>	0.94 ^b	1.37 ^{ab}	1.53 ^a	0.08	0.001	0.39 ^c	0.75 ^b	2.79 ^a	0.40	<0.001
Muscular glycolysis										
<i>hk1</i>	0.76 ^c	1.24 ^b	1.43 ^a	0.08	<0.001	0.90 ^b	0.92 ^b	1.53 ^a	0.15	<0.001
<i>hk2</i>	1.40 ^a	1.01 ^b	1.07 ^b	0.12	<0.001	1.08 ^b	0.87 ^c	1.25 ^a	0.15	<0.001
<i>pfkma</i>	0.66 ^c	1.07 ^b	1.25 ^a	0.05	<0.001	0.61 ^{ab}	0.54 ^b	0.66 ^a	0.04	<0.001
<i>pfkmb</i>	1.37 ^a	0.99 ^b	1.15 ^{ab}	0.13	<0.001	0.65 ^c	1.08 ^b	1.13 ^a	0.09	<0.001
<i>pkma</i>	0.84 ^b	1.06 ^{ab}	1.38 ^a	0.07	<0.001	0.74 ^c	0.83 ^b	1.04 ^a	0.05	<0.001
Amino acid catabolism										
<i>alat</i>	1.16 ^b	1.26 ^a	0.98 ^c	0.09	<0.001	1.47 ^a	1.11 ^b	0.42 ^c	0.09	<0.001
<i>asat</i>	1.27 ^a	1.04 ^a	0.73 ^b	0.20	0.001	1.26 ^a	1.09 ^a	0.80 ^b	0.16	<0.001
<i>gdh</i>	1.65 ^a	1.45 ^b	0.94 ^c	0.08	<0.001	1.02 ^a	1.09 ^b	0.70 ^c	0.11	<0.001

Abbreviations: LC/HP = low carbohydrate and high protein; HC/LP = high carbohydrate and low protein; *glut4* = glucose transporter 4; *hk* = hexokinase; *pfkm* = phosphofruktokinase in muscle; *pkm* = pyruvate kinase in muscle; *alat* = alanine aminotransferase; *asat* = aspartate aminotransferase; *gdh* = glutamate dehydrogenase; RSE = residual standard error.

¹ One-way ANOVA was used to determine the differences in gene expression in muscle obtained from juvenile and adult fish. Means with different superscripts in each row differ significantly ($P < 0.05$).

for 5 weeks could compensate for growth after fasting for 1 week; however, this period of refeeding could not compensate for growth after severe fasting for two or 4 weeks (Elbially et al., 2022). However, information on the influence of different levels of CHO in the diet (as an energy source) on CHO and its related metabolism is still limited. Previous study demonstrated that Nile tilapia adapt well its metabolism to dietary CHO during a long-term nutritional study (Boonanuntanasarn et al., 2018a, 2018b), but nothing is known after a short-term intake of CHO. In this context, a fasting-refeeding protocol with CHO is the best design to test the short-term effect of nutrient (CHO) intake, as shown previously with rainbow trout fasted 4 days and then refed 4 days (Marandel et al., 2015). Our results showed that 4 days of fasting reduced BW, and 4 days of refeeding with either a LC/HP or HC/LP diet was adequate to compensate for growth lost during fasting; these results were similar between juvenile and adult fish. Therefore, we selected the 4-day refeeding period to investigate the instantaneous metabolic responses of Nile tilapia to different dietary CHO levels for compensatory growth.

In fish, refeeding after fasting leads to the modulation of several intermediate metabolites in the plasma, including increased glucose, TAG, and cholesterol levels, depending on the fish species, as well as the duration of fasting and refeeding (Tian et al., 2013; Viegas et al., 2013; Yarmohammadi et al., 2012; Pérez-Jiménez et al., 2007). Different dietary refeeding conditions would have diverse effects on metabolic responses, particularly for intermediate metabolites in the plasma. Similarly, we observed that refeeding with different CHO levels had diverse effects in juveniles and adults: (1) Refeeding with a HC diet increased plasma glucose levels which was not observed in fish refed a LC diet; (2) Refeeding increased plasma TAG levels, irrespective of dietary CHO/protein contents; however, the effects of the HC diet on increased plasma TAG levels were superior to that of the LC diet; and (3) refeeding with a LC/HP diet increased plasma protein levels. Therefore, short-term refeeding with a HC diet had similar effects on plasma metabolites to that observed in Nile tilapia fed a high-CHO diet for the long term, including increased glucose and TAG levels (90 days), as well as reduced plasma protein levels (45 days) (Boonanuntanasarn et al., 2018a). In addition, long-term feeding of high dietary CHO for 40 weeks led to increased plasma glucose and TAG levels and decreased plasma protein levels in Nile tilapia (Boonanuntanasarn et al., 2018b). Overall, these findings suggest that refeeding dietary CHO content reflected plasma metabolites in Nile tilapia, which supports its sensitive responses to dietary CHO.

Previous studies have demonstrated that refeeding after fasting modulates the HSI and hepatic nutrient composition and that these modulations were affected by glucose homeostasis (Takahashi et al., 2011; Sakyi et al., 2020). Similarly, our results showed that, compared with the fasting state, short-term refeeding in juvenile and adult fish resulted in (1) an increased HSI, which was higher in fish fed the HC/LP diet than in those fed the LC/HP diet; (2) increased hepatic glycogen, lipid, and triglyceride contents, which was higher in fish fed with the HC/LP diet; and (3) increased hepatic protein levels in adults fed the LC/HP diet. These modulatory effects were also observed at the molecular level. The increase in plasma glucose levels and glycogen contents were linked to the upregulation of glycolytic genes in both adult (*pk1r* and *pfklr*) and juvenile (*gck* and *pfklr*) fish refed with the HC diet, suggesting that refeeding-induced glycolysis; this effect was greater in those fed the HC diet. In Nile tilapia, consistent and long-term feeding of high-CHO diets led to elevated hepatic glycogen levels and increased expression of some glycolytic genes (Boonanuntanasarn et al., 2018a, 2018b). Similarly, refeeding after fasting resulted in the recovery of glycogen content in European sea bass, gilthead seabream, and rainbow trout which was found to be linked with the induction of glycolytic glucokinase mRNA

and/or enzyme activities (Viegas et al., 2013; Metón et al., 2004; Soengas et al., 2006). Our results suggest that the increase in hepatic lipid and TAG levels is correlated with the upregulation of lipogenic genes, for which stronger effects were observed in fish fed the HC/LP diet. Furthermore, at the molecular level, our findings suggest that refeeding lowers the expression of gluconeogenic genes (except for *g6pca2*, *pck1*, and *pck2* in adults) and amino acid catabolism-related genes (except for *alat* and *asat* in adults) and the HC/LP diet has stronger effects than the LC/HP diet. Similarly, long-term feeding with a high-CHO diet led to the downregulation of genes involved in gluconeogenesis and amino acid catabolism-related genes in adult Nile tilapia (Boonanuntanasarn et al., 2018a, 2018b). In rainbow trout, a carnivorous fish, feeding with a high-CHO diet led to the downregulation of several gluconeogenic genes; nevertheless, the upregulation of *g6pcb2* orthologues due to high-CHO feeding resulted in glucose-intolerant characteristics (Marandel et al., 2015). These findings suggested that both juvenile and adult Nile tilapia expeditiously responded to refeeding HC diet for hepatic glycolysis and lipogenesis. Taken together, the refeeding status was related to CHO and its metabolism, and at the molecular level, tilapia can efficiently metabolise the HC/LP diet even when administered in the short term.

This study also investigated the effects of short-term refeeding on the chemical content, as well as CHO and its metabolism, in the muscle. Again, the effects appeared to be similar between juveniles and adults. Among the nutrient contents in the muscle, refeeding resulted in increased muscular TAG levels, and the effects of the HC/LP diet were superior to that of the LC/HP diet. These findings indicate that short-term HC/LP refeeding induces lipogenesis. Similarly, fasting and subsequent refeeding induced muscular lipid accumulation in Nile tilapia (Tian et al., 2013). At the molecular level, refeeding modulated gene expression with stronger effects observed in fish refed the HC/LP diet, including (1) the upregulation of *glut4* and several glycolytic genes in the muscle (except for *hk2* and *pfkmb* in juveniles and *pfkma* in adults) and (2) downregulation of genes related to amino acid catabolism. Overall, these findings suggest that short-term refeeding modulates glucose metabolism, including the induction of lipogenesis, glucose transport, and glycolysis, as well as the reduction of amino acid catabolism, and these effects were stronger in the HC/LP diet. Our results were similar to those observed in Nile tilapia and grass carp fed a long-term high-CHO diet (Boonanuntanasarn et al., 2018a, 2018b; Gaye-Siessegger et al., 2006; Cai et al., 2018; Xiong et al., 2014). Overall, the effects of short-term refeeding with a high-CHO diet appeared to only resemble the CHO metabolic response in Nile tilapia fed a long-term high-CHO diet, indicating that tilapia is highly susceptible to dietary CHO utilisation.

In conclusion, 4 days of fasting and subsequent refeeding could compensate for BW loss in the juvenile and adult stages. These findings suggest that refeeding with a high-CHO diet induced glucose metabolism which led to the induction of glycolysis and lipogenesis as well as the suppression of gluconeogenesis and amino acid catabolism. The effects of short-term CHO refeeding diets resembled that of long-term CHO refeeding diets, suggesting that Nile tilapia is a high responder to dietary CHO utilisation.

Supplementary material

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Ethics approval

All experimental procedures involving fish were approved by the Ethics Committee of the Suranaree University of Technology

Animal Care and Use Committee (Approval No. SUT-IACUC-001/2023).

Data and model availability statement

None of the data were deposited in an official repository. The data/models that support the study findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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S. Thongchaitriwat: Methodology. **S. Kumkhong:** Methodology. **E. Plagnes-Juan:** Methodology. **S. Panserat:** Data curation. **L. Marandel:** Conceptualization. **S. Boonanuntanasarn:** Data curation, Conceptualization.

Declaration of interest

None.

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