Aquaculture 543 (2021) 736948

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Spotted seabass, *Lateolabrax maculatus* can utilize the high-starch diet by effectively regulating the energy metabolism

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ARTICLE INFO

Keywords:
Lateolabrax maculatus
High-starch diet
Glucose metabolism
Lipid metabolism

ABSTRACT

Carbohydrates are the least cost feed source and conserve protein without affecting the growth of fish at a certain level. However, carnivorous fish fed with trophic levels >3.5 have a limited capability to utilize carbohydrates as an energy source. A 56-day feeding trial was conducted to evaluate the growth performance, hepatic histology, and glucose and lipid metabolism of spotted seabass, *Lateolabrax maculatus* (initial weight = 10.43 ± 0.01 g), a typical carnivorous fish, fed low - (130 g/kg) or high - starch (202 g/kg) diets (named the LS or HS diets) under satiety and starvation conditions (3 h and 24 h postprandial). The results showed that compared with the LS diet, the HS diet did not negatively affect growth but slightly increased the FR and FCR of spotted seabass. A well-regulated glucose metabolism response was observed in the HS group with significant downregulation of gluconeogenesis (*G6P*) and upregulation of glycolysis (*PK*) at 3 h postprandial (P3 h) and downregulation of glycolysis (*GK, PFK-1*) at the transcription level at 24 h postprandial (P24 h). However, hyperglycemic was still observed in the HS group with increased enzyme activities of gluconeogenesis (G6P) and glycolytic (PK) at P24 h, which indicated that the spotted seabass could not effectively regulate glycolytic under starvation conditions. Excessive starch intake significantly increased hepatic cAMP content, which enhanced the rate of basal metabolism to relieve hyperglycemia at P24 h. No hepatic glycogen accumulation was observed for both groups, and hepatic glycogen content was significantly decreased with upregulation glycogenolysis (*PYGL*) in the HS group under starvation conditions. In addition, and lipogenesis and lipolysis/β-oxidation were not affected under satiety conditions (P3 h) in the HS group, while the mRNA levels of lipogenesis genes (*FASN*, *ACC1*) and β-oxidation (*CPT1α*, *PPARα*) were downregulated under starvation conditions. These results indicated that spotted seabass could preferentially utilize glycogen and then lipids under starvation conditions. In conclusion, the spotted seabass could accelerate basal energy metabolism to relieve hyperglycemia and avoid glycogen accumulation and fatty liver after fed HS diet, and preferentially catabolized hepatic glycogen to provide energy under starvation conditions, which indicated that it could effectively regulate energy metabolism after feeding the HS diet.

Abbreviations: ACC1, acetyl-CoA carboxylase 1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; *ATGL*, adipose triglyceride lipase; cAMP, cyclic adenosine 3′, 5′-monophosphate; CF, condition factor; *CPT1α*, carnitine palmitoyltransferase 1α; *EF1α*, elongation factor 1α; *FASN*, fatty acid synthase; *FBP*, fructose-1,6-bisphosphatase; FBW, final body weight; FCR, feed conversion rate; FR, feeding rate; *G6P*, glucose-6-phosphatase; *GK*, glucokinase; *GLUT2*, glucose transporter type 2; *GLUT4*, glucose transporter type 4; *GYS*, glycogen synthase; HDL-C, high-density lipoprotein cholesterol; HSI, hepatosomatic index; *HSL*, hormone-sensitive lipase; NEFA, non-esterified fatty acids; *PDH*, pyruvate dehydrogenase; *PEPCK*, phosphoenolpyruvate carboxykinase; *PFK-1*, phosphofructokinase; *PK*, pyruvate kinase; *PPARα*, peroxisome proliferator activated receptor α; PPV, productive protein value; *PYGL*, glycogen phosphorylase L; SGR, specific growth rate; SR, survival rate; TC, total cholesterol; TG, triglyceride; TP, total protein; VAI, visceral adipose index; VSI, viscerasomatic index; WGR, weight gain rate..

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https://doi.org/10.1016/j.aquaculture.2021.736948
Received 29 December 2020; Received in revised form 20 May 2021; Accepted 21 May 2021
Available online 24 May 2021

1. Introduction

Carbohydrates are the cheapest energy source for animals to conserve dietary protein (Polakof et al., 2012; Kamalam et al., 2017). Fish fed with trophic levels (< 3) (such as common carp, *Cyprinus carpio*; major Indian carp, *Cirrhinus cirrhosis*; Nile tilapia, *Oreochromis niloticus*; and catfish *Silurus asotus*) are relatively tolerant to dietary starch, and herbivorous fish (gass carp, *Ctenopharyngodon idella*) can effectively use carbohydrates in the diet (Polakof et al., 2012; Wilson, 1994). Fish with feeding habits at trophic levels (> 3) respond differently to dietary carbohydrates (Marandel et al., 2018; Duarte et al., 2009; Zhang, 2005). Fish fed with high trophic levels (> 3.5) are usually considered to have poor dietary carbohydrate utilization (Stone, 2003), and persistent hyperglycemia is commonly observed after acute glucose loading and/or carbohydrate-rich diet consumption in rainbow trout (*Oncorhynchus mykiss*), Atlantic Salmon (*Salmo salar*), channel catfish (*Ictalurus punctatus*), silver European eels (*Anguilla anguilla*), etc. (Moon, 2001; Polakof et al., 2012; Palmer and Ryman, 1972; Ince and Thorpe, 1974; Ottolenghi et al., 1995; Geurden et al., 2014; Villasante et al., 2019). Although starch-rich diets cause persistent postprandial hyperglycemia, studies have shown that fish fed with high trophic levels (> 3.5) could compensate by increasing feed intake to maintain growth (Bergot, 1979; Bergot and Breque, 1983; Degani and Viola, 1987). Thus, improved feed utilization and protein retention were found in rainbow trout and Atlantic salmon fed high-starch diets (Bergot, 1979; Erfanullah, 1998; Hemre et al., 1995a; Hemre et al., 2002). Previous studies have shown that fish cannot control circulating glucose levels and lack the complex endocrine systems responsible to regulate blood glucose (Polakof et al., 2012). There are many hypotheses about the reasons why carnivorous fish cannot make good use of carbohydrates in diet. For example, the amino acids in fish meal diets have a stronger effect on promoting insulin secretion than glucose in high carbohydrates diets (Mommsen and Plisetskaya, 1991); the number of insulin receptors in fish muscles is lower than that in mammals (Párrizas et al., 1994); poor glucose phosphorylation in fish muscles (Cowey et al., 1977) and low numbers of glucose transporters (Wright Jr et al., 1998); and fish fed with high trophic levels (> 3.5) are unable to effectively regulate gluconeogenesis and the glycolysis pathway under starvation conditions and maintain a high endogenous hepatic glucose production after being fed a high-carbohydrate diet (Enes et al., 2009; Panserat et al., 2001; Gong et al., 2015).

Spotted seabass (*Lateolabrax maculatus*), in contrast to its sister species, Japanese seabass (*Lateolabrax japonicus*), has a limited range and little genetic variation (Liu et al., 2006). It is a higher trophic level (3.9) fish (Zhang et al., 2016), and its diet includes sardines, barracudas, anchovies, and shrimp, as well as any other small fishes and crustaceous in the wild, and has been widely farmed in China and Eastern Asia (Sun et al., 1994; FAO, 2018). Due to its dietary characteristics, the demand for protein in spotted seabass is relatively high. The protein content of the basic diet is 41%–47.8% (Ai et al., 2004; Wang et al., 2012; Wang et al., 2015; Cheng et al., 2010; Zhang et al., 2019), and fishmeal is the most significant protein in aquaculture; however, resources are limited. Increasing carbohydrate levels in feed and thus reducing the use of fish meal and the level of protein in artificial compound diets is an effective way to address fish meal shortages.

The latest research showed that largemouth bass (*Micropterus salmoides*) could not utilize more than 10% dietary starch; otherwise, it would induce serious metabolic liver disease related to glucose and lipid metabolic disorder (Yu et al., 2019; Zhang et al., 2020). However, this symptom has not yet been observed in spotted seabass. In most studies on spotted seabass, more than 15% of starch could be used in basal diets, and liver disease symptoms are seldom observed (Liang et al., 2019; Dou et al., 2014). Moreover, starch is commonly used as an important binder in extrusion processing (Sørensen et al., 2011). In subsequent studies, Japanese seabass gradually became less dependent on fish meal as they adapted to the plant protein diet, but a high plant protein diet still caused energy metabolism disorder and liver disease (Zhang et al., 2019). However, whether spotted seabass has a higher starch utilization ability to decrease protein content in feed has not been reported. The aim of the present study was to investigate the effects of dietary starch levels on growth performance and glucose and lipid metabolism in spotted seabass, important considerations for reducing the use of fish meal and conserving protein.

2. Materials and methods

During the entire experiment period, all of fish were maintained in compliance with the Laboratory Animal Welfare Guidelines of China (General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China, Standardization Administration of China, GB/T 35892—2018).

2.1. Experimental diets

The diets were formulated to be isonitrogenous and isoenergetic (Table 1). A basal diet containing 130 g/kg starch was used as the control (named LS), whereas a diet containing 202 g/kg starch was regarded as the high-starch (named HS) group. Wheat flour and dextrin were added to the diet as a digestible carbohydrate, and the control group was leveled with wheat flour, wheat middling and microcrystalline cellulose. Both groups of diet were extruded by a single-screwed extruder (EXT50A, Yang Gong Machine, China), then dried and kept frozen at −20 °C until using. Two experimental diets formulation and composition are showed in Table 1.

Table 1
Formulation and composition of experimental diets (g/kg).

Ingredient	LS	HS
Fish meal[a]	540	540
Tapioca flour	30	30
Wheat flour	180	280
Wheat middling	100	0
Microcrystalline cellulose	51	0
Dextrin	0	51
Fish oil[a]	30	30
Soybean oil[a]	20	20
Lecithin oil	20	20
$Ca(H_2PO_4)_2$	5	5
Yeast extract	10	10
Vitamin and mineral premix[b]	14	14
Total	1000	1000
Analyzed nutrients compositions (g/kg, in dry matter basis)		
Crude protein	463	460
Crude lipid	136	130
Crude ash	98.9	94.4
Moisture	95.9	102
Crude fiber	60.2	10.1
Nitrogen free extract[c]	145	203
Starch	130	202
Gross energy (MJ/kg)	22.6	23.0

[a] Fish meal (crude protein content was 68.8%) and fish oil were supplied by Triple Nine Fish Product Co., Esbjerg, Denmark; CPC (crude protein content was 61.5%, low free gossypol and raffinose) was supplied by Xinjiang Jinlan Plant Protein Co. Ltd., Xinjiang, China; soybean oil were supplied by Yi hai Kerry Investment Co. Ltd. Shandong, China.

[b] Vitamin premix ($mg \cdot kg^{-1}$ diet): Vitamin A 20; Vitamin D_3 10; Vitamin K_3 20; Vitamin E 400; Vitamin B_1 10; Vitamin B_2 15; Vitamin B_6 15; Vitamin B_{12} (1%) 8; Vitamin C 1000; Niacinaminde 100; Calcium pantothenate 40; Biotin (2%) 2; Folic acid 10; Inositol 200; Choline chloride (50%) 4000; Corn gluten meal 150; mineral premix ($mg \cdot kg^{-1}$ diet): $CuSO_4 \cdot 5H_2O$ 10; $FeSO_4 \cdot H_2O$ 300; $ZnSO_4 \cdot H_2O$ 200; $MnSO_4 \cdot H_2O$ 100; KI (10%) 80; $CoCl_2 \cdot 6H_2O$ (10% Co) 5; Na_2SeO_3 (10% Se) 10; NaCl 100; $MgSO_4 \cdot 5H_2O$ 2000; Antioxidant 200; Zeolite 4995.

[c] Nitrogen free extract (g/kg) = 1000 - (moisture + crude protein + crude lipid + crude ash + crude fiber).

2.2. Experimental fish, feeding and sampling

Juvenile spotted seabass were obtained from Weihai Yulong Aquafarm (Shangdong, China) and desalinated and acclimated to freshwater within 2 w. The experiment was conducted in the indoor circulating water system at the National Aquatic Feed Safety Evaluation Base (Nan Kou, Beijing). Prior to the formal experiment, the fish were adapted for 4 w by feeding the LS diet. Fish with initial average body weight (10.43 ± 0.01) g were randomly selected and transferred to 22 tanks (30 fish per tank); 8 tanks (4 tanks per group) were used for the growth trial, and 14 tanks (7 tanks per group) were used for sampling. The fish were fed until apparent satiation twice a day (8:00 am and 8:00 pm) for 56-day. At each feeding, the excess weighed feed was provided with the water flow stopped. One hour later, uneaten feed was removed, dried to constant weight at 70 °C and reweighed. Leaching loss of the uneaten diet was estimated by leaving each diet sample in five tanks without fish for 1 h, recovering, drying and reweighing. Feed intake of fish in each tank was calculated as the difference between the amount fed and uneaten diet recovered, corrected for leaching losses. The aquaculture water environmental conditions were the same as Liang et al. (2019).

Thirty fish were randomly collected at the beginning and end of the feeding trial and kept frozen (−80 °C) for the proximate analysis. The fish were batch weighed and measured body length, and viscera, liver, and abdominal adipose weights to analyze growth performance and morphometric parameters at the end of the 8 w after starvation for 24 h. Blood samples were sampled from the caudal vein and centrifuged at 4000 rpm for 10 min at 4 °C to obtain plasma, and livers and dorsal white muscles were sampled from twelve fish randomly selected from each treatment group in 14 other tanks after anesthetization with 300 mg L^{-1} chlorobutanol at 3 h and 24 h postprandial. About 5 mm^3 liver tissue around the bile duct (12 samples per group) were collected and half of which were fixed in 4% paraformaldehyde solution for histology examination, and the other half were fast frozen for subsequent analysis of genes expressions and enzymes activities related to glucose and lipid metabolism. All samples were kept frozen at −80 °C until analysis.

2.3. Chemical analysis

All chemical analyses referred to AOAC, 2006. The dry matter was analyzed by drying the samples to constant weight at 105 °C. Ash was analyzed by CWF 1100 muffle furnace (Carbolite, UK) using combustion at 550 °C for 6 h. Crude protein was analyzed by Kjeltec™ 2300 Unit (FOSS, Denmark) using Kjeldahl method. Crude lipid was analyzed by acid hydrolysis method with Soxtex System HT 1047 Hydrolyzing Unit (Foss, Hillerød, Denmark) using and Soxhlet extraction method with Soxtex System 1043 (Foss, Hillerød, Denmark).

The plasma glucose contents were analyzed by a commercial kit (Shanghai Rongsheng Biotech Co. Ltd., China, No. 361500). The enzymes activities related to glucose metabolism (glucose-6-phosphatase (G6P), pyruvate kinase (PK)) and the contents of glucagon, insulin, and cyclic adenosine 3′, 5′-monophosphate (cAMP) were analyzed using the enzyme-linked immunosorbent assay method with commercial ELISA kit (Jiangsu Meimian industrial Co. Ltd., China, No. MM-2206O1, MM-32939O1, MM-32948O1, MM-1909O1, MM-3259101). During the determination, we used a blank control and a negative IgG control (Dako, X0936), only target antibodies showed specialized reaction. Non-esterified fatty acids (NEFA) were analyzed by commercial kits (Wako Pure Chemical Industries, Ltd. Japan, No. KRB0081). Total protein (TP), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL—C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and glycogen were analyzed with commercial kits (Nanjing Jiancheng Bioengineering Institute, China, No. A045, A110, A111, A112, C009, C010, and A043). Glycogen content was expressed as mg of protein equivalent per g of fresh liver or muscle tissues.

2.4. RNA isolation, reverse transcription and mRNA level analysis

The sequences of genes involved in glucose metabolism and lipid metabolism, including *G6P*, fructose-1,6-bisphosphatase (*FBP*), phosphoenolpyruvate carboxykinase (*PEPCK*), phosphofructokinase (*PFK-1*), *PK*, glucokinase (*GK*), pyruvate dehydrogenase (*PDH*), glycogen synthase (*GYS*), glycogen phosphorylase L (*PYGL*), glucose transporter type 2 (*GLUT2*), glucose transporter type 4 (*GLUT4*), fatty acid synthase (*FASN*), acetyl-CoA carboxylase 1 (*ACC1*), adipose triglyceride lipase (*ATGL*), hormone-sensitive lipase (*HSL*), carnitine palmitoyltransferase 1α (*CPT1α*), and peroxisome proliferator activated receptor alpha (*PPARα*) in spotted seabass were obtained from an RNA-seq database. In this experiment, as an endogenous reference gene, elongation factor 1α (*EF1α*, GenBank accession no. KT827794) was stable expressed gene, which was not affected by the treatment. The gene-specific primers *E*-values range are shown in Table 2. Total RNA isolation, quantification, reverse transcription and mRNA quantification were conducted following the protocols in Zhang et al. (2019).

2.5. Histopathological examination of liver tissues

The liver tissue pieces (0.5 × 0.5 × 0.5 cm) were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 6 μm slices. Liver slices were stained following hematoxylin and eosin (H & E) and periodic acid-Schiff (PAS) staining protocols, observed by light microscopy (Leica DM2500, Leica, Solms, Germany) and photographed.

2.6. Statistics

Independent *t*-test was performed on the data of growth performance, morphometric parameters, and nutrient compositions of whole body. Two-way ANOVA was performed on the data of two starch levels (LS and HS) in different time points (3 h and 24 h postprandial) using SPSS Statistics 23.0. All data are presented as the mean value ± standard error of the mean (S.E.M). $P < 0.05$ was considered significantly different. The graphics were drawn using GraphPad Prism 7.0 (GraphPad Software Inc. USA).

3. Results

3.1. Growth performance and morphometric index

The effects of different starch levels after the 8 w growth trial on the growth performance and morphometric index of spotted seabass are presented in Table 3. Both the LS and HS groups showed a high survival rate (SR) (100%), and there were no significant differences between the two groups. No significant differences in specific growth rate (SGR) and weight gain rate (WGR) were observed between the two groups ($P > 0.05$) (Table 3). In addition, the fish in the HS group showed a significantly higher feeding rate (FR) and feed conversion rate (FCR) ($P < 0.05$) (Table 3) than the fish in the LS group over the entire 8 w period. The productive protein value (PPV) was significantly decreased in the HS group ($P < 0.05$) (Table 3). In addition, the viscerasomatic index (VSI) in the HS group was significantly higher than that in the LS group ($P < 0.05$) (Table 3). The condition factor (CF), hepatosomatic index (HSI), and visceral adipose index (VAI) were not significantly different between the two groups ($P > 0.05$) (Table 3). The nutrient compositions of whole body were also not significantly different between the two groups ($P > 0.05$) (Table 4).

3.2. Plasma and hepatic metabolism parameters

The results of the plasma biochemical parameter analysis are presented in Table 5. The plasma glucose levels at 24 h postprandial were significant lower than those at 3 h postprandial in the LS group ($P < 0.01$). However, The HS group showed persistent postprandial

Table 2
Primer sequences for real-time PCR.

Gene	Primers	Sequence 5′-3′	Target size (bp)	TM (°C)	*E*-Value (%)
EF1α	F[a]	AATCGGCGGTATTGGAACTG	205	58.5	102
	R[a]	TCCACGACGGATTTCCTTGA			
PEPCK	F	ACGCCAATGTGCCTTTTTAC	128	55.8	92.0
	R	GCTCCCTTCACAAACTCAGC			
FBP	F	CACGTCACGTCTGGTGCTAAGC	180	61.9	90.5
	R	TGGCATAGTGTGAGGCGGACTG			
G6P	F	TCTGTGTCCTACCTGGTGGTGTTC	164	60.7	93.5
	R	GAGATGAATCCTGCTGCGAGACTG			
GK	F	GGTGAAGAAGCGAATGAGTGAGGAG	119	60.7	97.1
	R	CATGCTCTGTTCCGTCTGGTGTG			
PFK-1	F	GATGAACGCAGCGGTGAGGTC	132	61.9	92.4
	R	CCAGCCACATTGTGCCAGTCC			
PK	F	CTCTCGTTCTGTGGATATGCTGAAGG	105	60.7	104.4
	R	AGGATGGTCTCTGCGTGGTACTC			
PDH	F	AAGTGCCGCCATATTGTAGG	225	56.7	92.9
	R	ACCGGCAGAAAAGGAGGTAT			
GYS	F	TCATAGTCCATCGGCAGCAGAG	190	60.0	90.9
	R	CCATCTACGCCACTCAGAGACA			
PYGL	F	AAGGACCTCAGCCAGCTAGACA	191	59.0	94.6
	R	TGCCGCTTGTACTCGTGGATTC			
GLUT2	F	GGCTCGAAAAAGTCTGCATC	132	59.0	94.5
	R	ACAGAGGAGCGGATCAAAGA			
GLUT4	F	GTGGCCTTCTTGAGTGCTTC	177	57.5	98.6
	R	ACAGATGGAGGATGGCTCAC			
ACC1	F	AATCAACATCCGCCTGACTCCAAC	176	59.0	90.2
	R	CCTGCTTGTCTCCGTATGCTTGG			
FASN	F	AGGCATTGTGGAGGGTGTAG	223	56.8	97.1
	R	CCAGTCCACCAGTGATGATG			
ATGL	F	CTTCCTCTCCGCAACAAGTC	211	55.8	100
	R	TGGTGCTGTCTGGAGTGTTC			
HSL	F	TGATGTTTGCCAAGAAGCTG	228	57.8	93.8
	R	CTGATGGACTGGTGCTCTGA			
CPT1α	F	TCCGTGGCAGTCTTCTGAGGTC	115	60.4	92.9
	R	GCAGCAGCAGACATACACCTACAG			
PPARα	F	CCACCGCAATGGTCGATATG	193	58.0	92.7
	R	TGCTGTTGATGGACTGGGAAA			

[a] F: forward primer; R: reverse primer.

hyperglycemia, and the plasma glucose levels were significantly higher in the HS group than those in the LS group at 24 h postprandial ($P < 0.01$) (Table 5). In addition, the plasma insulin and glucagon levels at 24 h postprandial were significantly higher than those at 3 h postprandial in both groups ($P < 0.01$) (Table 5). The plasma insulin and glucagon levels were not significantly different between the two groups in different postprandial time points ($P > 0.05$). Plasma lipid metabolism indicators (TP, TC, and HDL—C) at 24 h postprandial were significantly higher than those at 3 h postprandial in both groups ($P < 0.05$) (Table 5). Plasma lipid metabolism indicator (NEFA) and hepatic function indicators (ALT and AST) at 24 h postprandial were significantly lower than those at 3 h postprandial in both groups ($P < 0.001$) (Table 5). The contents of TG at 24 h postprandial were significantly higher than those at 3 h postprandial in the HS group ($P < 0.01$), and the contents were significantly increased in the HS group compared with LS group ($P < 0.05$) (Table 5).

The results of the hepatic lipid metabolism parameter analysis are presented in Table 6. The contents of TG and TC were not significantly different between the two groups at 3 h and 24 h postprandial ($P > 0.05$) (Table 6). The contents of NEFA in the HS group were significantly lower than those in the LS group at 3 h postprandial ($P < 0.05$), conversely, the contents in the HS group were significantly higher than those in the LS group at 24 h postprandial ($P < 0.05$). The contents of NEFA at 24 h postprandial were significantly higher than those at 3 h postprandial ($P < 0.01$) (Table 6).

3.3. Glycogen content in liver and muscle and mRNA levels of glycogen metabolism genes

The glycogen content in liver and muscle are presented in Fig. 1A. The contents of glycogen in liver were not significantly different between the two groups at 3 h postprandial ($P > 0.05$), but the contents in the HS group were significantly lower than those in the LS group at 24 h postprandial ($P < 0.05$). The contents of hepatic glycogen at 24 h postprandial were significantly higher than those at 3 h postprandial in the LS group ($P < 0.01$) (Fig. 1A). The contents of muscle glycogen at 24 h postprandial were significantly lower than those at 3 h postprandial in both groups ($P < 0.05$) (Fig. 1A), and the contents were not significantly different between the two groups at 3 h and 24 h postprandial ($P > 0.05$) (Fig. 1A).

The mRNA levels of genes that participated in glycogen metabolism and glucose transport in spotted seabass fed diets with different starch levels at different time points (3 h and 24 h postprandial) are shown in Fig. 1B. The expressions of glycogenesis-related gene (*GYS*) were not significantly different between the two groups at 3 h and 24 h postprandial ($P > 0.05$). The expressions of glycogenolysis-related gene (*PYGL*) were not significantly different between the two groups at 3 h postprandial ($P > 0.05$), but the expressions of *PYGL* were significantly upregulated in the HS group compared with the LS group at 24 h postprandial ($P < 0.05$) (Fig. 1B). The expression of the glucose transport gene *GLUT2* was significantly upregulated at 24 h postprandial compared with 3 h postprandial in both groups ($P < 0.05$) (Fig. 1B). The mRNA levels of the glucose transporter gene *GLUT4* were not significantly different between the two groups at 3 h and 24 h postprandial ($P > 0.05$).

3.4. The mRNA levels and enzymes activities related to glucose and lipid metabolism genes

The genes expressions and enzymes activities related to glucose

Table 3
Effects of dietary starch levels on growth performance and morphometric parameters in spotted seabass (means ± SEM, $n = 4$).

Items[1]	Diets	
	LS	HS
Growth performance		
SR (%)	100 ± 0.00	100 ± 0.00
FBW (g)	110 ± 0.94	117 ± 5.07
SGR (%/d)	3.57 ± 0.01	3.67 ± 0.06
WGR (%)	954 ± 8.48	1026 ± 48.5
FCR	0.94 ± 0.01[a]	0.99 ± 0.01[b]
FR (% bw/d)	2.35 ± 0.02[a]	2.52 ± 0.02[b]
PPV (%)	35.5 ± 0.30[b]	33.3 ± 0.22[a]
Morphometric parameters		
CF (g/cm^3)	1.37 ± 0.01	1.41 ± 0.02
VSI (%)	10.8 ± 0.11[a]	11.9 ± 0.35[b]
HSI (%)	1.38 ± 0.07	1.58 ± 0.08
VAI (%)	6.59 ± 0.41	6.97 ± 0.27

Values are means ± S.E.M of two groups and values within the same row with different letters are significantly different ($P < 0.05$).
[1] SR (survival rate, %) = 100 × final fish number/initial fish number; FBW: final body weight (g); SGR (specific growth rate, %/d) = 100 × [ln (FBW/initial body weight)]/days; WGR (weight gain rate, %) = 100 × ((final weight (g) - initial weight (g))/initial weight (g)); FCR (feed conversion rate) = 100 × feed intake (g)/(final weight (g) - initial weight (g); FR (feeding rate, % bw/d) = 100 × feed intake (g)/((initial weight (g) + final weight (g))/2)/time (days); PPV (productive protein value, %) = 100 × (final body weight (g) × C_t - initial body weight (g) × C_0)/feed consumption), in which, C_t (%) is final nitrogen content in whole fish body, C_0 (%) is initial nitrogen content in whole fish body; CF (condition factor, g/cm^3) = 100 × (body weight (g)/(body length^3 (cm^3)); VSI (viscerasomatic index, %) = 100 × (viscera weight (g)/body weight (g)); HSI (hepatosomatic index, %) = 100 × (liver weight (g)/body weight (g)); VAI (visceral adipose index, %) = 100 × (visceral adipose weight (g)/whole body weight (g)).

Table 4
Nutrient compositions of whole body in spotted seabass (means ± SEM. $n = 12$).

Items (%)	whole body	
	LS	HS
Moisture	69.0 ± 0.13	68.3 ± 0.27
Ash	3.79 ± 0.10	3.48 ± 0.08
Crude protein	15.4 ± 0.06	15.2 ± 0.07
Crude lipid	10.6 ± 0.12	11.0 ± 0.15

metabolism in spotted seabass fed diets with different starch levels at different time points (3 h and 24 h postprandial) are shown in Fig. 2. The expressions of the gluconeogenesis-related genes *PEPCK* and *FBP* were not affected by the high-starch diet, but the expressions of *G6P* were significantly downregulated in the HS group compared with the LS group at postprandial 3 h ($P < 0.01$) (Fig. 2A). The expressions of *G6P* at 24 h postprandial were significantly upregulated than those at 3 h postprandial in the HS group ($P < 0.01$). In the glycolysis pathway, the expressions of *GK* or *PFK-1* were not significantly different between the two groups, but the expressions of *PK* were significantly upregulated in the HS group compared with the LS group at 3 h postprandial ($P < 0.01$) (Fig. 2A). The expressions of *GK* and *PFK-1* were significantly downregulated in the HS group compared with the LS group at 24 h postprandial ($P < 0.01$) (Fig. 2A). The expressions of *GK*, *PFK-1*, and *PFK-1* were significantly downregulated at 24 h postprandial compare with 3 h postprandial in the HS group ($P < 0.05$). The expressions of the pyruvate aerobic oxidation gene *PDH* were not significantly different between the two groups at the different postprandial times ($P > 0.05$). Gluconeogenesis-related enzyme G6P and glycolysis-related enzyme PK activities were not significantly different between the two groups at 3 h postprandial ($P > 0.05$) (Fig. 2B), but G6P and PK activities were significantly increased in the HS group compared with LS group at 24 h postprandial ($P < 0.001$) (Fig. 2B). G6P and PK activities at 24 h postprandial were significantly higher than those at 3 h postprandial in the HS group ($P < 0.001$). The contents of cAMP in liver were not significantly different between the two groups at 3 h postprandial ($P > 0.05$), but the contents were significantly increased in the HS group compared with LS group at 24 h postprandial ($P < 0.001$) (Fig. 2C). The contents of cAMP at 24 h postprandial were significantly higher than those at 3 h postprandial in the HS group ($P < 0.001$).

The genes expressions related to lipid metabolism in spotted seabass fed diets with different starch levels at different time points (3 h and 24 h postprandial) are shown in Fig. 3. The expressions of lipogenesis-related gene *ACC1* were significantly downregulated in the HS group compared with the LS group at 3 h and 24 h postprandial ($P < 0.01$). The expressions of lipogenesis-related gene *FASN* were also significantly downregulated in the HS group compared with the LS group at 24 h postprandial ($P < 0.01$). The expressions of *FASN* were significantly downregulated at 24 h postprandial compared with 3 h postprandial in the HS group ($P < 0.01$). The expressions of lipolysis-related genes (*ATGL* and *HSL*) were not significantly different between the two groups at the different postprandial times ($P > 0.05$). The expressions of β-oxidation-related genes (*CPT1α* and *PPARα*) were not significantly different between the two groups at 3 h postprandial, but the expressions of *CPT1α* and *PPARα* were significantly downregulated in the HS group compared with the LS group at 24 h postprandial ($P < 0.01$). The expressions of *CPT1α* and *PPARα* were significantly downregulated at 24 h postprandial compared with 3 h postprandial in the HS group ($P < 0.05$, $P < 0.01$).

3.5. Phenotypic observation of liver tissue sections

Fish liver sections were examined after H & E and PAS staining. The results are shown in Fig. 4. The H & E staining results showed no obvious abnormity with well-shaped cells and rounded nuclei in either group. The PAS staining results showed a low glycogen accumulation signal, without extensive intracellular glycogen granules in hepatocytes in hepatic tissue in the LS and HS groups. Only 1/6 of the fish in the two groups showed a fatty liver phenotype, with high expression of hepatic glycogen, and the rest of the fish were normal. There was no difference between the two groups.

4. Discussion

4.1. High-starch diet did not negatively affect growth of spotted seabass

Carbohydrates can be used as energy sources to conserve dietary protein. As high trophic level (> 3.5) fish, a high-carbohydrate diet resulted in lower growth performances in largemouth bass (Yu et al., 2019; Zhang et al., 2020) and Asian sea bass (*Lates calcarifer*) (Catacutan and Coloso, 1997). However, similar to spotted seabass, European seabass (*Dicentrarchus labrax* L.) also had good feeding and growth performance after ingesting a high-starch diet (20%) (Lanari et al., 1999; Peres and Oliva-Teles, 2002; Enes et al., 2006; Enes et al., 2011). When starch accounted for a larger part of the available energy, fish fed with higher trophic levels (> 3.5) could compensate with increased feed intake to maintain growth, thus conserving protein (Erfanullah, 1998; Hemre et al., 2002). In the present study, the results showed that spotted seabass was well-adapted to the HS diet (20.2% starch) after an 8 w growth trial. Although the fish in the HS group showed a higher FR thereby negatively influenced FCR and PPV, feeding a HS diet did not negatively affect growth from the perspective of slightly higher final body weight (FBW), SGR, and WGR, which indicated that HS diet had the potential to conserve dietary protein in spotted seabass.

Table 5
Effects of the dietary starch levels on plasma biochemical parameters in spotted seabass at satiety (P3 h) and starvation (P24 h) conditions (means ± SEM. n = 8).

Items	P3 h		P24 h		ANOVA models significance		
	LS	HS	LS	HS	Period	Treatment	Period×Treatment
Glucose metabolism							
Glucose (mmol/L)	7.45 ± 0.53^{B}	7.39 ± 0.63	4.83 ± 0.48^{Aa}	8.86 ± 1.38^{b}	**	**	**
Insulin (nmol/L)	41.0 ± 4.14^{A}	59.9 ± 5.26^{A}	72.3 ± 7.18^{B}	72.8 ± 7.70^{B}	**	ns	ns
Glucagon (pg/mL)	409 ± 64.49^{A}	627 ± 58.14^{A}	683 ± 68.92^{B}	775 ± 54.65^{B}	**	ns	ns
Lipid metabolism							
TP (g prot/L)	16.1 ± 0.80^{A}	18.3 ± 0.37^{A}	18.8 ± 0.73^{B}	18.7 ± 0.41^{B}	*	ns	ns
TG (mmol/L)	3.39 ± 0.22	2.93 ± 0.21^{A}	3.16 ± 0.14^{a}	3.55 ± 0.23^{Bb}	**	*	*
NEFA (mEq/L)	0.33 ± 0.06^{B}	0.36 ± 0.07^{B}	0.16 ± 0.01^{A}	0.15 ± 0.01^{A}	***	ns	ns
TC (mmol/L)	7.05 ± 0.48^{A}	6.99 ± 0.37^{A}	8.60 ± 0.37^{B}	8.51 ± 0.68^{B}	**	ns	ns
HDL-C (mmol/L)	3.42 ± 0.60^{A}	3.40 ± 0.31^{A}	6.29 ± 0.18^{B}	5.95 ± 0.25^{B}	***	ns	ns
HDL-C/TC (%)	0.48 ± 0.08^{A}	0.49 ± 0.05^{A}	0.74 ± 0.03^{B}	0.76 ± 0.08^{B}	***	ns	ns
Liver function							
ALT (U/L)	48.3 ± 5.57^{B}	47.6 ± 8.32^{B}	8.33 ± 2.09^{A}	3.69 ± 0.48^{A}	***	ns	ns
AST (U/L)	115 ± 22.72^{B}	144 ± 27.39^{B}	9.46 ± 3.18^{A}	3.07 ± 0.62^{A}	***	ns	ns

The letters "A" and "B" indicate a significant difference between the two groups in the different test phases under the same starch level, while the letters "a" and "b" indicate a significant difference between the two groups in the different starch levels under the same test phase. "ns" indicates not significant; "*" indicates a significant difference ($P < 0.05$); "**" indicates a significant difference ($P < 0.01$); "***" indicates a significant difference ($P < 0.001$).
TP: total protein; TG: triglyceride; NEFA: non-esterified fatty acids; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

Table 6
Effects of the dietary starch levels on hepatic lipids metabolism in spotted seabass under satiety (P3 h) and starvation (P24 h) comditions (means ± SEM. n = 8).

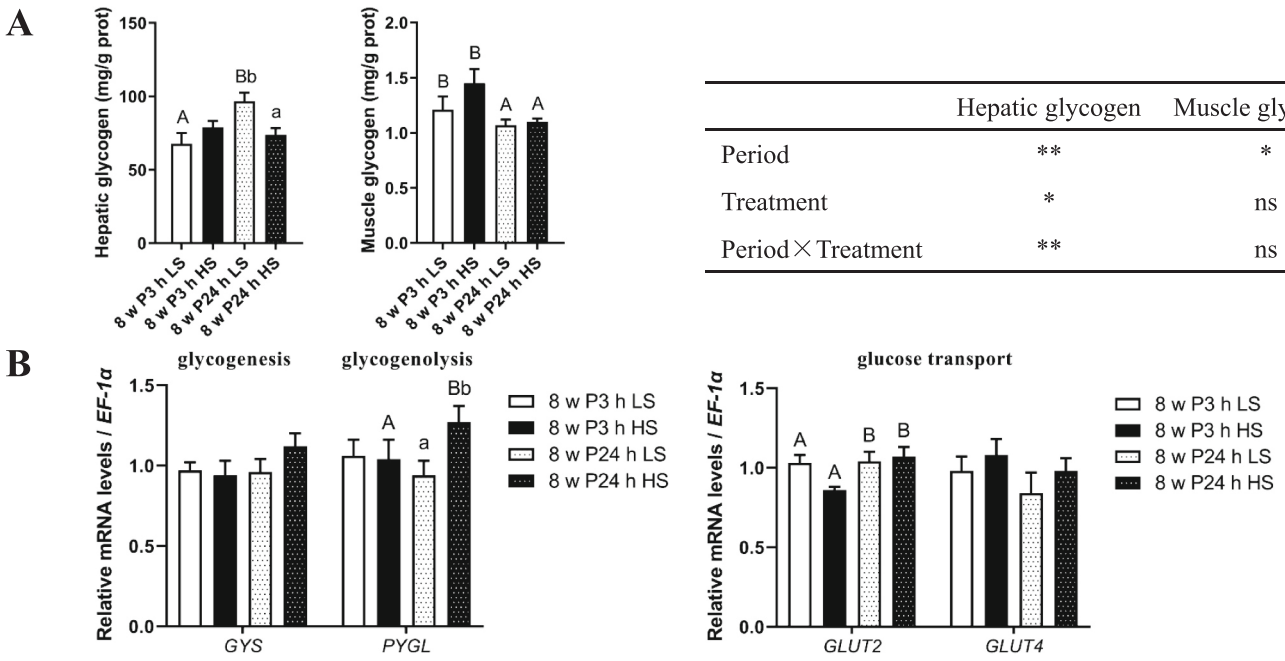
Items	LS	HS	LS	HS	Period	Treatment	Period×Treatment
TC (mmol/g prot)	0.29 ± 0.03	0.31 ± 0.03	0.31 ± 0.02	0.33 ± 0.02	ns	ns	ns
TG (mmol/g prot)	2.39 ± 0.09	2.29 ± 0.12	2.54 ± 0.09	2.52 ± 0.10	ns	ns	ns
NEFA (mEq/g prot)	2.07 ± 0.12^{Ab}	1.53 ± 0.11^{Aa}	2.89 ± 0.16^{Ba}	3.42 ± 0.23^{Bb}	**	*	*

The letters "A" and "B" indicate a significant difference between the two groups in the different test phases under the same starch level, while the letters "a" and "b" indicate a significant difference between the two groups in the different starch levels under the same test phase. "ns" indicates not significant; "*" indicates a significant difference ($P < 0.05$); "**" indicates a significant difference ($P < 0.01$).
The letter "prot" means total protein in liver tissue.
TC: total cholesterol; TG: triglyceride; NEFA: non-esterified fatty acids.

4.2. Spotted seabass can relieve hyperglycemia at P24 h by effectively regulating the basal energy metabolism

Although the fish owned all of the enzymatic machinery required for carbohydrate utilization, prolonged hyperglycemia was showed after fed high-carbohydrate diets (Moon, 2001; Enes et al., 2009; Rocha et al., 2015). The rate of glucose metabolism in fishes is one to two orders of magnitude lower than that in mammals (Hemre et al., 2002), so the glucose clearance is relatively slow. Many studies showed that regulating plasma glucose back to the normal levels was required 24 h postprandial-recovery time for rainbow trout and turbot (*Scophthalmus maximus*) (Bergot, 1979; Garcia-Riera and Hemre, 1996), and even longer time for Atlantic salmon (Hemre et al., 1995b; Villasante et al., 2019) and Atlantic cod (*Gadus morhua*) (Hamre, 2006). This phenomenon may be ubiquitous in higher trophic level (> 3.5) fish. The normal ranges of blood glucose are not fully defined in spotted seabass and many other aquaculture fish species (Hemre et al., 2002).

Fish fed with lower trophic levels (< 3, such as Nile tilapia) can regulate plasma glucose more effectively (Chen et al., 2017). This may be because the carbohydrate in the diet promoted glycolysis and lipogenesis but inhibited gluconeogenesis (Hemre et al., 2002). Fish fed with high trophic levels (> 3.5) usually cannot effectively regulate plasma glucose because they cannot regulate gluconeogenesis well during satiation status and regulate glycolysis well during starvation status (Panserat et al., 2001). G6P is a key enzyme involved in the last step of gluconeogenesis, which mainly regulates the process of the hydrolysis of glucose-6-phosphate to glucose and plays a crucial role in the homeostasis of blood glucose balance (Lochhead et al., 2000; Foster and Nordlie, 2002). Glycolysis is the essential pathway for glucose catabolism in all organisms, including fish (Cowey et al., 1977; Enes et al., 2009). GK, PFK-1, and PK are three key enzymes in the glycolytic pathway. GK can maintain homeostasis of blood glucose balance through the catalytic action of glucose phosphorylation (Polakof et al., 2011; Enes et al., 2009). PK is a rate-limiting enzyme in the aerobic oxidation of glucose that catalyzes phosphoenolpyruvate to pyruvate (Metón et al., 2003). In the current study, higher plasma glucose levels were observed between the two groups at 3 h postprandial. A well-regulated glucose metabolism response was observed that significant downregulation of gluconeogenesis (*G6P*) and upregulation of glycolysis (*PK*) in the HS group at under satiety conditions (P3 h). Under starvation conditions (P24 h), the plasma glucose of spotted seabass in the LS group decreased to normal level, while persistent hyperglycemic was still observed in the HS group. The gluconeogenesis (G6P) pathway was significantly upregulated, and the glycolytic (*GK, PFK-1*) at the transcription level was significantly down-regulated at 24 h postprandial (P24 h), which was supposed to be a normal glucose metabolic response. However, the activities of key enzymes PK in the glycolysis pathway were significantly increased, which indicated that high-starch intake could not control glycolysis well under starvation conditions (P24 h). As one of the most universal second messengers, cAMP plays a crucial role in regulating substance metabolism and biological function (Wahlang et al., 2018). In this study, excessive starch intake significantly increased hepatic cAMP content, which enhanced the rate of basal energy metabolism of spotted seabass. Thereby body energy metabolism was accelerated to relieve hyperglycemia under starvation conditions (P24 h). Excessive starch intake provided energy for rapid basal metabolism of spotted seabass, so no significant improvement in growth performance in the HS group during the whole growth phases.



	Hepatic glycogen	Muscle glycogen
Period	**	*
Treatment	*	ns
Period × Treatment	**	ns

	GYS	*PYGL*	*GLUT2*	*GLUT4*
Period	ns	*	*	ns
Treatment	ns	*	ns	ns
Period × Treatment	ns	*	ns	ns

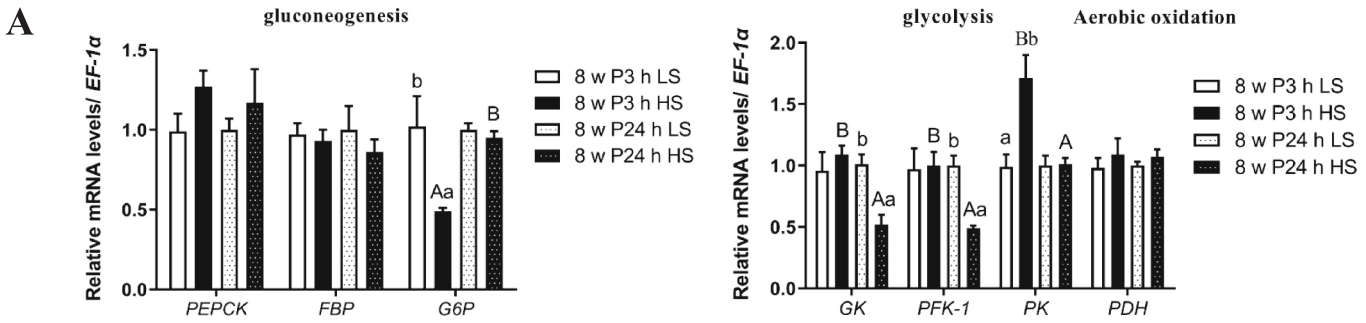
Fig. 1. Effects of high-starch diets on content of glycogen in liver and muscle and mRNA levels of glycogen metabolism and glucose transporters in liver of spotted seabass under satiety (P3 h) and starvation (P24 h) conditions (means ± SEM, $n = 8$).
The letters "A" and "B" indicate a significant difference between the two groups in the different test phases under the same starch level, while the letters "a" and "b" indicate a significant difference between the two groups in the different starch levels under the same test phase. "ns" indicates not significant; "*" indicates a significant difference ($P < 0.05$); "**" indicates a significant difference ($P < 0.01$).

4.3. Spotted seabass could preferentially catabolize glycogen to provide energy and then utilize lipid under starvation conditions

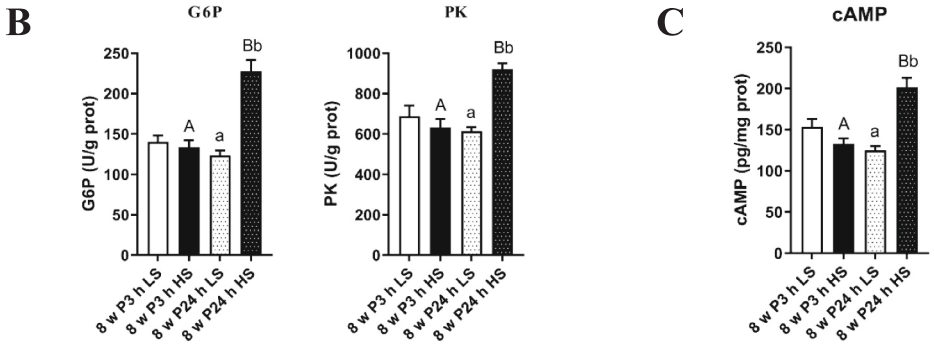
Carbohydrates can be converted into glycogen and stored in the liver and muscle tissue in fish. In mammals, under starvation conditions, the body will mobilize and rapidly decompose liver glycogen into glucose to maintain plasma glucose stability. In fish, the role of glycogen in this process varies depending on fish species (Enes et al., 2009). European sea bass, rainbow trout, and gilthead sea bream (*Sparus aurata*) can effectively mobilize glycogen and significantly deplete hepatic glycogen in the early stages of starvation (Metón et al., 2003; Pérez-Jiménez et al., 2007; Soengas et al., 2006). However, the content of glycogen in the liver of largemouth bass increased significantly after feeding a diet with approximately 10% starch (Yu et al., 2019; Zhang et al., 2020). In this study, there was no significant increase in glycogen content in the HS group after feeding the high-starch diet, and no glycogen accumulation was observed in liver tissue sections stained with PAS. In addition, under starvation conditions, hepatic glycogen content was significantly decreased, and the mRNA levels of glycogenolysis-related gene (*PYGL*) were significantly upregulated in the HS group compared with the LS group, indicating that the high-starch diet did not induce glycogen accumulation in spotted seabass and that spotted seabass can mobilize hepatic glycogenolysis to provide energy under starvation conditions.

Under aerobic conditions, glucose is completely catabolized through glycolysis, the Krebs cycle and the respiratory chain to produce ATP to provide energy or through the pentose phosphate pathway to produce nicotinamide adenine dinucleotide phosphate (NADP) for biosynthesis (Polakof et al., 2012). Excess glucose can be stored as glycogen by glycogenesis pathway or converted into lipids by lipogenesis pathway (Enes et al., 2009; Polakof et al., 2012). A previous study demonstrated that excessive dietary carbohydrates affect normal growth and cause excessive fat accumulation (Chen et al., 2012). The lipid deposition pattern in fish mainly includes subcutaneous adipose, visceral adipose, liver and intramuscular adipose tissue (Ren et al., 2018). In this study, there were no significant differences in the HSI or VAI between the two groups. According to the observation of liver tissue section phenotypes, the HS diet did not cause fatty liver symptoms, indicating that visceral adipose tissue was not affected by dietary starch levels. In contrast to largemouth bass (Yu et al., 2019), excessive dietary starch did not cause excessive accumulation of hepatic adipose tissue in spotted seabass. The contents of TG and TC in serum reflect a certain lipid metabolism state. In the present study, there were no significant differences in TC or TG between the LS and HS groups, and no significant differences in hepatic lipids were observed at different postprandial time points, which preliminarily indicated that hepatic lipid metabolism was normal.

The conversion of excess glucose into fatty acids may cause fat accumulation; thus, lipogenesis may play a vital role in glucose homeostasis balance. ACC1 and FASN are two key enzymes involved in fatty acid synthesis (Mckim et al., 1989; Menendez and Lupu, 2007). In this study, the high-starch diet did not cause significant upregulation of lipogenesis (*ACC1*, *FASN*) under satiety conditions (P3 h), and it was significantly downregulated under starvation conditions (P24 h). Excess glucose did not accelerate lipid accumulation. This observation was different from the response of largemouth bass to metabolic liver disease



	PEPCK	*FBP*	*G6P*	*GK*	*PFK-1*	*PK*	*PDH*
Period	ns	ns	**	**	*	*	ns
Treatment	ns	ns	**	**	*	**	ns
Period × Treatment	ns	ns	**	**	*	**	ns

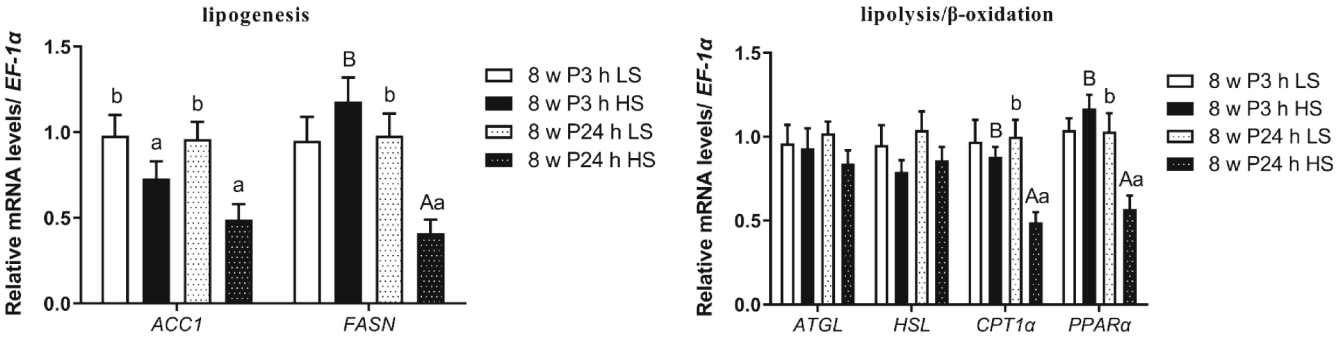


	G6P	PK	cAMP
Period	***	***	***
Treatment	***	***	***
Period × Treatment	***	***	***

Fig. 2. Effects of high-starch diets on glucose and energy metabolism of spotted seabass under satiety (P3 h) and starvation (P24 h) conditions (means ± SEM, n = 8). (A): The relatively mRNA levels of gluconeogenesis, glycolysis and aerobic oxidation related genes at different postprandial time points of spotted seabass fed LS or HS diets. (B): The enzymes activities related to gluconeogenesis and glycolysis at different postprandial time points of spotted seabass fed LS or HS diets. (C): The content of cAMP related to energy metabolism at different postprandial time points of spotted seabass fed LS or HS diets.
The letters "A" and "B" indicate a significant difference between the two groups in the different test phases under the same starch level, while the letters "a" and "b" indicate a significant difference between the two groups in the different starch levels under the same test phase. "ns" indicates not significant; "*" indicates a significant difference ($P < 0.05$); "**" indicates a significant difference ($P < 0.01$); "***" indicates a significant difference ($P < 0.001$).

caused by feeding a 10% starch diet (Yu et al., 2019). Lipogenesis and lipolysis/β-oxidation are pathways usually regulated in opposite directions (Bonacic et al., 2016). ATGL is a rate-limiting enzyme in the hydrolysis of triglycerides. As an intracellular neutral lipase, HSL can hydrolyze triacylglycerols, diacylglycerols, monoacylglycerols, and cholesteryl esters, as well as other lipids and water-soluble substrates (Luo et al., 2019; Lampidonis et al., 2011). CPT1α is involved in the fatty acid β-oxidation pathway, and PPARα is considered to be a main regulator of lipid metabolism and act as a fat sensor to regulate the response of fatty acid metabolism to diet (Rogue et al., 2011). In the present study, lipolysis/β-oxidation processes were not affected by the high-starch diet under satiety conditions (P3 h), but the β-oxidation process (*CPT1α* and *PPARα*) was inhibited in the HS group compared with the LS group under starvation conditions (P24 h). Since the hepatic glycogen content in the HS group was significantly reduced compared with that in the LS group at 24 h postprandial, fatty acid β-oxidation was down-regulated. Hepatic cAMP content increased by excessive starch intake accelerated the rate of basal metabolism to avoid glycogen accumulation and fatty liver. Under starvation conditions (P24 h), spotted seabass were able to preferentially catabolize glycogen during starvation to provide energy and then catabolize lipids.

In conclusion, feeding a high-starch diet did not negatively affect growth, but slightly increased the FR and FCR and decreased the PPV of spotted seabass. Although the HS group remained persistent hyperglycemia at 24 h postprandial, increasing hepatic cAMP content accelerated the rate of basal energy metabolism and decreased lipogenesis to relieve



	ACC1	*FASN*	*ATGL*	*HSL*	*CPT1α*	*PPARα*
Period	ns	**	ns	ns	*	**
Treatment	**	**	ns	ns	**	**
Period × Treatment	ns	**	ns	ns	*	**

Fig. 3. Spotted seabass effectively regulated the lipid metabolism in liver under satiety (P3 h) and starvation (P24 h) conditions fed LS or HS diets (means ± SEM, n = 8).
The letters "A" and "B" indicate a significant difference between the two groups in the different test phases under the same starch level, while the letters "a" and "b" indicate a significant difference between the two groups in the different starch levels under the same test phase. "ns" indicates not significant; "*" indicates a significant difference ($P < 0.05$); "**" indicates a significant difference ($P < 0.01$).

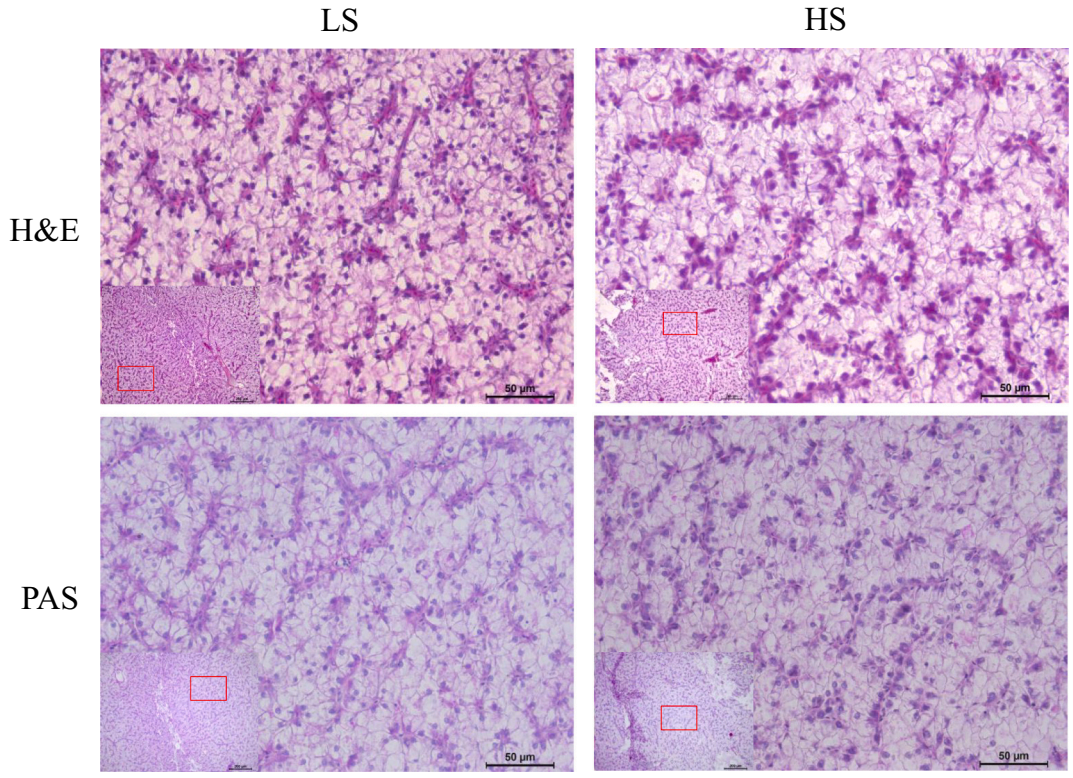


Fig. 4. Effects of the dietary starch levels on liver histopathology in spotted seabass.
Liver sections were stained following the protocols of hematoxylin and eosin (H & E) staining, periodic acid-Schiff (PAS) staining, two phenotypes were observed under 10× and 40× objective lens with bar = 200 μm and 50 μm, respectively. The H & E staining results showed no obvious abnormity with well-shaped cells and the PAS staining results showed a low glycogen accumulation signal either group. Only 1/6 of the fish in the two groups showed a fatty liver phenotype, with high expression of hepatic glycogen, and the rest of the fish were normal.

hyperglycemia and avoid glycogen accumulation and fatty liver. Spotted seabass could preferentially catabolize hepatic glycogen to provide energy and then utilize lipids under starvation conditions, which indicated that it could effectively regulate energy metabolism after feeding the HS diet.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the National Key R&D Program of China (2019YFD0900200 and 2018YFD0900400); National Natural Science Foundation of China (31902382 and 31802306); National Natural Science Foundation of Beijing (6204047); The Agricultural Science and Technology Innovation Program of CAAS, China (CAAS-ASTIP-2017-FRI-08); and Beijing Technology System for Sturgeon and Salmonids (BAIC08-2021).

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