Fish Physiol Biochem (2019) 45:1015–1028
https://doi.org/10.1007/s10695-019-00612-x

Nutritional regulation of pyruvate kinase and phosphoenolpyruvate carboxykinase at the enzymatic and molecular levels in cobia *Rachycentron canadum*

Ruixin Li · Hongyu Liu · Shuyun Li · Beiping Tan · Xiaohui Dong · Shuyan Chi · Qihui Yang · Shuang Zhang · Liqiao Chen

Received: 12 August 2018 / Accepted: 22 January 2019 / Published online: 20 February 2019

Abstract Despite being a carnivorous fish species, cobia (*Rachycentron canadum*) can utilize high levels of dietary carbohydrate (up to 360 g kg^{-1}). By contrast, rainbow trout (also carnivorous) cannot, due to the absence of molecular induction of glycolytic enzyme and inhibition of gluconeogenic enzyme gene expressions such as pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK). We hypothesized that this phenomenon is species-specific and will not be observed in cobia. Our results show that, at the molecular level, the mRNA abundance of the important glycolytic (PK) and gluconeogenic (PEPCK) enzymes in cobia liver are regulated by dietary carbohydrate-to-lipid (CHO:L) ratios and nutritional status (fed, unfed, and refed). Significantly upregulated hepatic PK and depressed PEPCK gene expressions were observed when the fish were fed with an increasing CHO/L-ratio diet or were refed. However, in contrast to gene expression, there was no significant effect of dietary CHO/L ratios on PK enzyme activity. The decrease in PEPCK activity was significantly found between low CHO/L ratio and high CHO/L ratio diets, whereas the moderate CHO/L ratio group showed intermediate values. But PEPCK activity appeared to be independent of nutritional status. These results suggest that nutritional regulation is obvious, at least at the molecular level, in the key hepatic enzymes (PK and PEPCK) of the glucose metabolism pathway, in response to different dietary CHO/L ratios and to the transition from being starved to fed. Determining whether other key enzymes involved in hepatic glucose metabolism contribute to glucose tolerance in cobia is necessary for further investigation of this phenomenon at the enzymatic and molecular levels.

Keywords *Rachycentron canadum* · Pyruvate kinase · Phosphoenolpyruvate carboxykinase · Nutritional regulation · Carbohydrate-to-lipid ratios · Starvation and refeeding

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10695-019-00612-x) contains supplementary material, which is available to authorized users.

R. Li · H. Liu · S. Li · B. Tan · X. Dong · S. Chi · Q. Yang · S. Zhang
Laboratory of Aquatic Animal Nutrition and Feed, College of Fisheries, Guangdong Ocean University, Zhanjiang 524088 Guangdong, People's Republic of China

R. Li · H. Liu (✉) · S. Li · B. Tan (✉) · X. Dong · S. Chi · Q. Yang · S. Zhang
Aquatic Animals Precision Nutrition and High Efficiency Feed Engineering Research Center of Guangdong Province, Zhanjiang, Guangdong, China
e-mail: liu_hong_yu@163.com
e-mail: bptan@126.com

L. Chen
Life Science College, East China Normal University, Shanghai, China

Introduction

Unlike carnivorous mammals, carnivorous fish usually exhibit poor utilization of carbohydrates. However, the basis of such apparent glucose intolerance in

carnivorous fish remains unknown (Moon 2001; Wilson 1994). One hypothesis is that there is an imbalance between hepatic glucose uptake (glycolysis) and production (gluconeogenesis), leading to inadequate regulation of glucose homeostasis (Panserat et al. 2001c).

In rainbow trout (*Oncorhynchus mykiss*), a typical carnivorous fish, the poor utilization of dietary carbohydrate is reflected by prolonged postprandial hyperglycemia after high carbohydrate intake (Cowey and Walton 1989; Wilson 1994). Regarding nutritional regulation, there is no specific effect of dietary carbohydrate on hepatic pyruvate kinase (PK) gene expression (Panserat et al. 2001a), as this is not controlled by nutritional status, i.e., being food-deprived or well-fed (Kirchner et al. 2003; Panserat et al. 2001a). In gilthead sea bream (*Sparus aurata*), short-term refeeding after starvation is not sufficient to recover its earlier hepatic PK activity (Metón et al. 2003). For the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK), in gilthead sea bream and rainbow trout, the hepatic PEPCK gene is expressed at the same level independent of dietary composition and nutritional status (Panserat et al. 2001b, 2002). The absence of the nutritional regulation of PEPCK gene expression leads to speculation that the PEPCK gene cloned in rainbow trout liver codes for a mitochondrial isoform. In mammals, hepatic glycolysis PK and gluconeogenesis PEPCK activity and mRNA abundance are primarily regulated by dietary carbohydrate intake and are major contributors to glucose homeostasis (Hanson and Reshef 1997; Yamada and Noguchi 1999).

Cobia (*Rachycentron canadum*) is a carnivorous fish species that mainly preys on squid and fish (Franks et al. 1996). Because of their carnivorous nature, juvenile cobia can utilize large amounts of proteins and lipids without any impact on production characteristics. Food given to cultured cobia generally contains more than 45% crude protein and 15–16% crude lipid (Craig et al. 2006; Liao et al. 2004); however, Chou et al. (2001) reported that high levels of lipid (up to 18%) showed no growth or efficiency benefit over a 6% lipid diet. Interestingly, being carnivores, cobia should be unable to utilize high levels of carbohydrates, but research has demonstrated that they can tolerate up to 360 g kg^{-1} of dietary dextrin (Schwarz et al. 2007). Ren et al. (2011) also reported that the appropriate dietary starch supplementation for juvenile cobia was estimated to be about 20%, along with 42% protein and 10% lipid. These data suggest that it is highly feasible for fish farms to reduce food costs by replacing lipids with cheaper carbohydrates; however, no information on the effect of different dietary carbohydrate-to-lipid ratios (CHO/L) on intermediary metabolism is available for this fish.

Rainbow trout have been found to lack molecular induction of glycolytic enzymes and inhibition of gluconeogenic enzyme gene expressions (such as PK and PEPCK), which can at least partially account for their poor utilization of dietary carbohydrate (Panserat et al. 2001a, b). Given that cobia seem to be able to utilize high levels of dietary carbohydrates, we did not expect to find these same phenomena in this species. Thus, the first step was to clone full-length cDNA for PK since full-length cDNA of cytosolic PEPCK (accession no. FJ645270) has already been cloned in a previous study (Lee 2009). Then, we assessed the resulting changes in the gene expression and activity of key enzymes (PK and PEPCK) in the glucose metabolism pathway in cobia liver in response to various diets (various CHO/L ratios and the starved-to-fed transition).

Materials and methods

Feeding trial, experimental conditions, and sample collection

Dextrin was used as the main carbohydrate source in three isonitrogenous and isoenergetic diets containing different CHO/L ratios (low CHO/L ratio 0.68%, moderate CHO/L ratio 1.91% and high CHO/L ratio 5.64%). Compositions of the experimental diets are shown in Table 1.

Before the feeding trial, six healthy fish were randomly captured from a pool and were sacrificed to obtain heart, whole kidney, white muscle, liver, and intestine for PK tissue expression tests. Then, triplicate groups (20 fish in each cage) of juvenile cobia with an average of body weight of 23.53 ± 0.39 g were reared in a farm of the South Island, Zhanjiang, Guangdong. They were fed twice a day (08:00 and 16:30) to apparent satiation with formulated dry diets containing different CHO/L ratios during the feeding trial period (8 weeks). After the 8-week feeding experiment, fish were starved for 24 h. Next, four fish (two fish for the measurement of gene expression, the other two for the measurement of enzyme activity) were randomly captured from each cage and were deeply anesthetized with eugenol

(200 mg L^{-1}). The fish were then euthanized by immediate spinal dislocation, and liver tissue was promptly removed from the fish and immediately frozen in liquid nitrogen and stored at − 80 °C until required.

In addition, 120 fish of similar size divided into another six cages (20 fish per cage) were starved for a period of 4 weeks. Three of these cages (four fish per cage) were sampled at the end of the food-deprivation period. At the same time, fish in the other three cages were refed over the 4-week period, and the same number fish were then sampled at the end of trial experiment. The fish fed with a moderate CHO/L ratio diet for 8 weeks were used as a control, according to Ren et al. (2011) in comparison with fish during the starved-to-fed transition. During the experiment, the water temperature remained between 26 and 33 °C, and dissolved oxygen was maintained above 6.0 mg/L. The procedures of this study involving animals and their care was conducted in conformity with NIH guidelines (NIH Pub. No.85–23, revised 1996) and were approved by the Animal Care and Use Committee of Guangdong Ocean University.

Table 1 Formulation and proximate composition of cobia experimental diets (%dry matter)

Ingredients (%)	CHO/L ratio		
	Low (L)	Moderate (M)	High (H)
Fish meal (CP:65%)	22	22	22
Casein	20	20	20
Vital wheat gluten	15	15	15
Dextrin	12	24	36
Fish oil	13.8	8.19	2.48
Soybean lecithin	1.5	1.5	1.5
Vitamin premix[a]	0.6	0.6	0.6
Minerals premix[b]	0.6	0.6	0.6
Vitamin C	0.1	0.1	0.1
Choline chloride	0.5	0.5	0.5
Ethoxyquin	0.03	0.03	0.03
Attractant[c]	0.1	0.1	0.1
CaH_2PO_4	1	1	1
Microcrystalline cellulose	12.77	6.38	0.09
Total	100	100	100
Crude protein	43.42	44.04	43.85
Crude lipid	17.17	11.66	6.16
Nitrogen-free extract	11.69	22.31	34.73
Carbohydrate-to-lipid ratio	0.68	1.91	5.64
Gross energy (MJ/kg)	19.06	18.85	18.76

[a.] Vitamin premix obtained from DSM (China) Limited (mg·kg^{-1} diet): vitamin A, 10 mg; vitamin D_3, 50 mg; vitamin E, 99 mg; vitamin K, 5.0 mg; vitamin B_1, 25.50 mg; vitamin B_2, 25 mg; vitamin B_6, 50 mg; vitamin B_{12}, 0.1 mg; calcium pantothenate, 61 mg; nicotinic acids, 101 mg; biotin, 25 mg; inositol, 153.06 mg; folic acid, 6.25 mg; microcrystalline cellulose, 389.09 mg

[b.] Minerals premix supplied by Guangdong Tianke Biotechnology (Guangzhou, China) (mg·kg^{-1} diet): KIO_4, 0.03 mg; $CoCl_2 \cdot 6H_2O$, 4.07 mg; $CuSO_4 \cdot 5H_2O$, 19.84 mg; ferric citrate, 13.71 mg; $ZnSO_4 \cdot 7H_2O$, 28.28 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; $MnSO_4 \cdot H_2O$, 12.43 mg; KCl, 15.33 mg; Na_2SeO_3, 2 mg; zeolite powder, 824.19 mg

[c.] Attractant composition: taurine: glycine: betaine = 1:3:3; they are obtained from Hangzhou King Techina Technology (Hangzhou, China)

Analytical methods

The proximate composition of the experimental diets was analyzed by the standard methods of AOAC (AOAC 1995). For the measure of PK and PEPCK in livers, a frozen sample of cobia liver (500 mg) was homogenized (dilution 1/10) in ice-cold buffer (80 mM Tris; 2 mM DTT; 5 mM EDTA;1 mM benzamidine; 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, pH 7.6). The homogenate was centrifuged at 10,000×*g* for 20 min at 4 °C. The resultant supernatant was separated, and the activity of hepatic PK was determined following the procedure described by Kirchner et al. (2003), monitoring the decrease in absorbance (β-NAD, reduced form disappearance) using purified lactate dehydrogenase (LDH, 300 unit/mg protein, Sigma) in excess as the coupling enzyme. The PEPCK enzymatic activity was assessed with the method of Polakof et al. (2008), briefly (in mM): 50 Tris–HCl (pH 7.5), 1 $MnCl_2 \cdot 4H_2O$, 20 $NaHCO_3$, 1.5 PEP, 0.3 NADH, excess malate dehydrogenase (MDH, 400 unit/mg protein, Sigma), and 12 deoxyguanosine-5′-diphosphate was used. The reaction was started with deoxyguanosine-5′-diphosphate, and the absorbance at 340 nm was recorded in a microtiter plate reader (SLT).

RNA isolation and reverse transcription

Total RNA was extracted from liver samples according to the RNAiso Plus Kit manual (TaKaRa, Dalian, China). The tissues were lysed with RNAiso Plus buffer, extracted with chloroform (Sangon, Shanghai, China), and precipitated with 2-propanol (Sangon) to obtain total RNA. The extracted RNA was digested with RNase-free DNase I (TaKaRa).

The quantity and purity of the isolated RNA was later determined by absorbance measurements at 260 and 280 nm using a NanoDrop ND-1000 (Thermo Scientific, USA), and its integrity was tested by electrophoresis in 1.5% formaldehyde denaturing agarose gels. Only high-quality samples in which OD 260/OD 280 > 1.8, OD 260/OD 230 > 2.0, and 28S/18S rRNA > 1 were used for subsequent complementary DNA (cDNA) synthesis. cDNA was obtained by annealing 1 μg of total RNA with 1 μg of random primers and incubating with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for 1 h at 42 °C.

Molecular clone of cobia PK genes

PK sequences from *Homo sapiens* (GenBank accession number CAA39849.1), *Rattus norvegicus* (GenBank accession numbers AAB93666.1), *Danio rerio* (GenBank accession number AAH45421.1), and *Trachinotus ovatus* (GenBank accession numbers AJD07313.1) were compared using the Clustal-W multiple alignment algorithm (Thompson et al. 2002). Degenerate primers were chosen on the basis of the most conserved coding regions of the PK gene. Specific primers were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) under default parameters. The primers for BLAST searches were conducted to confirm the specificity of the primer sequences. The degenerate (upstream and downstream) primers and specific primers are presented in Table 2. The polymerase chain reaction (PCR) was conducted to amplify PK cDNA with 1 μl cDNA template and 0.5 μM degenerate primers in a reaction mixture containing 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 20 mmol/L Tris–HCl, 0.25 mmol/L dNTP and 2.5 units of Taq polymerase (TransTaq® DNA Polymerase High Fidelity Boehringer, TransGen Biotech, China). The PCR program contained 33 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s followed by an extension at 72 °C for 2 min. PCR products were separated by electrophoresis in 1.5% agarose gels in Tris-acetate (TAE) buffer and photographed after staining with ethidium bromide. A fragment of approximately 1400-bp bright band was purified using an GeneJET Gel Extraction Kit (Fermentas, Vilnius, Lithuania) following the manufacturer's protocol. The purified fragment was ligated into the pMD18-T Vector (TaKaRa, Dalian, China) and transformed into *Escherichia coli* Cast & Chalm (strain DH5a) cells (Sangon Biotech, Shanghai, China). Recombinant plasmids were extracted using the Plasmid Mini Kit (QIAGEN, Valencia, CA, USA), and cDNA fragments were sequenced by Shanghai Sangon Biotech Service Co. Ltd. (Shanghai, China).

According to the core PK cDNA fragments sequence, specific primers (Table 2) for RACE-PCR were designed to obtain the full-length cDNAs by using the SMARTer™ RACE cDNA Amplification Kit (Clontech). The first strand of cDNA from the 3′ end was obtained with reverse transcription of total RNA and Oligo $(dT)_{16}$AP as the primer. This was used as a template for PCR with a gene-specific forward primer 3′ PK-RACE1 and a reverse primer AP. After that, the product was applied to the nested PCR with a specific forward primer 3′ PK-RACE2. For 5′ rapid-amplification of the cDNA ends, reverse transcription of total RNA was conducted with a specific reverse primer 5′ PK-RACE1 to obtain the first-strand cDNA. Then, an Oligo (dA) tail at the 5′ end was added using terminal deoxynucleotidyl transferase. That was used for the first PCR with a universal forward primer Oligo $(dT)_{16}$AP and a specific reverse primer 5′ PK-RACE2. The resulting product was diluted for the nested PCR with a specific reverse primer, 5′ PK-RACE3, and an anchor forward primer AP. PCR products were separated by electrophoresis in 1.5% agarose gels in Tris-acetate (TAE) buffer and photographed after staining with ethidium bromide. A fragment of approximately 1500-bp bright band was purified using an GeneJET Gel Extraction Kit (Fermentas, Vilnius, Lithuania) following the manufacturer's protocol. The purified fragment was ligated into the pMD18-T Vector (TaKaRa, Dalian, China) and transformed into *Escherichia coli* Cast & Chalm (strain DH5a) cells (Sangon Biotech, Shanghai, China). Recombinant plasmids were extracted using the Plasmid Mini Kit (QIAGEN, Valencia, CA, USA), and cDNA fragments were sequenced by Shanghai Sangon Biotech Service Co. Ltd. (Shanghai, China).

Real-time quantitative PCR analysis

The expression of PK mRNA in different tissues was analyzed by using real-time quantitative PCR. Total RNA was isolated from five tissues (heart, whole kidney, white muscle, liver and intestine) of six cobia prior to the trial experiment. The cDNA, obtained as described above, was amplified by PCR using specific

Table 2 Primers used for PK cDNA cloning by RT-PCR (degenerate primers) and RACE-PCR and for PK and PEPCK gene expression analysis by RT-PCR

Primer name	Sequence (5′-3′)	Tm	length
PK ORF F	GTGYCKNCTGGAYATYGAYTC	56 °C	1468 bp
PK ORF R	AGRNCCDGGRCGCCABCCBGT		
5′ PK-RACE1	AYTCATGTGTGCCATG	62 °C	335 bp
5′ PK-RACE2	CGTATCCACAGAACGAGAGG	60 °C	270 bp
5′ PK-RACE3	ATKGTGCAGATRATGCCAGTGT	55 °C	294 bp
3′ PK-RACE1	CTCTACACCAAGCCTGCCAACGACG	61 °C	363 bp
3′ PK-RACE2	CATGGATATGGGCAAAGTCCGTGGC	53 °C	301 bp
RT-PCR	Sequence(5′-3′)	Tm	Accession No.
PK F	TCCTCTGGCTCGACTACAA	60 °C	KM262818
PK R	CGTTCTCAATCTCGCACA		
PEPCK F	CTCTGACGAGGAGAACCG	59 °C	FJ645270
PEPCK R	TGTCTTACTTTCTACACGAGCC		
β-actin F	AGGGAAATTGTGCGTGAC	58 °C	EU266539
β-actin R	AGGCAGCTCGTAGCTCTT		

N = A/C/G/T; D = A/G/T; B=C/G/T; S = C or G; Y = C or T; R = A or G; W = A or T; K = G or T. Tm, melting temperature

primers chosen from the full-length sequence for cobia PK cDNA (Table 2). The RT-PCR analysis (ABI 7500, Applied Biosystems, America) was performed with the following program: 1 cycle at 95 °C for 30 s and 40 cycles of 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s. The melting curves of the qPCR products were performed at the end of each PCR to confirm that only one product was amplified and detected. PK mRNA expression levels were normalized to the equivalent RNA levels of the reference gene β-actin (accession number EU266539) using the optimized comparative Ct ($2^{-\Delta\Delta Ct}$) value method (Livak and Schmittgen 2001). β-actin from cobia was chosen as the reference gene to normalize expression levels of targets between different samples. Among the reference genes tested, β-actin did not reveal changes in expression levels and presented the lowest standard deviation within different tissues (0.79) and conditions studied (0.88) using the BestKeeper. In addition, the Cq values of the candidate reference gene were used for comparison of expression stability in the NormFinder and GeNorm programs. β-actin was also found to be a stable reference gene within different tissues and conditions. The PCR efficiency (E) and correlation coefficients (R^2) were determined based on the slopes of the standard curves generated from the Cq values of serial dilutions of the cDNA. The E values of several references ranged from 96.7 to 101.1%, and the R^2 values ranged from 0.991 to 0.997. PK (accession number: KM262818) and PEPCK (accession number: FJ645270) gene expressions of cobia in response to different dietary CHO/L ratios and nutritional status were also determined following the procedures described above. There were six fish ($N = 6$) for each treatment, each fish was analyzed for each sample, and each sample was run in triplicate.

Sequence alignment and phylogenetic analysis

Nucleotide sequences were compared with DNA sequences from the GenBank database using the basic local alignment search tool (BLAST) algorithm. Multiple amino acid sequence alignments and percentages of amino acid conservation were evaluated with the ClustalX 2.1 and Genedoc Package 4.0. The neighbor-joining phylogenic trees were constructed using MEGA 4.0 software based on amino acid sequences.

Statistical analysis and sequence analysis

The results were presented as means ± standard deviation (SD, $N = 6$). Data in this study were subjected to one-way analysis of variance (ANOVA) after testing the homogeneity of variances using the SPSS 20.0 software package (SPSS Inc. Wacker Drive, Chicago, IL USA). Statistical differences among means were determined

using Duncan's multiple-range test. Differences were considered significant at 5%.

Results

Molecular characterization of cobia PK

The full length of cobia PK (accession no. KM262818) gene cDNA covered 1869 bp with a 1599 bp open reading frame (ORF) encoding 532 amino acids (Fig. 1). This AA sequence contained several conserved functional sites, including an ADP/ATP binding site, a phosphoenolpyruvate binding site, an effector site, cation requirement sites, and an additional N-terminal sequence (Fig. 2). Complete AA sequence alignment analysis showed that cobia PK had similar structural features to those of other species (Fig. 2) and showed the greatest similarity of amino acid residues to zebra fish PKM_1 (up to 89%), but lower identity with human, rat, and zebra fish PKL (about 68%), suggesting that cobia PK is likely to be the PKM gene.

Phylogenetic analysis

The evolutionary relationships between the PKs were investigated by the construction of phylogenetic trees (Fig. 3). Alignments were made based on the amino acid sequences studied. MEGA 4.0 neighbor-joining software was used to produce an un-rooted tree. The tree represented a 50% consensus from 1000 bootstrap replicates. The sequences fell into two clusters: vertebrate PKLR and PKM, with a bootstrap value of 100%. In each cluster, mammalian sequences formed a group distinct from the others, as shown by the branch bifurcations. The second cluster comprised a branch with one group formed by *Homo sapiens* and *Rattus norvegicus* PKM. For another part of the cluster, a close evolutionary relationship was observed between the zebra fish (PKM_1) and cobia PK sequences. The fact that the cobia PK sequence fell into the cluster of PKM suggested that cobia PK was expected to be the PKM gene (Fig. 3).

Tissue-specific expression of PK mRNA in cobia

Distributions of PK mRNA were detected in a range of tissues, including the heart, intestine, liver, kidney, and white muscle of cobia (Fig. 4). The results showed that the cobia PK gene was predominantly expressed in the liver and heart, followed by white muscle, while significantly lower expression levels were observed in the kidney and intestine ($P < 0.05$).

The effect of dietary CHO:L ratios and nutritional status on mRNA expression and hepatic PK and PEPCK activity in cobia

In mammalian PK enzymes, there are four distinct isozymes, M_1, M_2, L, and R, encoded by two genes: PKM and PKL. The PEPCK enzyme possesses two distinct isoforms (a constitutive mitochondrial isozyme and an inducible cytosolic isozyme) encoded by two different PEPCK genes (Imamura and Tanaka 1982; Hanson and Reshef 1997). Given that it is unknown how many PEPCK isozymes there are in cobia and which ones are inducible, we decided to only examine total PEPCK activities and the cytosolic PEPCK gene expression in cobia liver.

The relative expression levels of hepatic PK and PEPCK in cobia in response to different dietary CHO/L ratios and nutritional status are presented in Figs. 5 and 7. Significantly induced hepatic PK gene expression was observed when the fish were fed an increasing CHO/L-ratio diet. Such an effect was also observed in fish in the refed diet category in comparison with the food-deprived fish ($P < 0.05$), and after 4 weeks of refeeding PK mRNA abundance restored to the same level of the control group (Fig. 5). However, there was no significant effect of CHO/L ratio on PK enzyme activity ($P > 0.05$), and PK activity was significantly higher in food-deprived and refed than fish in control group (Fig. 6).

As to the gluconeogenic enzyme PEPCK, there was a significant effect of the dietary CHO/L ratio and nutritional status on PEPCK gene expression, with downregulated PEPCK gene expression when the fish were fed an increasing CHO/L-ratio diet. Such effect was also observed in fish when refed the diet in comparison with the food-deprived fish, but after 4 weeks of refeeding, it did not restore the same values from the control group (Fig. 7). In addition, the decrease in PEPCK activity was significant between the L-CHO/L and the H-CHO/L group, whereas the M-CHO/L group showed intermediate values. However, no significant difference was observed in fish with different nutritional status (Fig. 8).

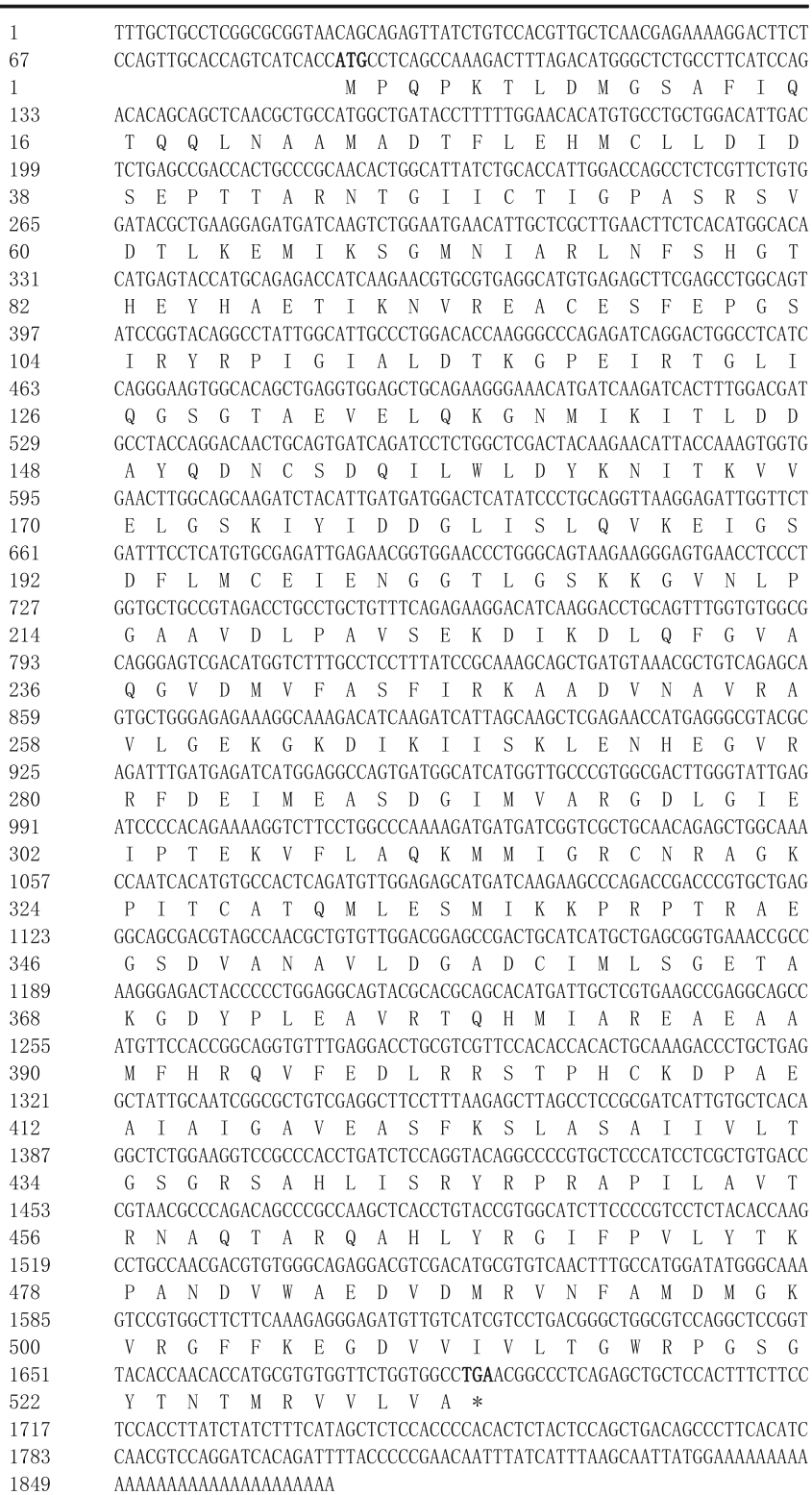

Fig. 1 Nucleotide and deduced amino acid sequences of cobia PK cDNA. The start and stop codons (ATG, TGA) are indicated with bold letters. The deduced amino acid sequence is displayed above the nucleotide sequence. This nucleotide sequence was submitted to the NCBI GenBank with an accession number of KM262818

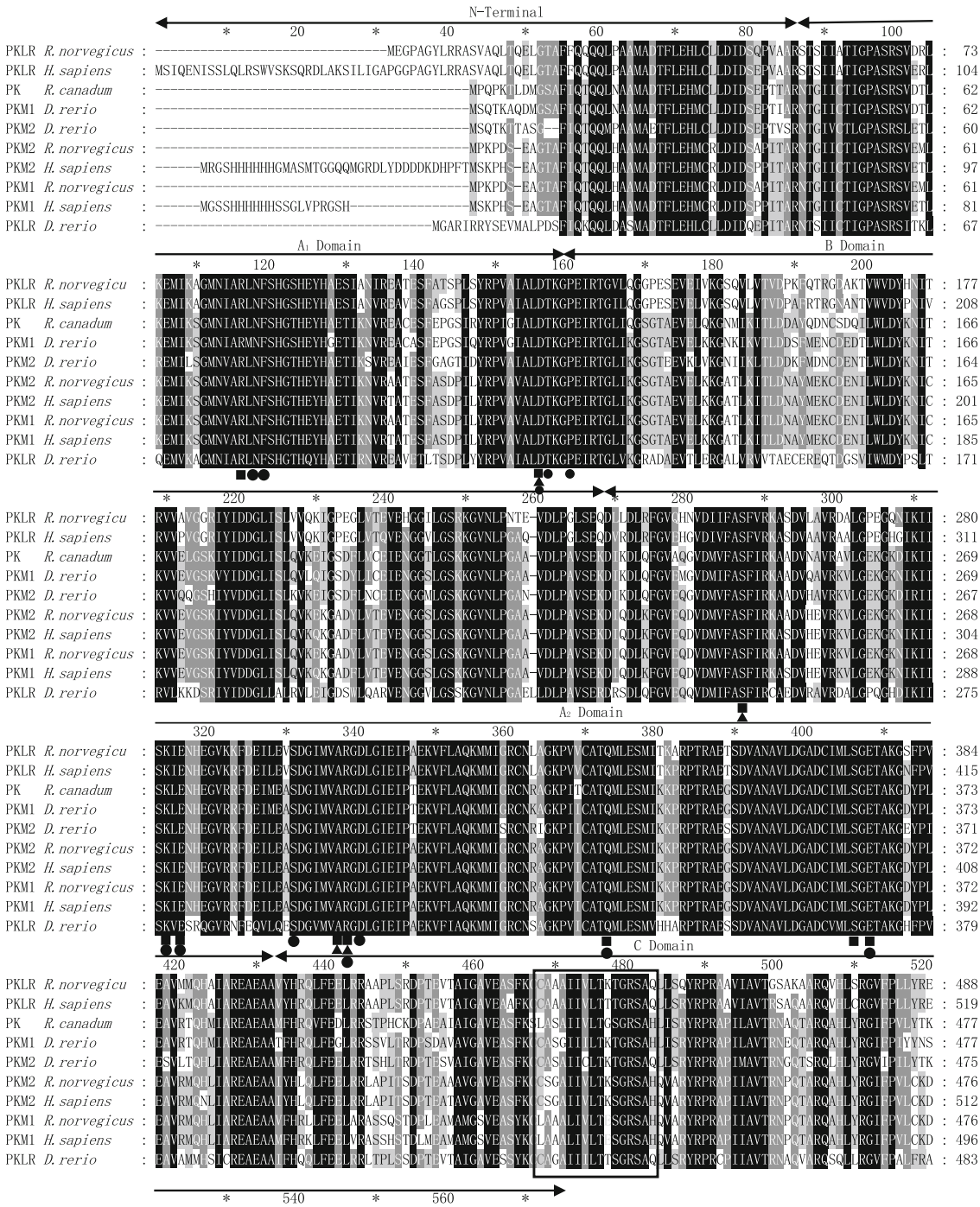
N-Terminal
A1 Domain
B Domain
A2 Domain
C Domain
PKLR R.norvegicus
PKLR H.sapiens
PK R.canadum
PKM1 D.rerio
PKM2 D.rerio
PKM2 R.norvegicus
PKM2 H.sapiens
PKM1 R.norvegicus
PKM1 H.sapiens
PKLR D.rerio

Fig. 2 Comparison of the deduced AA sequence of PK from cobia with that from human, zebra fish, and mice. The AA sequences were aligned using ClustalX and Genedoc. Identical residues are shaded black, and similar residues are shaded gray. The three domains of the PK subunit (A, B, and C), and the N-terminal sequences, are marked by a continuous line in the upper part of the sequences. The residues involved in binding PEP and ADP/ATP are indicated below the sequence with square and triangle, respectively. Circle indicates ligands for the cations. The 16 residues involved in the formation of binding site for F1, 6BP in animals are shown in the black box, in which the residue Cys involved in the allosteric regulation of PKM_2 is shown in red, and the residue Glu of the non-allosteric-type human and rat PKM_1 is shown in orange

Discussion

Glycolysis and gluconeogenesis are opposing metabolic pathways involved in glucose uptake and production and play vital roles in the regulation of glucose homeostasis. In the present study, the full-length PK cDNA was cloned and characterized. The complete AA sequence alignment analysis showed that cobia PK had similar structural features to those of other species, with the greatest similarities with zebra fish PKM_1 (up to 89%), but with a lower identity with human, rat, and zebra fish PKL (about 68%), suggesting that the cobia PK is expected to be the PKM gene. The conserved sequence and characteristic motifs of PKs were identified in it, including an ADP/ATP binding site, phosphoenolpyruvate binding site, effector site, and cation requirement sites (Muñoz and Ponce 2003). Based on the results from sequence alignments, these sites were all highly conserved in all species examined in this study, with only minor AA changes observed. In the present study, the amino acid residues Cys-423 (Fig. 2, residue in red) involved in the allosteric regulation of the enzyme were not found in the cobia PK at this position, which was the same in the non-allosteric-type rat and human PKM_1 (Ikeda and Noguchi 1998). This indicated that the cobia PK might be a cytosolic enzyme clearly distinct from allosteric-type PKM_2. However, the structural function of the replacement of Glu-432 of the non-regulated PKM_1 (Fig. 2, residue in orange by Gly) remains unknown (Ikeda and Taniguchi 2000). In addition, the deduced amino acid sequence of cobia PK was organized into three principal domains: A—subdivided into A1 and A2; B and C; and an additional N-terminal domain (Muirhead et al. 1986). Finally, the analysis of PK mRNA tissue distributions showed that high abundance of PK was expressed in the liver, heart, and muscle of cobia, while lower expression levels were observed in the intestine and kidney. The presence of the highly expressed PK gene in the liver was to be expected, given the known tissue expression profiles of PK in other species (Panserat et al. 2001a; Yuan et al. 2013). It is well known that the liver plays a central role in the production and catabolism of glucose to maintain glucose homeostasis (Kamalam et al. 2016). Most enzymes involved in the regulation of glucose metabolism, including PK and PEPCK, are found in the liver. However, the lower expression of PK in the cobia kidney and intestinal tissues may be species-dependent, as a previous study found the PK gene to be highly expressed in both rainbow trout and mammals (Panserat et al. 2001a; Imamura and Tanaka 1982). Currently, limited data on tissue-specific expressions of PK in fish are available, hindering further comparative analysis.

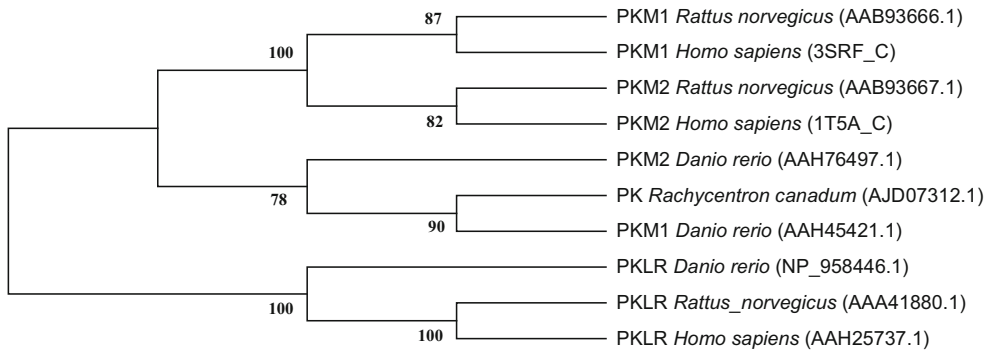

Fig. 3 Phylogenetic tree based on PKs AA sequences was made with MEGA 4.0 software using the neighbor-joining method. The numbers represent bootstrap percentages. The topology was tested using bootstrap analyses (1000 replicates). GenBank accession numbers of the sequences are indicated in the figure

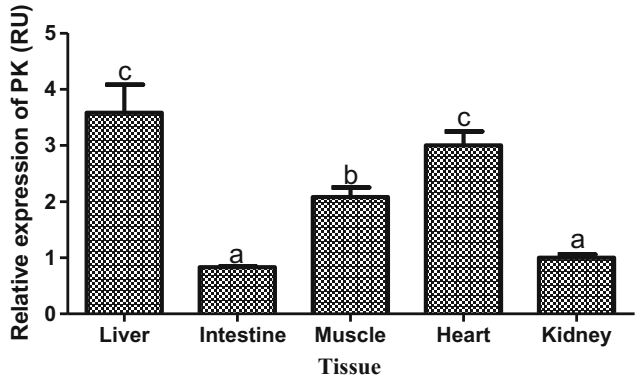

Fig. 4 Relative expression of PK in various tissues of cobia. For tissue expressions, data are referred to the relative expression value (Relative units, RU) obtained in the kidney. Each data represents the mean ± SD ($N = 6$). Bars assigned with different superscripts are significantly different ($P < 0.05$)

The glycolytic enzyme PK is also associated with the regulation of nutrients and hormones (Gali et al. 2012; Yokozawa et al. 2008). In fish, previous studies have mostly focused on the regulation of PK activity by dietary composition (Dias et al. 2004; Enes et al. 2006; Fernández et al. 2007; Couto et al. 2008), but molecular-level analyses of the regulation

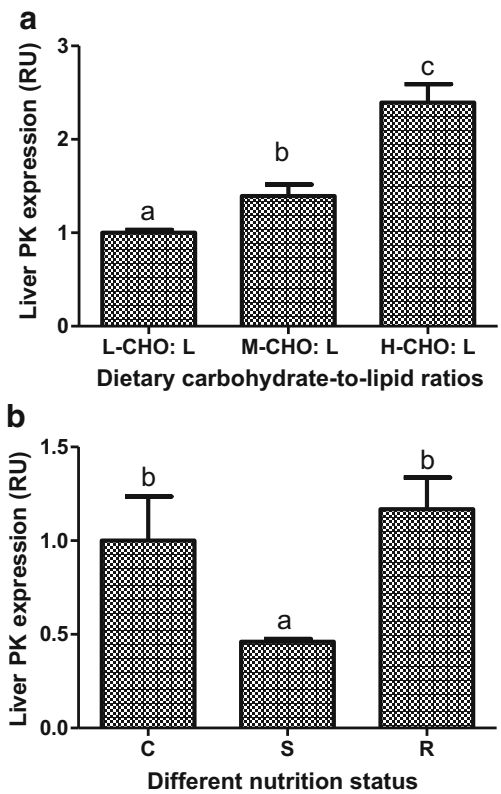

Fig. 5 The expression of liver PK in response to **a** different dietary carbohydrate-to-lipid (CHO/L) ratios (L: low, M: moderate, H: high) for 8 weeks and **b** different nutritional status (C: feeding, S: starvation, R: refeeding) in cobia. For different dietary CHO/L ratio, data are referred to the relative expression value obtained in the L-CHO/L ratio diet. For different nutritional status, data are referred to the relative expression values obtained in the control group (M-CHO/L ratio diet). Bars bearing different letters were significantly different ($P < 0.05$) and values are means ± SD ($N = 6$)

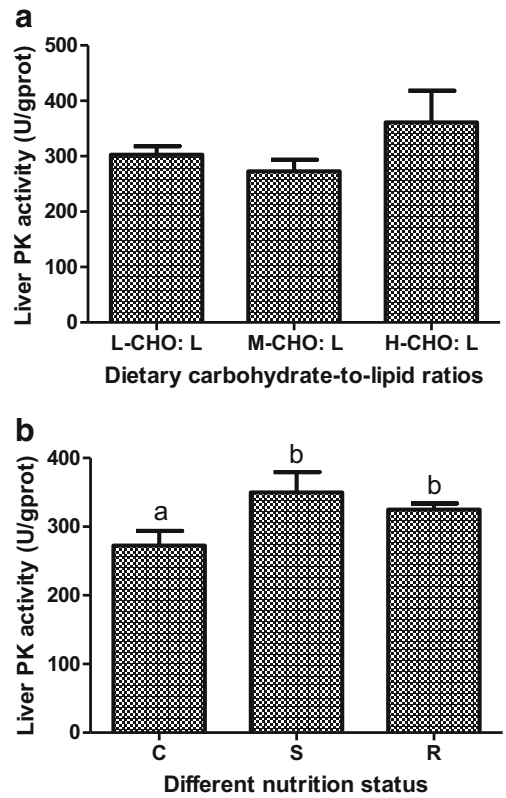

Fig. 6 The activity of liver PK in response to **a** different dietary carbohydrate-to-lipid (CHO/L) ratios (L: low, M: moderate, H: high) for 8 weeks and (**b**) different nutritional status (C: feeding, S: starvation, R: refeeding) in cobia. Bars bearing different letters were significantly different ($P < 0.05$) and values are means ± SD ($N = 6$)

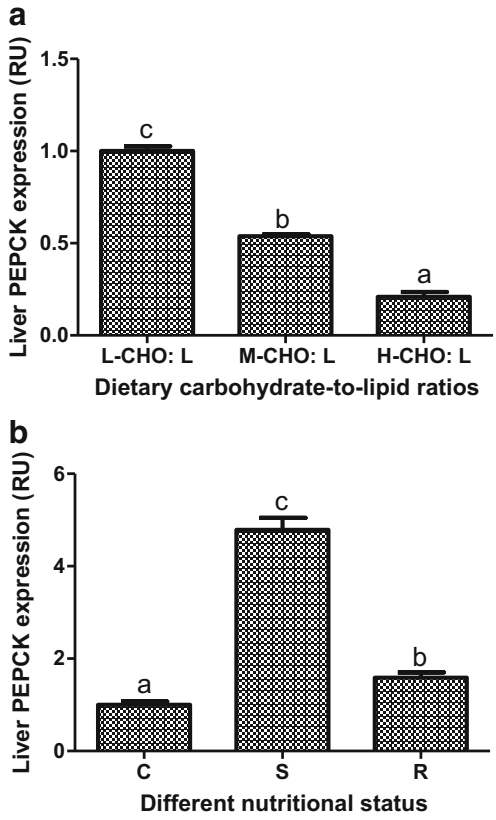

Fig. 7 The expression of liver PEPCK in response to **a** different dietary carbohydrate-to-lipid (CHO/L) ratios (L: low, M: moderate, H: high) for 8 weeks and **b** different nutritional status (C: feeding, S: starvation, R: refeeding) in cobia. For different dietary CHO/L ratio, data are referred to the relative expression value obtained in the L-CHO/L ratio diet. For different nutritional status, data are referred to the relative expression values obtained in the control group (M-CHO/L ratio diet). Bars bearing different letters were significantly different ($P < 0.05$) and values are means ± SD ($N = 6$)

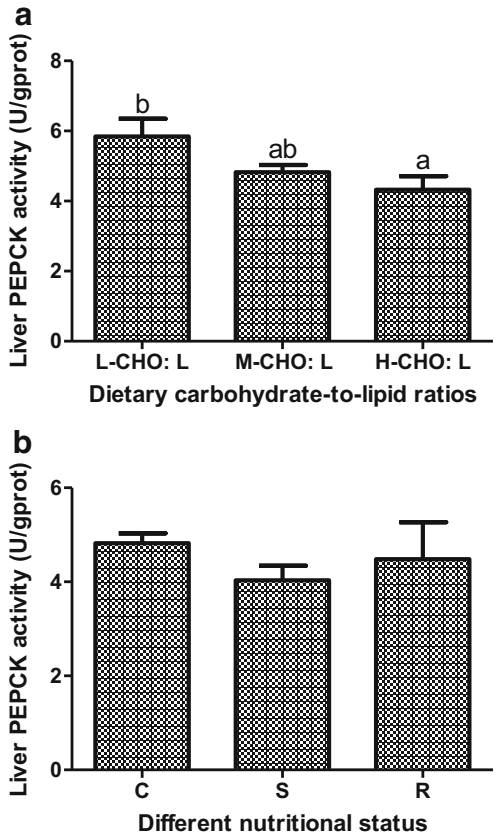

Fig. 8 The activity of liver PEPCK in response to **a** different dietary carbohydrate-to-lipid (CHO/L) ratios (L: low, M: moderate, H: high) for 8 weeks and **b** different nutritional status (C: feeding, S: starvation, R: refeeding) in cobia. Bars bearing different letters were significantly different ($P < 0.05$) and values are means ± SD ($N = 6$)

of this gene are relatively limited. In the present study, the nutritional regulation of hepatic PK was analyzed at the enzymatic and molecular levels. The results showed that the abundance of PK mRNA was under nutritional regulation. A significantly increased expression of hepatic PK genes was observed when the fish were fed an increasing CHO/L-ratio diet, and such an effect was also found in fish when refed the diet in comparison with the food-deprived fish, and after 4 weeks of refeeding, PK mRNA abundance restored to the same level of control group. This contrasted with rainbow trout, in which no specific effect of dietary carbohydrate on hepatic PK gene expression was observed (Panserat et al. 2001a). In fact, PK is known to occur as four distinct isozymes, M1, M2, L, and R, encoded by two genes: PKL and PKM (Imamura and Tanaka 1982). In rainbow trout, the PK gene is cloned coding for L-type isoform, which is different from our cloning of cobia PK cDNA that coded for a M1 isoform enzyme. However, the discrepancy in nutritional regulation between these two fish species cannot simply be explained by the different isoforms assessed, as it was recently reported that, in grass carp, PKL gene expression and activity are both induced by high dietary carbohydrate levels (Yuan et al. 2013). In addition, an induction of hepatic PK activity by dietary carbohydrates was also observed in gilthead sea bream and European sea bass (*Dicentrarchus labrax*) (Enes et al. 2006;

Fernández et al. 2007; Couto et al. 2008) but not in other fish species (Dias et al. 2004; Enes et al. 2008). In the present study, in contrast to gene expression, there was no significant effect of various CHO/L-ratio diets on PK enzyme activity. In fact, PK activity was significantly higher in food-deprived fish than those fed with the M-CHO/L-ratio diet. An explanation for the increase in gene expression but not in enzymatic activity could be that there is no direct relationship between gene expression and activity, possibly linked to post-transcriptional regulation or to qualitative enzyme modifications, such as the phosphorylation/dephosphorylation of PK (Panserat et al. 2001a). During starvation, no dietary glucose is available, but because animals still require energy, it is possible that they were performing glycolysis at high levels compared to fish that were fed a moderate glucose content. Moreover, these results could be in agreement with the results for PEPCK, the gluconeogenic enzyme, which showed an increase in gene expression in the food-deprived fish but did not present differences in activity, since that would have provided glucose for PK.

As for the gluconeogenesis pathway, previous studies have demonstrated that dietary carbohydrates do not inhibit certain hepatic gluconeogenic enzymes in some teleosts, like PEPCK and G6Pase in rainbow trout (Panserat et al. 2000 2001c; Coutinho et al. 2016) as well as FBPase in European sea bass (Moreira et al. 2008) and gilthead sea bream (Couto et al. 2008). Here, we observed for the first time that there was a significant effect of the dietary CHO/L ratio and nutritional status on PEPCK (cytosolic isoform) gene expression, which was downregulated when fish were fed increasing CHO/L-ratio diets. Such effects were also observed in fish when refed the diet in comparison with fish that were food-deprived. This result is in agreement with that generally observed in mammals (Hanson and Reshef 1997). In fact, there are two distinct isoforms (mitochondrial and cytosolic) encoded by separate PEPCK genes with different regulation patterns (Hanson and Reshef 1997). The gene for the cytosolic form is acutely under the regulation of hormones and nutritional status, whereas the mitochondrial form is largely constitutively expressed. In Siberian sturgeon (*Acipenser baerii*) and turbot (*Scophthalmus maximus*), liver cytosolic PEPCK expression was significantly higher in fish fed a high carbohydrate diet than fed a carbohydrate-free diet, while mitochondrial PEPCK expression was similar in both groups (Nie 2003; Gong et al. 2015). In rainbow trout and gilthead sea bream, the fact that PEPCK gene expression is not regulated by nutritional has led to speculation that the PEPCK gene cloned in these species codes for a mitochondrial isoform (Panserat et al. 2001b, 2002). This has been confirmed by a previous finding of rainbow trout, that the mitochondrial isoform dominates PEPCK activity (> 80%; Walton and Cowey 1979). Thus, nutritional regulation of the expression and activity of cytosolic PEPCK could have been masked in rainbow trout, although the cytosolic form is tightly controlled by nutrients. In the present study, we only determined the total PEPCK activities and the cytosolic PEPCK gene expressions in cobia liver. In contrast to gene expression, the decrease in PEPCK activity was significant between the L-CHO/L and H-CHO/L groups, whereas the M-CHO/L group showed intermediate values. But PEPCK activity remained at the same level independent of the fishes' nutritional status (fed, unfed, or refed). This result can be interpreted as the PEPCK gene in the present study coding for a cytosolic enzyme that is nutritionally regulated, whereas hepatic total PEPCK activity in cobia might be mainly mitochondrial, so that nutritional regulation of cytosolic PEPCK activity could have been masked.

Overall, in accordance with our hypothesis, at least at the molecular level, the mRNA abundance of the important glycolytic (PK) and gluconeogenic (PEPCK) enzymes are regulated by dietary composition and nutritional status in cobia liver, which is not the case in rainbow trout (Panserat et al. 2001a, b). However, in contrast to the regulation occurring at the molecular level, we did not observe the same nutritional regulation at the enzymatic level. Further studies that include data from other key enzymes involved in hepatic glucose metabolism are necessary to reveal the truth about whether cobia effectively utilize carbohydrates. In addition, the issue of PK and PEPCK isoforms is important in the study of the nutritional regulation of PK and PEPCK gene expression. As the proportions of the PK and PEPCK isoforms in liver cells are species-specific, future studies will need to begin by defining the proportion of each isoform in each species under investigation.

Funding information This work was supported by the National Natural Science Foundation of China (No. 31772864), the National Basic Research Program of China (2014CB138600), China Agriculture Research System (CARS-47), Natural Science Foundation of Guangdong Province (2018A030313154), Zhanjiang Science and Technique Foundation (2016A03011), and Project of Enhancing School with Innovation of Guangdong Ocean University (2015KTSCX055).

References

AOAC (1995) Agricultural chemicals; contaminants, drugs, in: official methods of analysis of AOAC international. AOAC International, Arlington, p 1298

Chou RL, Su MS, Chen HY (2001) Optimal dietary protein and lipid levels for juvenile cobia (*Rachycentron canadum*). Aquaculture 193:81–89

Coutinho F, Peres H, Castro C, Pérez-Jiménez A, Pousão-Ferreira P, Oliva-Teles A, Enes P (2016) Metabolic responses to dietary protein/carbohydrate ratios in zebra sea bream (*Diplodus cervinus*, Lowe, 1838) juveniles. Fish Physiol Biochem 42:343–352

Couto A, Enes P, Peres H, Oliva-Teles A (2008) Effect of water temperature and dietary starch on growth and metabolic utilization of diets in gilthead sea bream (*Sparus aurata*) juveniles. Comp Biochem Physiol A Mol Integr Physiol 151:45–50

Cowey CB, Walton MJ (1989) Intermediary metabolism. In: Halver JE (ed) Fish Nutrition. Academic, San Diego, pp 260–321

Craig SR, Schwarz MH, Mclean E (2006) Juvenile cobia (*Rachycentron canadum*) can utilize a wide range of protein and lipid levels without impacts on production characteristics. Aquaculture 261:384–391

Dias J, Rueda-Jasso R, Panserat S, Conceição LECD, Gomes EF, Dinis MT (2004) Effect of dietary carbohydrate-to-lipid ratios on growth, lipid deposition and metabolic hepatic enzymes in juvenile Senegalese sole (*Solea senegalensis*, Kaup). Aquac Res 35:1122–1130

Enes P, Panserat S, Kaushik S, Olivateles A (2006) Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in European sea bass (*Dicentrarchus labrax*) juveniles. Comp Biochem Physiol A Mol Integr Physiol 143:89–96

Enes P, Panserat S, Kaushik S, Oliva-Teles A (2008) Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (*Sparus aurata*) juveniles. Aquaculture 274:101–108

Fernández F, Miquel AG, Córdoba M, Varas M, Metón I, Caseras A, Baanante IV (2007) Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. J Exp Mar Biol Ecol 343:1–10

Franks JS, Garber NM, Warren JR (1996) Stomach contents of juvenile cobia, *Rachycentron canadum*, from the northern Gulf of Mexico. Bull Jpn Soc Sci Fish 94:374–380

Gali P, Broer Y, Rosselin G, Hartmann L (2012) Hormone dependence of the L and M isozymes of pyruvate kinase in isolated rat hepatocytes. Biol Cell 48:133–141

Gong G, Xue M, Wang J, Wu XF, Zheng YH, Han F, Liang XF, Su XO (2015) The regulation of gluconeogenesis in the Siberian sturgeon (*Acipenser baerii*) affected later in life by a short-term high-glucose programming during early life. Aquaculture 436:127–136

Hanson RW, Reshef L (1997) Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu Rev Biochem 66:581–611

Ikeda Y, Noguchi T (1998) Allosteric regulation of pyruvate kinase M2 isozyme involves a cysteine residue in the intersubunit contact. J Biol Chem 273:12227–12233

Ikeda Y, Taniguchi NT (2000) Dominant negative role of the glutamic acid residue conserved in the pyruvate kinase M1 isozyme in the heterotropic allosteric effect involving fructose-1,6-bisphosphate. J Biol Chem 275:9150–9156

Imamura K, Tanaka T (1982) Pyruvate kinase isozymes from rat. Methods Enzymol 90:150–165

Kamalam BS, Medale F, Panserat S (2016) Utilisation of dietary carbohydrates in farmed fishes: new insights on influencing factors, biological limitations and future strategies. Aquaculture 290:2–3

Kirchner S, Kaushik S, Panserat S (2003) Effect of partial substitution of dietary protein by a single gluconeogenic dispensable amino acid on hepatic glucose metabolism in rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol 134: 337–347

Lee LH (2009) Studies on the enzyme activity and gene expression of lipid and triacylglycerol biosynthesis of cobia (*Rachycentron canadum*). Dissertation National Sun Yat-sen University, Guangzhou

Liao IC, Huang TS, Tsai WS, Hsueh CM, Chang SL, Leano EM (2004) Cobia culture in Taiwan: current status and problems. Aquaculture 237:155–165

Livak K, Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Metón I, Fernández F, Baanante IV (2003) Short- and long-term effects of refeeding on key enzyme activities in glycolysis–gluconeogenesis in the liver of gilthead seabream (*Sparus aurata*). Aquaculture 225:99–107

Moon TW (2001) Glucose intolerance in teleost fish: fact or fiction? Comp Biochem Physiol B Biochem Mol Biol 129: 243–249

Moreira IS, Peres H, Couto A, Enes P, Oliva-Teles A (2008) Temperature and dietary carbohydrate level effects on performance and metabolic utilisation of diets in European sea bass (*Dicentrarchus labrax*) juveniles. Aquaculture 274: 153–160

Muirhead H, Clayden DA, Barford D, Lorimer CG, Fothergill-Gilmore LA, Schiltz E, Schmitt W (1986) The structure of cat muscle pyruvate kinase. EMBO J 5:475–481

Muñoz ME, Ponce E (2003) Pyruvate kinase: current status of regulatory and functional properties. Comp Biochem Physiol 135:197–218

Nie Q (2003) Effects of dietary carbohydrates on the activities and gene expression of carbohydrate metabolic enzymes in turbot (*Scophthalmus maximus*) Dissertation, Ocean University of China

Panserat S, Médale F, Brèque J, Plagnesjuan E, Kaushik S (2000) Lack of significant long-term effect of dietary carbohydrates on hepatic glucose-6-phosphatase expression in rainbow trout (*Oncorhynchus mykiss*). J Nutr Biochem 11:22–29

Panserat S, Plagnesjuan E, Kaushik S (2001a) Nutritional regulation and tissue specificity of gene expression for proteins involved in hepatic glucose metabolism in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 204:2351–2360

Panserat S, Plagnes-Juan E, Brèque J, Kaushik S (2001b) Hepatic phosphoenolpyruvate carboxykinase gene expression is not repressed by dietary carbohydrates in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 204:359–365

Panserat S, Capilla E, Gutierrez J, Frappart PO, Vachot C, Plagnesjuan E, Aguirre P, Brèque J, Kaushik S (2001c) Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (*Oncorhynchus mykiss*) by a single meal with glucose. Comp Biochem Physiol B 128:275–283

Panserat S, Plagnes-Juan E, Kaushik S (2002) Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*). Biochim Biophys Acta Gene Struct Exp 1579:35–42

Polakof S, Míguez JM, Soengas JL (2008) Dietary carbohydrates induce changes in glucosensing capacity and food intake of rainbow trout. Am J Phys Regul Integr Comp Phys 295: R478–R489

Ren M, Ai Q, Mai K, Ma H, Wang X (2011) Effect of dietary carbohydrate level on growth performance, body composition, apparent digestibility coefficient and digestive enzyme activities of juvenile cobia, *Rachycentron canadum* L. Aquac Res 42:1467–1475

Schwarz MH, McLean E, Craig SR (2007) Research experience with cobia: larval rearing, juvenile nutrition, and general physiology. In: Liao IC, Leano EM (eds) Cobia Aquaculture: Research, Development, and Commercial Production. World Aquaculture Society, Baton Rouge, pp 1–17

Thompson JD, Gibson TJ, Higgins DG (2002) Multiple sequence alignment using ClustalW and ClustalX. Current protocols in bioinformatics. Wiley, Hoboken

Walton MJ, Cowey CB (1979) Gluconeogenesis from serine in rainbow trout *Salmo gairdneri* liver. Comp Biochem Physiol 62:497–499

Wilson RP (1994) Utilization of dietary carbohydrate by fish. Aquaculture 124:67–80

Yamada K, Noguchi T (1999) Nutrient and hormonal regulation of pyruvate kinase gene expression. Biochem J 337:1–17

Yokozawa T, Okuda S, Oura H (1978) Influence of dietary protein, carbohydrate, and fat on pyruvate kinase activity in rat liver and kidney. Agric Biol Chem 42:1895–1900

Yuan X, Zhou Y, Liang XF, Li J, Liu L, Li B, He Y, Guo X, Fang L (2013) Molecular cloning, expression and activity of pyruvate kinase in grass carp *Ctenopharyngodon idella*: effects of dietary carbohydrate level. Aquaculture 410:32–40