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Molecular Characterization and Identification of Facilitative Glucose Transporter 2 (GLUT2) and Expression of Related Glycometabolism in Response to Different Starch Levels in Blunt Snout Bream (*Megalobrama amblycephala*)

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Abstract: Facilitative glucose transporters (GLUT) are transmembrane transporter of proteins involved in glucose transport across the plasma membrane. To date, there is no information about glucose transporter 2 (GLUT2) in blunt snout bream (*Megalobram aamblycephala*). In this study, GLUT2 were cloned and characterized from blunt snout bream, and its-expression in response to diets with different carbohydrate levels (17.1%; 21.8%; 26.4%; 32.0%; 36.3% and 41.9% of dry matter). In this study, the full-length cDNA of GLUT2 was 2577 bp, including a 5'-untranslated region (UTR) of 73 bp, a 3'-UTR of 992 bp, and an open reading frame of 1512 bp, encoding a polypeptide of 503 amino acids with predicted molecular weight of 55.046 kDa and theoretical isoelectric point of 7.52. GLUT2 has twelve across the membrane area locating at 7-29; 71-93; 106-123; 133-155; 168-190; 195-217; 282-301; 316-338; 345-367; 377-399; 412-434; 438-460 aimo acids respectively. Conservative structure domains located at 12-477 aimo acids belonging to sugar porter family major facilitator superfamily (MFS) transporter. The blunt snout bream GLUT2 showed high identity to their orthologs in other fish species and mammals. Quantitative real-time (qRT)-PCR assays revealed that GLUT2 expression was high in the liver, intestine and kidney; highest in the liver. Compared with the control group (17.1%), high dietary carbohydrate levels (32.0%; 36.3% and 41.9%) resulted in high plasma glucose at 3h after

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feeding, but high plasma glucose were back to basal at 24h after feeding. Furthermore, high dietary carbohydrate levels significantly improved the glycolysis and inhibitied gluconeogenesis by augmentation of GK and PK expression, inhibition of PEPCK and G6P mRNA levels ($P<0.05$). However, GLUT2; GK; PK; PEPCK and G6P mRNA levels were back to basal.

Keywords: blunt snout bream (*megalobrama amblycephala*); glucose transporter 2; glycometabolism; starch

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1. Introduction

Sufficient quantities of protein to meet anabolic requirements should be supplied in the diet to gain the protein-sparing effect of the carbohydrate [1], but the utilization of carbohydrate by fish is varies [2], and different from mammals, the utilization of carbohydrate in fish is limited; feeding with high level of carbohydrate, not only caused the reduction of fish growth, but also could lead to prolonged hyperglycemia [3-6]. In recent years, facilitative glucose transporter (GLUT) has become a hotspot in research in mammalian and fish. Growing evidences found that GLUTs across the plasma membrane of mammalian cells is the first rate-limiting step for glucose metabolism and involved in regulation of glucose metabolism and energy homeostasis [7-11].

Glucose concentrations in the liver are maintained at equilibrium with glucose in blood due to the existence of a specific diffusion glucose transporter called facilitative glucose transporter 2 (GLUT2) [12-15]. Compared to GLUT1, 3, 4, GLUT2 is the major glucose transporter in hepatocytes and exhibits several distinctive characteristics [16,17]. In mammalian, at least 13 different GLUT isoforms have been thus far identified [18,19], which are categorized into class I (GLUT1-4), class II (GLUT5, 7, 9, and 11), and class III (GLUT6, 8, 10, 12 and the myo-inositol transporter HMIT1) [9,20,21]. In recent years, the four mammalian GLUT homologues belonging to class I have also been identified in fish: GLUT1 in rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*) and Atlantic cod (*Gadus morhua*) [13,22-25]; GLUT3 in Atlantic cod and grass carp (*Ctenopharyngodon idellus*) [26,27]; and GLUT4 in brown trout (*Salmo trutta*) and

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Atlantic cod [13,28]. There is only a few information concerning cloning and identification of GLUT2 in rainbow trout Atlantic cod and sea bass (*Dicentrarchus labrax*) [13-15].

Blunt snout bream, *Megalobrama amblycephala*, is an important cultured freshwater fish species in China, because of its excellent flesh quality, rapid growth performance and high larval survival rate [29-31]. Furthermore, it is also distributed in North America (northern Canada to southern Mexico), Africa and Eurasia [32]. Commercial production has a fast increase and approximately 0.79 million tons in 2015 [33]. In the previous studies, we found high level of carbohydrate intake could reduce fish growth and result in prolonged hyperglycemia in blunt snout bream [6,34]. However, there is no information concerning identification of GLUT2 in blunt snout bream, and the mechanisms by which glucose transporters in this fish species are regulated to maintain to plasma glucose levels have not been identified. Therefore, the aim of this study was to clone and identify GLUT2 in blunt snout bream, and to investigate the expression of GLUT2 and related gene of glycometabolism in response to different starch levels.

2. Result

2.1. Cloning and characterization of blunt snout bream GLUT2 cDNA

GLUT2 cDNA sequence of blunt snout bream predicted a start codon at nucleotide 73, a stop codon at nucleotide 1288. The cDNA is 2577 bp in length and contains a 5'-untranslated region (UTR) of 73 bp, a 3'-UTR of 992 bp, and an open reading frame of 1512 bp (GenBank under accession no: KC513421.2) (Fig. 1). Blunt snout bream GLUT2 encoded polypeptide of 503 amino acids with predicted molecular weight of 55.046 kDa and theoretical isoelectric point of 7.52. GLUT2 has twelve across the membrane area locating at 7-29; 71-93; 106-123; 133-155; 168-190; 195-217; 282-301; 316-338; 345-367; 377-399; 412-434; 438-460 aimo acids respectively. Conservative structure domains located at 12-477 aimo acids belonging to sugar porter family major facilitator superfamily (MFS) transporter.

Phylogenetic analysis was performed to study the relation of blunt snout bream GLUT2 to class I GLUTs from other vertebrates, with special attention to GLUTs in fish, which clearly showed that it clustered with fish GLUT2 sequences, as well as other vertebrate GLUT2, but not with other class I GLUTs (Fig. 2).

The alignment of the complete amino acid sequence of blunt snout bream GLUT2 with

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zebrafish (*Danio rerio*; GenBank under accession no: NP_001036186.1); chicken (*Gallus gallus*; GenBank under accession no: NP_997061.1); human (*Homo sapiens*; GenBank under accession no: NP_000331.1); Mouse (*Mus musculus*; GenBank under accession no: NP_112474.2) are shown in Fig. 3. At the amino acid level, blunt snout bream GLUT2 had the high degree of sequence identity to three GLUT2s from zebra fish, chicken, human and mouse, with 91%, 63%, 57% and 54% identity, respectively. (Fig.2).

```
1    ATTGGGAAAGCAGCGAGAACCTTGGAGCCACTATTAATGACCAAGAGCGGCTTTTGCTGACACTTCATGCAAA
74   ATGGAGAAGCAGTTAACAGGCACACTCGCTCTGGCAGTGTTCACAGCTGCACTTGGCTCTCTGCAGATGGGATAC
       M  E  K  Q  L  T  G  T  L  A  L  A  V  F  T  A  A  L  G  S  L  Q  M  G  Y
149  AGCCTGGGTGTCATCAATGCCCCACAGAAGGTCATTGAGAGGCACTATGCAAGATCTCTCGGTGTCTATGATGAA
       S  L  G  V  I  N  A  P  Q  K  V  I  E  R  H  Y  A  R  S  L  G  V  Y  D  E
224  AATCTGTCCCGTAGAGAAGGAGGAAATGCCACAGAACATGAAGAACCTTCCGATCCTTCTGTGGTCATGTACTGG
       N  L  S  R  R  E  G  G  N  A  T  E  H  E  E  P  S  D  P  S  V  V  M  Y  W
299  TCCTTGTCTGTGGCCATCTTCTCCATTGGAGGCATGGTGTCCTCCTTTCTAGTGAGCTTTGTTAGCGACTTCCGT
       S  L  S  V  A  I  F  S  I  G  G  M  V  S  S  F  L  V  S  F  V  S  D  F  R
374  GGAAGGATCAAAGGCATGCTGGCAATAAATGTCCTTGCCATAACAGCTGGGCTGCTCATGGGCCTGGCTAAGATG
       G  R  I  K  G  M  L  A  I  N  V  L  A  I  T  A  G  L  L  M  G  L  A  K  M
449  GGCACACCTTACCTCATGGTGATAGCAGGACGTGCTATCATGGGACTGTACTGTGGTCTGTCATCTGGCCTGGTG
       G  T  P  Y  L  M  V  I  A  G  R  A  I  M  G  L  Y  C  G  L  S  S  G  L  V
524  CCCCTGTACATTGGAGAGATTTCTCCAATGAAGTTCAGAGGGGCTATGGGAGCGCTCCACCAGCTGGCTATTGTA
       P  L  Y  I  G  E  I  S  P  M  K  F  R  G  A  M  G  A  L  H  Q  L  A  I  V
599  ATTGGCATCCTTATTAGTCAAGTCATTGGTCTGGAGTTCCTGCTGGGAAATGATGACATGTGGCACGTACTGCTG
       I  G  I  L  I  S  Q  V  I  G  L  E  F  L  L  G  N  D  D  M  W  H  V  L  L
674  GGTCTTTCTGGAGCTCCTGCCATCCTGCAGAGTCTGCTGCTGCTTCTGTGTCCAGAGAGTCCTCGATACCTCTAC
       G  L  S  G  A  P  A  I  L  Q  S  L  L  L  L  L  C  P  E  S  P  R  Y  L  Y
749  ATCAAACAGGGCAAAGTGGAAGAAGCATGCAAGAATCTGAAAAGGCTGAAGGGAGATTACGACACCTCAAAAGAC
       I  K  Q  G  K  V  E  E  A  C  K  N  L  K  R  L  K  G  D  Y  D  T  S  K  D
824  ATAGCAGAGATGCAGGCAGAGAAGGAGGAGGCCATGAAAGAGGCGAAAATGTCCATCTGGCGGCTACTCCGTTCC
       I  A  E  M  Q  A  E  K  E  E  A  M  K  E  A  K  M  S  I  W  R  L  L  R  S
899  TCGGTGTACCGCCAGCAGCTCTTTGTGGCCCTCATGATGCACTTTTCCCAGCAGTTCTCTGGGATCAACGCTATC
       S  V  Y  R  Q  Q  L  F  V  A  L  M  M  H  F  S  Q  Q  F  S  G  I  N  A  I
974  TTTTATTACTCTACTTCGATCTTCCAGACTGCTGGTGTTGGTCAGCCTGTGTATGCCACTATTGGAGTGGGAGTT
       F  Y  Y  S  T  S  I  F  Q  T  A  G  V  G  Q  P  V  Y  A  T  I  G  V  G  V
1049 ATAAACATCATTTTCACCCTTGTGTCGGTGATCTTGGTGGACAGAACGGGCAGACGAACTCTTACTCTGGTTGGG
        I  N  I  I  F  T  L  V  S  V  I  L  V  D  R  T  G  R  R  T  L  T  L  V  G
1124 TTGGGAGGAATGTGCTGCTGTGCAGTGGCCATGACAGTGGGCCTGGCATTTCAGGATGTTTACTCATGGATGAGC
        L  G  G  M  C  C  C  A  V  A  M  T  V  G  L  A  F  Q  D  V  Y  S  W  M  S
1199 TACCTCAGCATGACGGCCATATTCTTGTTCGTGTCTTTCTTTGAGATCGGGCCTGGACCAATCCCATGGTTCATC
        Y  L  S  M  T  A  I  F  L  F  V  S  F  F  E  I  G  P  G  P  I  P  W  F  I
1274 GTGGCAGAGCTCTTCAGTCAGGGGCCACGTCCAGCAGCCATAGCATTAGCTGGGTGTTGCAACTGGACATGCAAT
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     V  A  E  L  F  S  Q  G  P  R  P  A  A  I  A  L  A  G  C  C  N  W  T  C  N
1349 TTCATAATTGGCATGTTTTTTCCTTATTTAGAGGGTCTTTGTGGGAGCTACGTCTTTGTCATTTTTGCAGTACTC
     F  I  I  G  M  F  F  P  Y  L  E  G  L  C  G  S  Y  V  F  V  I  F  A  V  L
1424 CTGTTCGGTTTCACCGTTTTTATCTATTTGCGTGTACCTGAAACAAAAGGGAAGACTTTTGAGGAGATAGCAGCG
     L  F  G  F  T  V  F  I  Y  L  R  V  P  E  T  K  G  K  T  F  E  E  I  A  A
1499 GTTTTCCACCACAAACGTGGAGCCCCTCCTTCCAAACCACAAGAAGAGGCTGAGATGGTGCAGCTCAAGAGCTCT
     V  F  H  H  K  R  G  A  P  P  S  K  P  Q  E  E  A  E  M  V  Q  L  K  S  S
1574 ACAGAGGCCTGA
     T  E  A  *
1586 AGGAGGACCGTTAGTTTATGGCAACTGTGGAATGAACGATGTTACTGCAGGCACAGAACTTAACTGCTATGACAGAAC
1664 TGAACTGCTGTATGTTCCACTGAACCTCATGCAGAACTCATCATTTGACAGGAAATAATTAGTTTAGAGATTAACTAA
1742 ACACAACTGCATAATCGTGAGAGATGGTGCACAATATAGACATGCACTGGCCTTGGCGCAGCTAATGTTGAATTTAGA
1820 TGATTGTATATTATTTTTATATTCCCAAAGTTATATATCTGTTAAAGATATTTTTTAAAGGTATCATTTTCTTCAGTG
1898 TATTTAATTAGTGATATTGATGGTGGCAAATTTAAAGTGATTTGTCCTTTCAGTTCAATATGGAGAGAATTGTATGTG
1975 TCAACTGAGATTTGTGAGATATAAATCAACTGAAAGAAGGTTATACAAACTTCAGTGCATTACATAATTAATATGGTA
2054 GTATGGTTATATGATAGATATTTAAAATTAAATATTCAAAGACAAAATGTAAGAATTTATTACATTTTGTTTTAGAGT
2132 TACAATGGTTGGATTACATTTGTGTATTTTTTCCCATTATAAAATTACCCATGTTTTAAAGTAAACCATTCCAACTTC
2210 AAAACAACCTTAATTATTTGCTTTGCTAGATATCTAAAAATCCAAAGATTGTTAACAATTACTTTATAATTTTGCAGA
2288 ATAGAAGACCCTTGAATTATATTATATTAAATGATATAGGAAAGCCTTTGAAAACACAATGGTGCTGCAACCCTTTTT
2366 TTGCTGTGTCTTTTAATCTGTTGCTATTATACTTCATGGCAGTTTGATGATTGTCAAAGTACCTGGAAACTTATTGCA
2243 AATATGCAGTGAATGTTACGTTTAGGCCTACTCTATTGTTAAAAAGTTTAGGGTCGGTAAGTGTCTTATTTAGCTACT
2522 CACCAAGGCTGCATTTAGTTGATCAAAAATACTGTAAAAACAGTAATATTGTGAAA
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Fig. 1. Nucleotide and predicted amino acid sequences of GLUT2 cDNA from the blunt snout bream (*Megalobrama amblycephala*) (GenBank accession no: KC513421.2). Amino acid sequences are shown by a capital letter below the nucleotide sequences. Start codon (ATG) is boxed. Stop codon (TGA) is shaded in gray.

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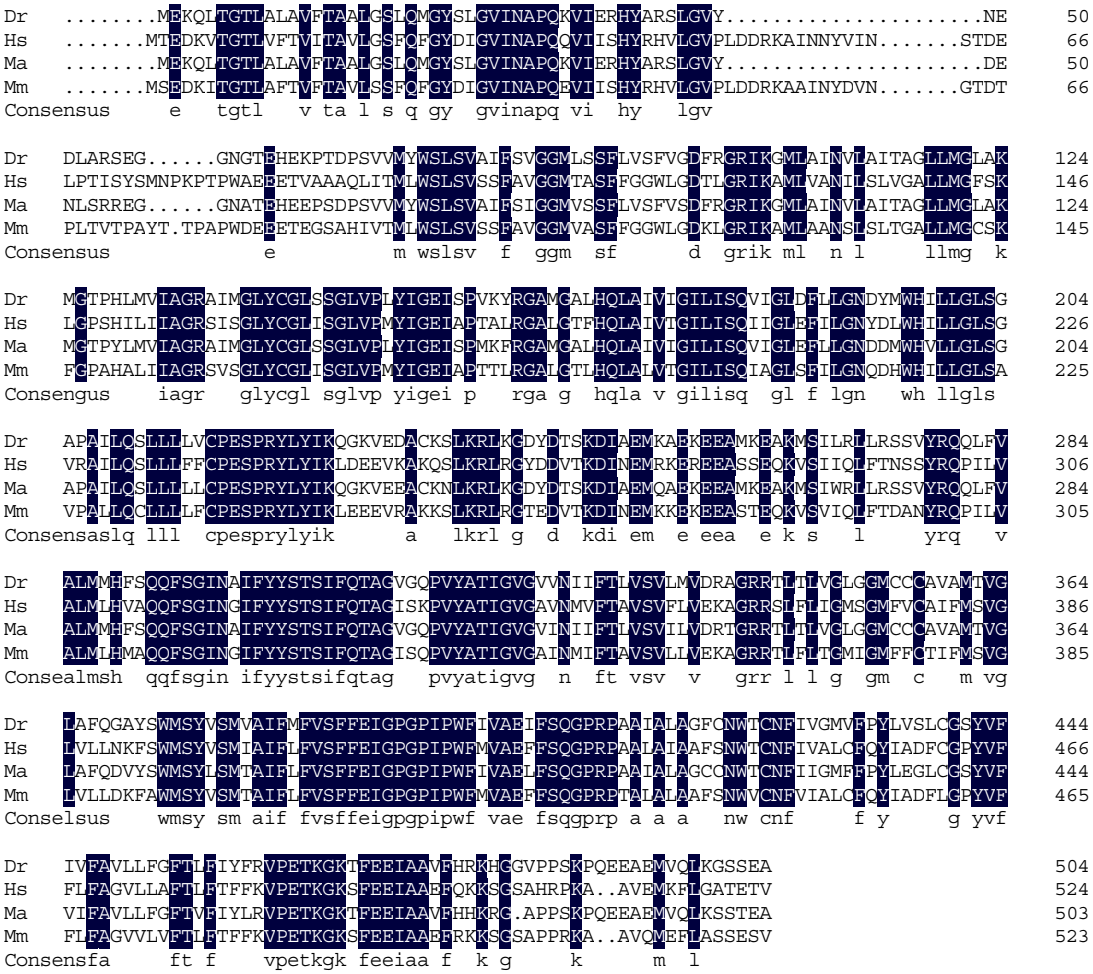

Fig 2. Comparison of the deduced amino acid sequences of GLUT2 with those of other known GLUT2 proteins, which from zebrafish; human; chicken; mouse; blunt snout bream. Identical residues are shaded black. Dr: *Danio rerio*, NP_001036186.1; Gg: *Gallus gallus*, NP_997061.1; Hs: *Homo sapiens*, NP_000331.1; Mm: *Mus musculus*, NP_112474.2; Ma: *Megalobrama amblycephala*, AGK44960.2

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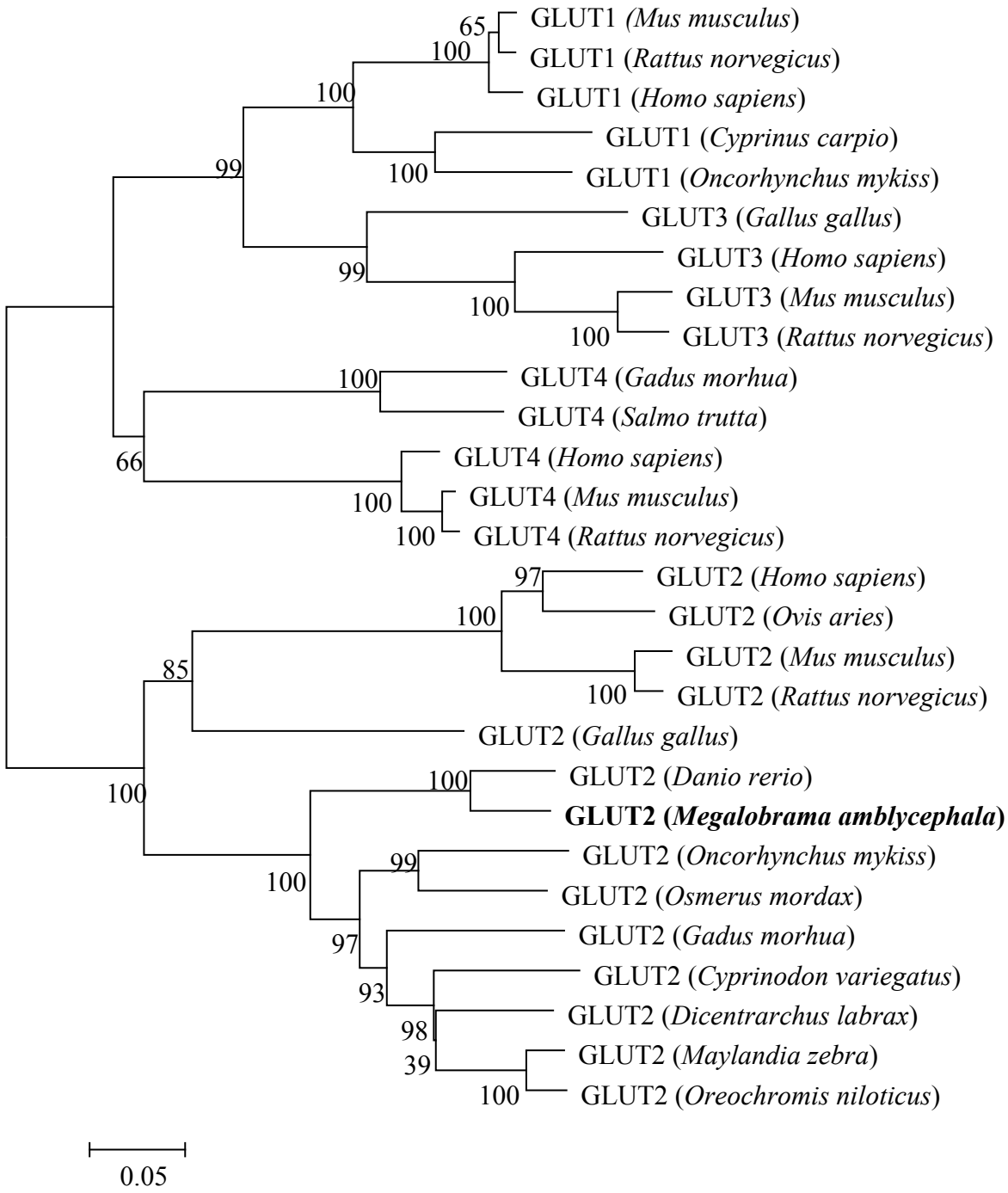

Fig 3. Phylogenetic tree of known vertebrate GLUT protein sequences. A phylogenetic tree was constructed with the complete protein sequence of blunt snout bream (*Megalobrama amblycephala*) GLUT2, and a number of protein sequences corresponding to various vertebrate GLUTs. GeneBank accession no: GLUT1 *(Mus musculus)*: AAA37752.1; GLUT1 (*Rattus norvegicus*): P11167.1; GLUT1 (*Homo sapiens*): AAA52571.1; GLUT1 (*Cyprinus carpio*): AAF75683.1; GLUT1 (*Oncorhynchus mykiss*): AAF75681.1; GLUT3 (*Gallus gallus*):

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NP_990842.1; GLUT3 (*Homo sapiens*): AAF82116.1; GLUT3 (*Mus musculus*): AAH34122.1; GLUT3 (*Rattus norvegicus*): AAA62503.1; GLUT4 (*Gadus morhua*): AAZ15731.1; GLUT4 (*Salmo trutta*): AAG12191.1; GLUT4 (*Homo sapiens*): NP_001033.1; GLUT4 (*Mus musculus*): BAB03251.1; GLUT4 (*Rattus norvegicus*): NP_036883.1; GLUT2 (*Homo sapiens*): NP_000331.1; GLUT2 (*Ovis aries*): XP_004003211.1; GLUT2 (*Mus musculus*): NP_112474.2; GLUT2 (*Rattus norvegicus*): P12336.1; GLUT2 (*Gallus gallus*): NP_997061.1; GLUT2 (*Danio rerio*): NP_001036186.1; GLUT2 (*Megalobrama amblycephala*): KC513421.2; GLUT2 (*Oncorhynchus mykiss*): AAK09377.1; GLUT2 (*Osmerus mordax*): ACO34844.1; GLUT2 (*Gadus morhua*): AAV63984.1; GLUT2 (*Cyprinodon variegatus*): XP_015230748.1; GLUT2 (*Dicentrarchus labrax*): ABJ98775.2; GLUT2 (*Maylandia zebra*): XP_004540234.1; GLUT2 (*Oreochromis niloticus*): XP_003442932.1. The scale bar refers to evolutionary distances in substitutions per site. The numbers at tree nodes refer to percentage bootstrap values after 1000 replicates.

2.2. Tissue distribution of GLUT2

Quantitative real-time reverse transcription polymerase chain reaction analysis (qRT-PCR) was used to quantify GLUT2 expression in the tissues of the liver, intestine, heart, spleen, muscle, red blood cell, kidney and gill. GLUT2 mRNA was found to be constitutively expressed in liver, intestine and kidney tissues, and highest level expression of GLUT2 was observed in the liver. There were negligible expression levels in spleen, muscle, and undetected expression levels in heart, red blood cell, gill (Fig. 4).

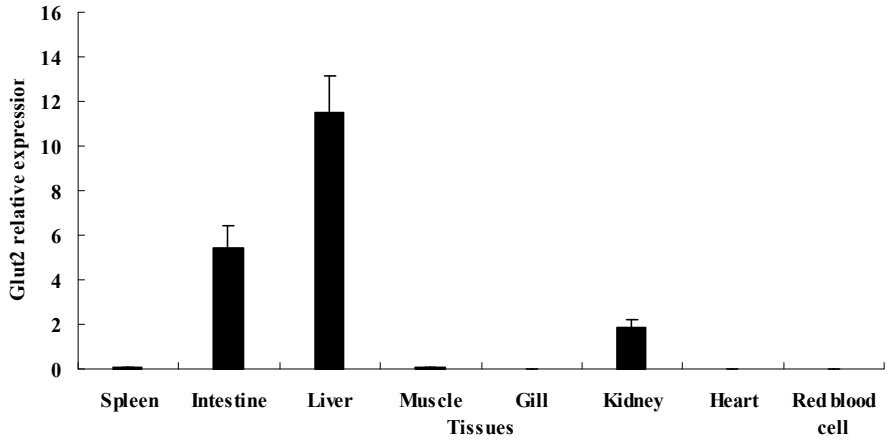

Fig 4. Tissue-specific mRNA expression of GLUT2 determined using quantitative real-time PCR. Vertical bars represent mean ± SE values for triplicate samples.

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2.3. *Chronic starch feeding trial*

Compared to control group (17.10%), feeding 32.0%; 36.3% and 41.9% starch levels groups significantly raised plasma glucose levels at 3h after feeding, but there were no significant differences among the groups throughout the experiment at 24h after feeding (Fig. 5). 21.8%; 36.3% and 41.9% starch levels significant up-regulated relative expression of GLUT2 in liver at 3h after feeding (Fig. 6). High dietary carbohydrate levels (32.0%; 36.3% and 41.9%) significantly improved the relative expression of GK and PK (Fig. 7; 8), but significantly inhibitied the relative expression of PEPCK and G6P in liver at 3h after feeding (Fig. 9; 10). GLUT2; GK; PK; PEPCK and G6P mRNA levels were back to basal in liver at 24h after feeding (Fig. 6; 7; 8; 9; 10).

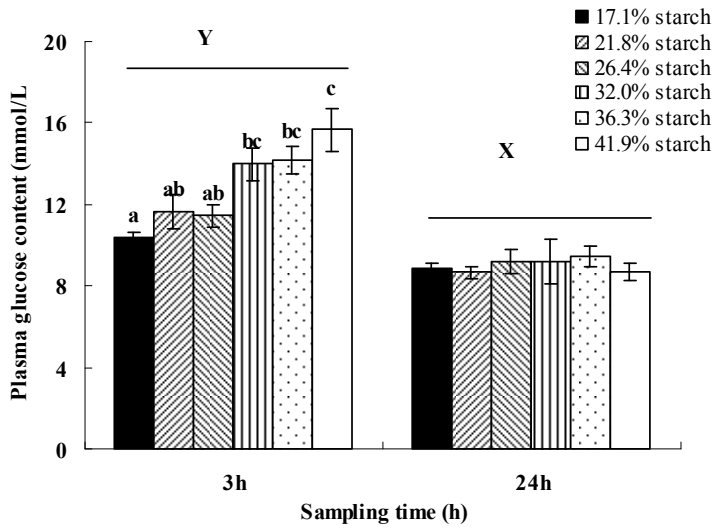

Fig 5. Serum glucose levels of feeding different experimental feed at 3h and 24h after feeding. Vertical bars represent mean ± SE values for triplicate samples. Value with different superscripts are significantly different ($P<0.05$).

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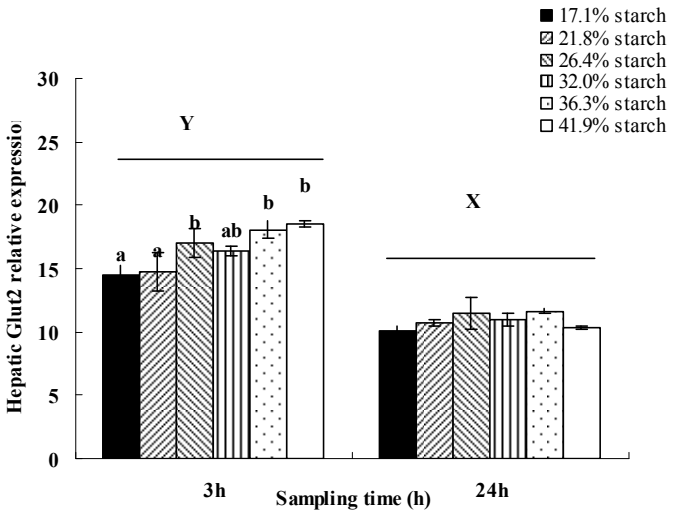

Fig 6. Relative expression of GLUT2 in liver in response to different carbohydrate levels at 3h and 24h after feeding. Vertical bars represent mean ± SE values for triplicate samples. Value with different superscripts are significantly different (*P<0.05*)

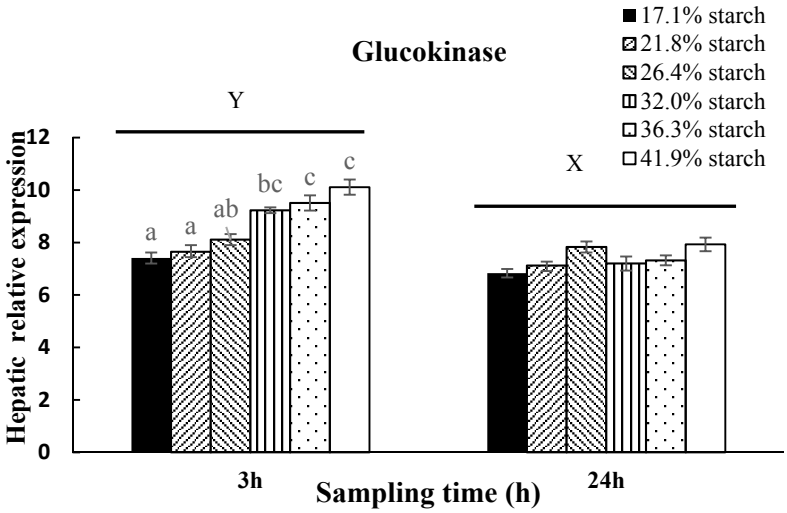

Fig 7. Relative expression of glucokinase in liver in response to different carbohydrate levels at 3h and 24h after feeding. Vertical bars represent mean ± SE values for triplicate samples. Value with different superscripts are significantly different (*P<0.05*)

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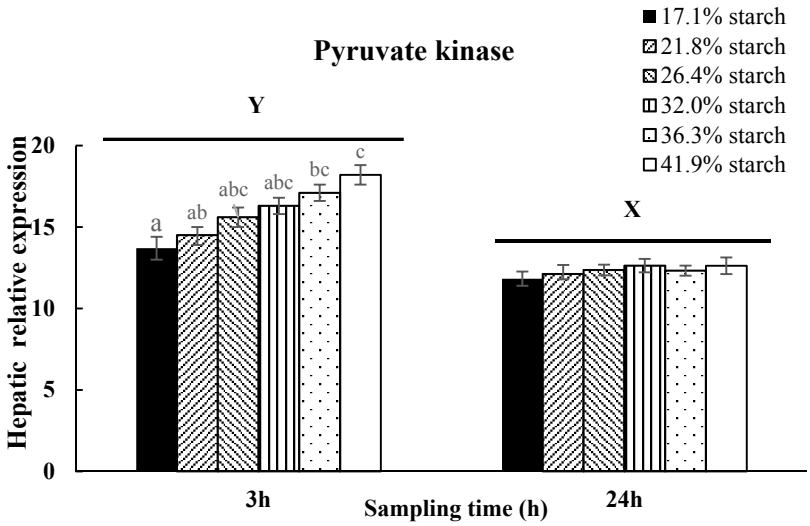

Fig 8. Relative expression of pyruvate kinase in liver in response to different carbohydrate levels at 3h and 24h after feeding. Vertical bars represent mean ± SE values for triplicate samples. Value with different superscripts are significantly different (*P<0.05*)

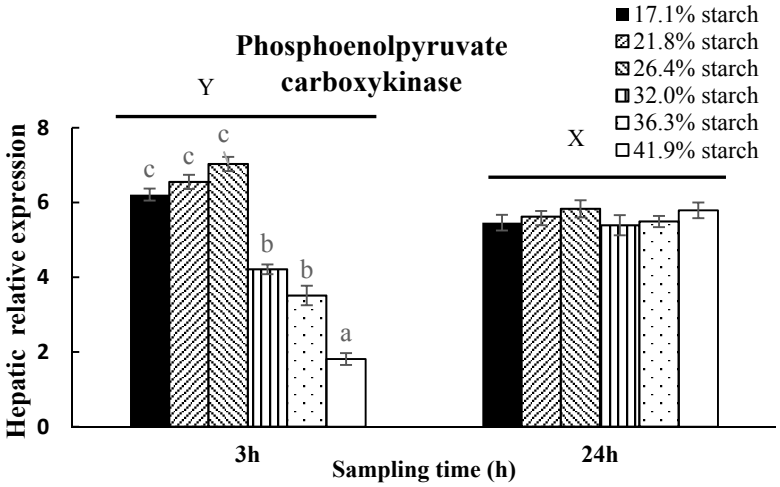

Fig 9. Relative expression of phosphoenolpyruvate carboxykinase in liver in response to different carbohydrate levels at 3h and 24h after feeding. Vertical bars represent mean ± SE values for triplicate samples. Value with different superscripts are significantly different (*P<0.05*)

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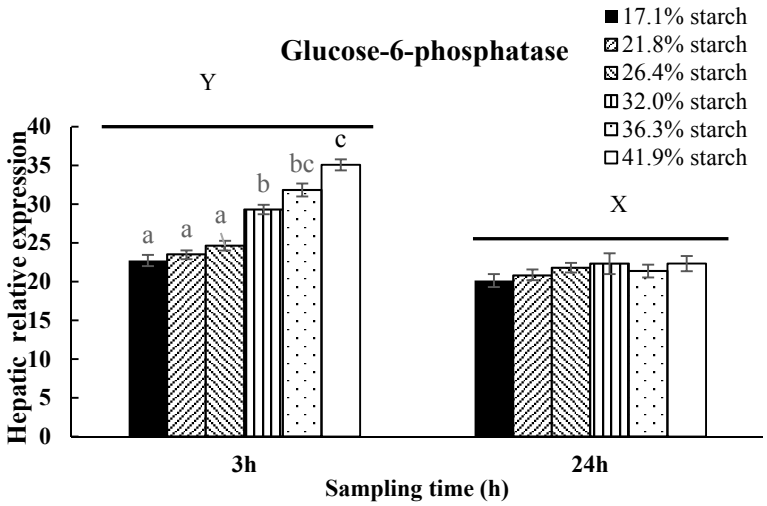

Fig 10. Relative expression of glucose-6-phosphatase in liver in response to different carbohydrate levels at 3h and 24h after feeding. Vertical bars represent mean ± SE values for triplicate samples. Value with different superscripts are significantly different ($P<0.05$)

3. Discussion

GLUT2 was the first characterized by cDNA cloning the Slc2a2/SLC2A2 gene from rat and human liver cDNA libraries [35,36]. To the best of our knowledge, we firstly cloned and identified GLUT2 in blunt snout bream. In the present study, a full-length cDNA clone representing the GLUT2 in blunt snout bream was obtained. By phylogenetic analysis, blunt snout bream GLUT2 clustered with fish GLUT2 sequences and other vertebrate GLUT2, not with other class I GLUTs. Therefore, the deduced blunt snout bream protein sequence was considered the blunt snout bream ortholog of GLUT2. GLUT2 has twelve across the membrane area locating at 7-29; 71-93; 106-123; 133-155; 168-190; 195-217; 282-301; 316-338; 345-367; 377-399; 412-434; 438-460 aimo acids respectively. Conservative structure domains located at 12-477 aimo acids belonging to sugar porter family major facilitator superfamily (MFS) transporter. Quantitative real-time reverse transcription polymerase chain reaction analysis indicated that GLUT2 expression of blunt snout bream was high in the liver, intestine and kidney, and highest in the liver. Tissue-specific expression of GLUT2 in blunt snout bream is generally consistent with that reported for GLUT2 in Atlantic cod and rainbow trout [13,14,37]. Different from sea bass and human, the highest expression of GLUT2 mRNA was observed intestinal tissue, followed by liver [15,38].

In general, fish, especially carnivorous fish have limited ability to metabolize glucose, and high digestible carbohydrate intake results in postprandial hyperglycemia for many hours

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[2]. A positive correlation between plasma glucose concentration and dietary carbohydrate level was found in cobia *(Rachycentron canadum* L); rainbow trout; silver perch (*Bidyanus bidyanus*) [1,39-41]. In our study, plasma glucose levels increased with an increasing dietary carbohydrate level at 3h after feeding, and were back to basal in the experiment at 24h after feeding. In the study of fish nutrition found that different fish species have different the capacity of glucose regulation. In omnivorous fish, the capacity of glucose regulation of carp only needed 5h to restore plasma glucose levels [3,42]. Tilapia and catfish (*Ictalurus punctatus*) needed 6h and 8h to return to normal levels respectively [43]. For carnivorous fish, rainbow trout and white sturgeon (*Acipenser transmontanus*), a 24h period was needed before plasma glucose levels were back to basal [40,44,45]. The present study confirmed the findings in fish which dietary carbohydrate level improved plasma glucose concentrations in juvenile blunt snout bream.

GLUT2 mRNA level can affect the capacity of glucose transfer between liver and blood, thereby affects glucose metabolism in the liver [46]. In our study, GLUT2 expression was raised in liver with increasing carbohydrate levels at 3h after feeding, highest expression in 41.9% carbohydrate level, however, GLUT2 expression were back to basal at 24h after feeding, indicating optimal dietary carbohydrate supplementation could enhance the capacity of glucose transfer between liver and blood in this juvenile fish. The present results suggest GLTU2 clould be regulated by dietary carbohydrate as mammals [7]. In fish, literature data in this area are relative scarcity. Thorens et al. (1990) indicated that the expression of GLUT2 increases after feeding a high-carbohydrate diet [47]. In rainbow trout, dietary carbohydrate did not induce a specific effect; expressions of GLUT2 mRNA were heightened at 24h after feeding [37]. Different from our study, dietary carbohydrate did not affect expression of GLUT2 mRNA at 6 h after feeding [37].The reason could be due to species differences, furthermore, carbohydrate level, feeding procedure and sample collection time could affect result of the trail.

Glycolysis is important in the glycometabolism pathway, furthermore, glucokinase (GK) and pyruvate kinase (PK) are two important rate-limiting enzymes in this pathway [48-50]. In this study, dietary carbohydrate improved expression of GK with increasing dietary carbohydrate level at 3h after feeding, furthermore, GK expression levels were back to basal

at 24h after feeding. In rainbow trout and gilthead sea bream (*Sparus aurata*), Panseratet al. (2000b) also found that carbohydrate feeding induced a high expression of GK gene, and there is a time-dependent decrease of hepatic GK mRNA levels in fish fed dietary carbohydrate 24h after a meal compared with 6h after a meal [51]. So high GK expression associated with dietary carbohydrate intake, one consequence of which is the relatively high levels of glycemia [40,41,52], which supported our experimental results. PK is the final step of glycolysis. In this study, the relative expression of PK showed a similar trend as plasma glucose level at 3h after feeding and were back to basal at 24h after feeding. This result was consistent with the previous researches in mammals [53]. In aquatic animals, some similar researches were also reported such as gilthead sea bream [54,55], carp [56]; grass carp [57] and channel catfish (*Ictalurus punctatus*) [58]. At the same time, other researchers have demonstrated that the above fact has not been observed in Senegalese sole (*Solea senegalensis*, Kaup) and eel (*Anguilla anguilla*) [59,60]. The present results suggested that dietary carbohydrate levels may improve hepatic glucose utilized by glycolysis pathway including up-regulation of GK and PK mRNA levels at 3h after feeding at least in juvenile blunt snout bream liver.

The rate of gluconeogenesis is principally controlled by the activities of certain unidirectional enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) [61]. In the present study, high dietary carbohydrate (32.0%; 36.3% and 41.9%) level decreased the relative expression of PEPCK and G6P at 3h after feeding and were back to basal at 24h after feeding, which was similar to those generally observed in mammals [62]. Several papers have reported the inhibition of PEPCK gene expression by glucose [63,64]. In aquatic animals, similar researches were also reported such as common carp (*Cyprinus carpio*) [65]; gilthead sea bream; dark barbel catfish (*Pelteobagrus vachelli*) [66]. However, Panserat et al. (2001) observed that there was no significantly effect of dietary carbohydrate on PEPCK gene expression [67]. G6P catalyzes the final step of gluconeogenesis, the production of free glucose from glucose-6-phosphate [61]. Panserat et al. (2002) and Caseras et al. (2002) also observed lower G6P expression in gilthead sea bream fed a diet with digestible carbohydrate comparatively to fish fed a carbohydrate-free diet [65,68]. In rainbow trout and sea bass, G6P expression was unaffected

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by the dietary carbohydrate levels tested [69,70]. The present results suggested that dietary carbohydrate levels inhibited gluconeogenesis by inhibition of PEPCK and G6P expression at 3h after feeding in juvenile blunt snout bream. However, the mechanism that dietary carbohydrate regulated by gluconeogenesis including PEPCK and G6P is still unclear and need to be further investigated.

4. Materials and methods

4.1. *Chronic carbohydrate feeding trial*

2.1.1 Experimental diets

Experimental diets were formulated to contain 30.0% crude protein, 8.5% crude lipid. Dietary protein was supplied by Casein, Gelatin and White fish meal, soybean oil and lecithin as lipid sources, wheat starch as sugar sources (Table 1). Experimental diets were formulated to contain graded carbohydrate levels (17.1 (control), 21.8, 26.4, 32.0, 36.3 and 41.9% of dry diet). Ingredients were ground into powder through 60 mash sieve and mixed uniformly with soybean oil, lecithin and water to make sinking pellet feed. The pellet feed was forced though a pelletizer (F-26 (II), South China University of Technology, China), which were then dried at 45°C overnight and then stored at-20°C for further use.

Table 1 Formulation and proximate composition of experimental diets for starch feeding trial

Ingredients	Groups					
	1	2	3	4	5	6
Casein[1]	22.0	22.0	22.0	22.0	22.0	22.0
Gelatin[1]	5.0	5.0	5.0	5.0	5.0	5.0
White fish meal[1]	11.0	11.0	11.0	11.0	11.0	11.0
Soybean oil	6.0	6.0	6.0	6.0	6.0	6.0
Soybean lecithin	1.0	1.0	1.0	1.0	1.0	1.0
Wheat starch[2]	18.0	23.0	28.0	33.0	38.0	43.0
Microcrystalline cellulose[3]	29.0	24.0	19.0	14.0	9.0	4.0
Carboxyl-methyl cellulose	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin premix[4]	2.0	2.0	2.0	2.0	2.0	2.0
Mineral premix[5]	4.0	4.0	4.0	4.0	4.0	4.0
Proximate analysis(% dry weight basis)						
Moisture	8.0	7.7	7.6	7.9	7.4	7.8
Crude protein	30.3	30.1	29.7	29.7	29.6	29.2
Crude lipid	8.5	8.7	8.4	8.5	8.5	8.6

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Starch	17.1	21.8	26.4	32.0	36.3	41.9
Ash	5.5	5.5	5.4	5.6	5.4	5.2

Notes: [1]Csein, obtained from Hua' an Biological Products Lit. (Gansu, China), crude protein 90.2%; gelatin, obtained from Zhanyu chemical Lit. (Shanghai, China), crude protein 91.3%; white fish meal, obtained from Copeinca (Lima, Peru), crude protein 67.4%, crude lipid 9.3%.

[2]wheat starch obtained from Guangsheng starch Lit. (Jiangsu, China).

[3]microcrystalline cellulose, obtained from Xinwang chemical Lit. (Zhejiang, China).

[4]vitamin premix (per kg diet): Vitamin A, 9 000 IU; Vitamin D, 2 500 IU; Vitamin E, 45 mg; Vitamin K_3, 2.2 mg; Vitamin B1, 3.2 mg; Vitamin B2, 10.90 mg; Vitamin B6, 50 mg; Vitamin B12, 1.16 mg; Biotin, 0.50 mg; Pantothenate, 10 mg; Folic acid, 1.65 mg; Inositol, 150 mg; Niacin acid, 25 mg.

[5]mineral premix (g /kg of diet): calcium biphosphate, 20 g; sodium chloride, 2.6g; potassium chloride, 5 g; magnesium sulphate, 2 g; ferrous sulphate, 0.9 g; zinc sulphate, 0.06 g; cupric sulphate, 0.02; manganese sulphate, 0.03 g; sodium selenite, 0.02 g; cobalt chloride, 0.05 g; potassium iodide, 0.004 g.

4.1.2 Experimental procedure

Pre-adult blunt snout bream were obtained from the breeding farm of Freshwater Fisheries Research Centre (FFRC) of Chinese Academy of Fishery Sciences. During the acclimation period, fish were hand-fed three times daily at 8:00, 12:00 and 16:00 until apparent satiation on the basis of visual observation of fish feeding behavior. At the initiation of the experiment, fish were weighed and counted 24h after last feeding. The pre-adult blunt snout bream (161 ± 2.7g) were randomly sorted into eighteen tanks (1000 L) with 20 fish. Each diet was randomly assigned to triplicate tanks for 9 weeks. Fish were hand-fed three times daily at 8:00, 12:00 and 16:00 until apparent satiation on the basis of visual observation of fish feeding behavior. During the experimental period, water temperature ranged from 27 to 29°C, pH from 7.1 to 7.8, dissolved oxygen from 6.3 to 7.2mg/L, ammonia nitrogen from 0.006 to 0.008mg/L and hydrogen sulfide from 0.007 to 0.009mg/L.

4.1.3 Sample collection

At the end of the feeding trial, six experimental blunt snout bream from each tank were collected, anesthetized with 100 mg L^{-1} MS-222, blood samples were collected immediately from the caudal vein with disposable medical syringes and then liver samples were collected from the sampled fish. Plasma was separated by centrifugation (3500×g, 10 min, 4°C). Plasma and liver samples were stored at -80°C until analysis.

4.2. Cloning of GLUT2 cDNAs

GLUT2 cDNA were cloned as described in our previous studies [71]. In briefly, according to the manufacturer's protocols, total RNA was extracted from mixed tissues of the

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blunt snout bream by using the RNAiso Plus Reagent (Takara, Dalian, China). First-strand cDNA was synthesized using the reverse transcriptase M-MLV Kit (Takara, Dalian, China). 3'-Rapid amplification of cDNA ends (RACE) and 5'-RACE were performed by the 3'-full RACE Core Set Ver.2.0 Kit and 5'-full RACE Kit (Takara, Dalian, China). All primers used for cloning are listed in Table 2. The PCR products were purified using a gel extraction kit (Sangon, China) and sequenced on the ABI3730 DNA analyzer (ABI, USA) after insertion into the PMD-18T vector (Takara, Dalian, China).

Table 2 Sequences of the PCR primers used in this work

Primer	Primer sequence (5'-3')
[1]GLUT2-F CDS amplification	ATGGAGAAGCAGTTAACAGGC
[1]GLUT2-R CDS amplification	TCAGGCCTCTGTAGAGCTC
[1]GLUT2-F1 (3'RACE out primer)	GCAAGAATCTGAAAAGGCTGAAGGG
[1]GLUT2-F2 (3'RACE in primer)	GGGAGATTACGACACCTCAAAAG
[1]GLUT2-R1 (5'RACE out primer)	TCATCCATGAGTAAACATCCTG
[1]GLUT2 -R2 (5'RACE in primer)	CCACTGTCATGGCCACTG
[1]GLUT2-F (Real-time primer)	CGGTGAAACCGAACAGGAGT
[1]GLUT2-R (Real-time primer)	TTCTTTGAGATCGGGCCTGG
β-actin-F (Real-time primer)	TCGTCCACCGCAAATGCTTCTA
β-actin-R (Real-time primer)	CCGTCACCTTCACCGTTCCAGT
[2]GK-F (Real-time primer)	GCTTCCACTGGGATTCACCT
[2]GK-R (Real-time primer)	CGACGTTATTGCCTTCAGCG
[3]PK-F (Real-time primer)	CGAGATTGAGAACGGAGGCA
[3]PK-R (Real-time primer)	GTCCTTCTCAGACACTGCGG
[4]PEPCK-F (Real-time primer)	TCGCCTGGATGAAGTTCGAC
[4]PEPCK-R (Real-time primer)	GTCTTGGTGGAGGTTCCTGG
[5]G6P-F (Real-time primer)	TTCAGTGTCACGCTGTTCCT
[5]G6P-R (Real-time primer)	TCTGGACTGACGCACCATTT

Note: [1]GLUT2: Glucose transporter 2; [2]GK: Glucokinase; [3]PK: Pyruvate kinase; [4]PEPCK: Phosphoenol pyruvate carboxykinase; [5]G6P: Glucose-6-phosphatase

4.3. Nucleotide sequence and bioinformatic analyzes

Similarity searches of the sequenced cDNA of GLUT2 were done by blastn (www.ncbi.nlm.nih.gov/blast/). The multiple-sequence alignments of amino acids were performed by DNAman. The deduced amino acid sequences were analyzed with DNAman and ExPASy Compute pI/MW (http://web.expasy.org/compute_pi/). SMART program

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(http://smart.embl-heidelberg.de/) and PROSITE program (http://kr.expasy.org/prosite/) were used to predict the functional sites or domains in the amino acid sequence. Phylogenetic analyses were carried out based on amino acid sequences using the neighbour-joining method, and the trees were constructed using MEGA 5.1. Transmembrane structure analyses were carried out based on amino acid sequences by TMHMM 2.0. The sequenced cDNA of GLUT2 were done by CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to predict Conservative structure domains.

4.4. Quantitative real-time PCR analysis of GLUT2 expression

Relative gene expressions of GLUT2 was determined using Real-time PCR analysis as described in our previous study [72]. Briefly, total RNA was extracted from the liver of juvenile blunt snout bream using an RNAiso plus kit (Takara, Dalian, China). After quality and quantity of RNA were checked, complementary DNA (cDNA) was synthesized using a PrimeScript TM RT reagent kit (Takara, Dalian, China). We designed specific primers according to the partial cDNA sequences of these genes and cloned sequences (Table 3) [73]. β-actin was employed as a nonregulated reference gene, aspreviously used in blunt snout bream studies [72] (Habte-Tsion et al., 2015). No changes in β-actin gene expression were observed in our investigations. Relative quantification of target gene expression was performed using the Pfaffl's mathematical model [74].

4.5 Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) using the software of SPSS 16.0 for Windows. Significant differences between means were evaluated by Turkey's Multiple Range Test. $P<0.05$ was considered significant. Data are expressed as means with SEM (X± SEM).

5. Conclusion

The present study demonstrated that GLUT2 was 2577 bp, encoding a polypeptide of 503 amino acids with predicted molecular weight of 55.046 kDa and theoretical isoelectric point of 7.52. GLUT2 has twelve across the membrane area locating at 7-29; 71-93; 106-123; 133-155; 168-190; 195-217; 282-301; 316-338; 345-367; 377-399; 412-434; 438-460 aimo acids respectively. Conservative structure domains located at 12-477 aimo acids belonging to sugar porter family major facilitator superfamily (MFS) transporter. Blunt snout bream has a

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positive correlation between plasma glucose concentration and dietary carbohydrate level at 3h after feeding. At 24h after feeding, plasma glucose concentration returned to basal level. Furthermore, high dietary carbohydrate levels improved the glycolysis and inhibited gluconeogenesis by augmentation of GK and PK expression, inhibition of PEPCK and G6P expression. However, when plasma glucose levels were back to basal at 24h, GLUT2; GK; PK; PEPCK and G6P were independent of dietary starch levels.

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Author Contributions: Xianping Ge and Mingchun Ren designed the study, Hualiang Liang carried out the experiments and wrote the manuscript. Ogwok-Manas Wilson-Arop reviewed the manuscript. Ke J performed data analysis. Haifeng Mi provided technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

Facilitative Glucose Transporter: GLUT

Glucokinase: GK

Pyruvate kinase: PK

Phosphoenol pyruvate carboxykinase: PEPCK

Glucose-6-phosphatase: G6P

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