General and Comparative Endocrinology 266 (2018) 9–20

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Comparatively study on the insulin-regulated glucose homeostasis through brain-gut peptides in Japanese flounder *Paralichthys olivaceus* after intraperitoneal and oral administration of glucose

Dong Liu [a,1], Benyue Guo [a,1], Dongdong Han [a,1], Kangyu Deng [a], Zhixiang Gu [a], Mengxi Yang [a], Wei Xu [a], Wenbing Zhang [a,b,*], Kangsen Mai [a,b]

[a] The Key Laboratory of Mariculture, Ministry of Education; The Key Laboratory of Aquaculture Nutrition and Feeds, Ministry of Agriculture; Ocean University of China, Qingdao 266003, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Wen Hai Road, Qingdao 266237, China

ARTICLE INFO

Article history:
Received 2 January 2018
Revised 9 February 2018
Accepted 14 February 2018
Available online 15 February 2018

Keywords:
Glucose
Insulin
Brain-gut peptides
Paralichthys olivaceus

ABSTRACT

The present study comparatively analyzed the blood glucose and insulin concentration, the temporal and spatial expression of brain-gut peptides and the key enzymes of glycolysis and gluconeogenesis in Japanese flounder by intraperitoneal injection (IP) and oral administration (OR) of glucose. Samples were collected at 0, 1, 3, 5, 7, 9, 12, 24 and 48 h after IP and OR glucose, respectively. Results showed that the hyperglycemia lasted for about 10 h and 21 h in OR and IP group, respectively. The serum insulin concentration significantly decreased at 3 h (1.58 ± 0.21 mIU/L) after IP glucose. However, it significantly increased at 3 h (3.37 ± 0.341 mIU/L) after OR glucose. The gene expressions of prosomatostatin, neuropeptide Y, cholecystokinin precursor and orexin precursor in the brain showed different profiles between the OR and IP group. The OR not IP administration of glucose had significant effects on the gene expressions of preprovasoactive intestinal peptide, pituitary adenylate cyclase activating polypeptide and gastrin in intestine. In conclusion, brain-gut peptides were confirmed in the present study. And the serum insulin and the brain-gut peptides have different responses between the IP and OR administration of glucose. The OR could stimulate the brain-gut peptide expressions, which have effects on the insulin secretion and then regulate the blood glucose levels. However, in IP group, there is little chance to stimulate brain-gut peptide expression to influence the insulin secretion, which leads to a longer hyperglycemia.

1. Introduction

Glucose acts as an important source of energy for most species of fish. However, carnivorous fish species like rainbow trout have been considered as "glucose intolerant" as hyperglycemia after a glucose load can last for several hours, even more than one day due to their limited ability in using glucose efficiently (Aguilar, 2010). Glycolysis and gluconeogenesis act as the physiological functions of carbohydrate decomposition and synthesis. Enzymes in the two pathways coordinate with each other to ensure the glucose homeostasis in fish. Previous studies in fish found that the hepatic glucokinase (GK) activity could be induced by dietary carbohydrates in rainbow trout, gilthead seabream and common carp (Panserat et al., 2000). Activity of pyruvate kinase (PK) was significantly influenced by high levels of dietary carbohydrate in perch and European seabass (Borrebaek and Christophersen, 2000; Enes et al., 2006). However, 6-phosphofructokinase-1 (PFK) activity was not induced by dietary carbohydrates in gilthead sea bream and turbot (Couto et al., 2008; Nie et al., 2013). A review of studies on carbohydrate utilization in fish (Luo and Xie, 2010) showed that some carnivorous fish species (i.e., rainbow trout, tilapia) showed a higher blood glucose without suppression on the activity and gene expression of glucose-6-phosphatase, fructose-1,6-bisphosphatase (FBPase) and phosphoenolpyruvate carboxykinase when fed diets containing carbohydrates. However, the findings were not always unanimous.

Although glucose is not the main energy substrate for carnivorous fish species, glucose metabolism is important for the function of specific tissues in fish, such as brain (Soengas and Aldegunde, 2002). The brain has the highest glucose utilization rates per unit mass in all tissues examined in rainbow trout (Washburn et al., 1992). In mammals, the brain is responsible for neuroregulation,

* Corresponding author.
E-mail address: wzhang@ouc.edu.cn (W. Zhang).
[1] These three authors contributed equally to this work

https://doi.org/10.1016/j.ygcen.2018.02.013
0016-6480/

meanwhile, gut cells responsible for perceive nutrients. The brain and gut have complicated connections in nutrients, hormone and nerve, thus a gut–brain axis has been generated (Romijn et al., 2008). Pearse and Takor (Pearse and Takor, 1979) pointed out that gastrointestinal peptide secreting cells and peptidergic neurons in the brain are all originated from the neuroectoderm. Some studies put forward the concept of enteric nervous system, and it has close ties to brain systems (Wood, 1996). With the discovery of brain-gut peptides, researchers produced the hypothesis of a brain-intestinal connection (Zhang, 2001). Although the function of the brain-gut axis in fish is not totally clear, the brain-gut peptides like gastrin (Pereira et al., 2015), cholecystokinin (Polakof et al., 2011) and somatostatin (SS) (Sheridan et al., 1987), has been demonstrated to play a crucial role in glucose homeostasis in mammals.

In previous studies in mammals, it was confirmed that somatostatin promotes glycogen breakdown and the release of glucose from liver to plasma (Eilertson et al., 1991). And it can also inhibit hormones secretion, including insulin (Eilertson and Sheridan, 1993; Sheridan et al., 1987; Very et al., 2001). Neuropeptide Y (NPY) is also involved in regulating insulin and glucose metabolism in fish. It was found that plasma insulin levels decreased in fasted European sea bass co-injected with NPY plus glucose, but remained stable when NPY was administrated alone to fed and fasted animals (Cerdá-Reverter et al., 1999). Studies in mammals showed that cholecystokinin (CCK) can stimulate insulin secretion and it can also make influence on β cell proliferation (May et al., 2016). Orexin (OX) can promote insulin secretion and enhance the capacity of glucose-stimulated insulin (Park et al., 2015). In addition, the functions of vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) are similar in animal reproduction, nutrient digestion and energy balance. And the two hormones can stimulate insulin secretion (Bredkjoer et al., 1997; Vaudry et al., 2000). Gastrin has a function similar to that of VIP and PACAP in glucose metabolism, and it can also stimulate insulin production (Yue et al., 2007).

However, little related information has been reported in fish. There are two kinds of glucose tolerance tests in published studies. They are the intraperitoneal injection glucose test and oral glucose test. It has been shown that after injected intraperitoneally with 1 g glucose/kg body weight, blood glucose levels reached a peak at 3 h in sea bream and 6 h in sea bass (Peres et al., 1999). The blood glucose in both the two fish species returned to the normal level at 12 h after injection. The concentration of plasma glucose in tilapia peaked at 3 h after oral administration of glucose and starch, and the plasma insulin concentration was higher at 1 h after oral administration of glucose than those orally administrated with starch (Lin et al., 1995). With regards to the absorption pattern of glucose, it was absorbed through coeliaco-mesenteric capillaries after intraperitoneal injection and through the digestive tract canal after oral administration. In mammals, the intestine can be stimulated by high glucose, which in turn triggers the secretion of intestinal hormones activating a hypoglycemic mechanism, such as the incretin (Hayes et al., 2014). But there is little chance for the intestinal hormones to be stimulated by the injection glucose. However, little related reports were found in fish.

Japanese flounder *Paralichthys olivaceus* is one of the typical marine carnivorous fish species. After a 45-day feeding trial with different dietary carbohydrate sources, a previous study found that the Japanese flounder utilized dextrin more efficiently than glucose, and dextrin was a better source of energy than lipid. In that study, however, only the apparent parameters including growth performance, feed utilization and body compositions were analyzed (Lee et al., 2003). The present study was conducted to analyze the compositions of the brain-gut peptides and their gene expression profiles after oral administration and injection of glucose, furthermore, to investigate the relationship between these gene expression and the changes of insulin and glucose concentration in blood, and the expressions of the key enzymes involved in glycolysis and gluconeogenesis in liver of Japanese flounder. The aim is to assess whether there is a negative feedback mechanism in the insulin-regulated glucose homeostasis through brain-gut peptides in Japanese flounder.

2. Materials and methods

2.1. Experimental animals

Japanese flounders (body weight: 225 ± 50 g) were provided by a local fish farm in Haiyang, Shandong Province, China. They were randomly distributed into cylindrical fiberglass tanks (300-L) in a re-circulating water system. During the 4-week acclimation, Japanese flounders were fed with commercial feeds (Qingdao Great Bio-tech Co., Ltd) to satiation twice daily. All animal care and handling procedures were approved by the Animal Care Committee of Ocean University of China.

2.2. Intraperitoneal injection of glucose

There were two intraperitoneal injection (IP) groups. One was IP injected with phosphate buffer solution (PBS), and one was injected with glucose. There were eight tanks per injection group, and eight fish per tank. Before injection, Japanese flounders were fasted for 48 h to eliminate the effect of residual food in the digestive tract, and then were anaesthetized with MS-222 (50 mg/l). After being weighed, fish were IP injected with 1 g glucose/kg body weight (Booth et al., 2006). The glucose was purchased from Sigma (500 mg/ml, Sigma, USA). Meanwhile, the other group of animal was injected with the same volume of PBS (0.01 mol/L) as the glucose injection group, and was used as the control. The operations of injection for each tank were completed within 5 min. After injection, Japanese flounders were put back into the tanks. Samples of blood, brain, intestinal, muscle and liver were collected just before (time 0 h) and 1 h, 3 h, 5 h, 7 h, 9 h, 12 h, 24 h and 48 h after injection (Yang et al., 2012; Liu et al., 2015). Blood was collected by 1 ml syringes from caudal vein. Serum was obtained after centrifugation (10,000*g* for 10 min at 4 °C) of blood. All samples were frozen in liquid nitrogen immediately and stored at -80 °C.

2.3. Oral administration of glucose

Before oral administration, Japanese flounders were fasted for 48 h to eliminate the effect of residual food in the digestive tract, and then were anaesthetized with MS-222 (50 mg/l). There were two oral administration groups, and eight tanks per group, eight fish per tank. One group received glucose (500 mg/ml, Sigma, USA) with 1.67 g/kg body weight (Huang et al., 2005). The other group received the same volume of PBS (0.01 mol/L) and was used as the control. After oral administration, Japanese flounders were put back into the tanks. Samples of blood, brain, intestinal and liver were collected just before (time 0 h), and 1 h, 3 h, 5 h, 7 h, 9 h, 12 h, 24 h and 48 h after OR. These samples were collected and stored as described above.

2.4. Analysis of glucose and insulin concentrations in serum

Concentration of glucose in serum was determined with the automatic biochemical analyzer (Hitachi, 7600-210, Japan). Concentration of insulin in serum was analyzed with double antibody sandwich enzyme-linked immunosorbent assays. The common carp insulin was used as the reference insulin (ELISA, MIbio, China).

2.5. Analysis of glycogen contents

The glycogen concentrations in muscle and liver were determined using the anthrone chromogenic method with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). They were measured with a UV spectrophotometer (UV-2401PC, Shimadzu, Kyoto, Japan).

2.6. Tissue distribution of the selected brain-gut peptides

Tissue distributions of the brain-gut peptides were detected in stomach, gill, spleen, muscle, brain, liver, kidney, intestine and eye by the real-time quantitative RT-PCR (q-PCR). Specific primers for peptides and β-actin (reference gene) are shown in Table 1. Isolation of total RNA and synthesis of the first strand cDNA were carried out following the instructions of the kit (Trizol and PrimeScript Reverse Transcriptase, Takara, Japan).

2.7. Gene expression analysis by real-time quantitative RT-PCR

Gene expression levels were determined by q-PCR using the iCycler iQ™ (Bio-Rad,Hercules, CA, USA). Analyses were using SYBR Green I (CWbiotech, China) according to the manufacturer's instructions. The total reaction volume was 25 μl, including 1.0 μl cDNA, 12.5 μl 2 × UltraSYBR Mixture, 1 μl each of gene-specific primer (10 μM, Table 2) and 9.5 μl DEPC water. Thermal cycling was initiated with incubation at 95 °C for 10 min using hot-start iTaq™ DNA polymerase activation, 40steps of PCR were performed, each one consisting of heating at 95 °C, 10 s for denaturing, and at specific annealing and extension temperatures is Tm for 30 s, 72 °C for 32 s. Following the final PCR cycle, melting curves were systematically monitored (58 °C temperature gradient at 0.5 °C/s from 58 to 95 °C) to ensure that only one fragment was amplified. Relative quantification of the target gene transcript was done using β-actin gene expression as reference (Olsvik et al., 2005) which was stably expressed in this experiment. Samples without reverse transcriptase and samples without RNA were run for each reaction as negative controls. Relative quantification of the genes were calculated using "$2^{-\Delta\Delta Ct}$" meth (Livak and Schmittgen, 2001) with β-actin as reference gene. All q-PCR reactions were performed in triplicate biological replicates.

2.8. Analysis of carbohydrate metabolism related enzymes activities

The analysis of the activities of GK, PK, PFK and FBPase were conducted by the methods of Nie et al. (2015). Briefly, a frozen sample of tissues (500 mg) was homogenized (dilution 1/10) in ice-cold buffer (pH 7.6) consisting of 50 mmol l^{-1} Tris, 5 mmol l^{-1} EDTA, 2 mmol l^{-1}, 1,4-dithiothreitol and 1% protease inhibitor cocktail (Sigma, USA). The homogenate was centrifuged at 10,000 r/min for 30 min and the supernatant was collected. The analysis was measured at 340 nm using a microplate reader (Thermo Scientific Multiskan Spectrum, USA) at 30 °C. The total protein content in crude extracts was determined at 30 °C using bovine serum albumin as a standard based on the method of Bradford (1976). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of substrate per mg protein per minute at 30 °C. Enzyme activities were expressed in per milligram of total protein (specific activity).

2.9. Statistics analysis

All data were expressed as means ± standard error and performed using SPSS 17.0. A two-way analysis of variance (ANOVA) was used to compare the differences in the values of concentrations of blood glucose and insulin, activities of enzyme, and gene expressions of peptides among the different sampling times. Values on these parameters on 0 h in PBS group are the same in glucose group. So these two groups share the values on 0 h. When overall differences were considered statistically significant at $P < 0.05$, Tukey's test was used to compare the means among individual treatments.

Table 1
Expression levels of prosomatostatin (Pro-SS), neuropeptide Y (NPY), cholecystokinin precursor (Pro-CCK), orexin precursor (Pro-OX), preprovasoactive intestinal peptide (Pro-VIP), pituitary adenylate cyclase activating polypeptide (PACAP) and gastrin mRNA in Japanese flounder.

	Pro-SS	NPY	Pro-CCK	Pro-OX	Pro-VIP	PACAP	Gastrin
Eye	1.33 ± 0.19^{a}	24.42 ± 0.33^{e}	0.06 ± 0.02^{a}	1.03 ± 0.27^{a}	1.01 ± 0.19^{b}	1.04 ± 0.39^{b}	1.01 ± 0.12^{a}
Stomach	0.23 ± 0.04^{a}	0.32 ± 0.03^{ab}	1.08 ± 0.33^{b}	0.20 ± 0.10^{a}	4.19 ± 0.61^{c}	0.02 ± 0.00^{a}	0.27 ± 0.14^{a}
Gill	0.96 ± 0.12^{a}	4.87 ± 1.72 ^{cd}	0.01 ± 0.00^{a}	0.34 ± 0.09^{a}	1.19 ± 0.03^{b}	0.01 ± 0.00^{a}	1.03 ± 0.28^{a}
Spleen	0.18 ± 0.12^{a}	5.90 ± 1.20^{d}	0.00 ± 0.00^{a}	0.16 ± 0.03^{a}	1.03 ± 0.01^{b}	0.01 ± 0.00^{a}	0.13 ± 0.05^{a}
Muscle	0.50 ± 0.09^{a}	1.13 ± 0.08^{ab}	0.00 ± 0.00^{a}	0.19 ± 0.08^{a}	0.02 ± 0.01^{a}	0.00 ± 0.00^{a}	0.43 ± 0.21^{a}
Brain	22.90 ± 2.12^{b}	36.82 ± 3.32^{f}	24.17 ± 5.65^{d}	5.84 ± 1.59^{b}	8.38 ± 1.01^{c}	8.47 ± 0.77^{c}	1.14 ± 0.32^{a}
Liver	0.36 ± 0.18^{a}	1.54 ± 1.02^{ab}	0.03 ± 0.02^{a}	0.26 ± 0.12^{a}	0.22 ± 0.24^{a}	0.01 ± 0.01^{a}	0.35 ± 0.17^{a}
Kidney	0.22 ± 0.06^{a}	0.04 ± 0.01^{a}	0.00 ± 0.00^{a}	0.12 ± 0.06^{a}	0.03 ± 0.01^{a}	0.00 ± 0.00^{a}	0.17 ± 0.12^{a}
Intestine	0.9 ± 0.20^{a}	2.18 ± 0.72^{bc}	5.01 ± 0.69^{c}	0.20 ± 0.06^{a}	14.54 ± 1.44^{d}	17.81 ± 2.77^{d}	21.63 ± 2.66^{b}

Those are relative expression levels. Different letters indicate significant differences ($P < 0.05$).

Table 2
Primers used for gene mRNA quantification by RT-PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′0–3′)	Target size (bp)	Annealing temperature (°C)	E value
β-actin	GGAAATCGTGCGTGACATTAAG	CCTCTGGACAACGGAACCTCT	155	58	1.011
NPY	AAGACAGAGGTATGGGAAGAG	CTTGACTGTGGAAGCGTGT	99	58	0.922
Pro-SS	AAACTCCGCCTGTTGCTG	CAGAGCCTCGTTCTCCACC	117	58	0.932
Pro-CCK	CATCTCGTCCAGGAAAGGT	TCCATCCAGCCCAAGTAG	105	57	0.913
Orexin	ATGCTCATCCTCCTTCCG	ACCATCTCGCTCCTGTCG	127	59	0.966
PACAP	CCCTCCCTGGATTATGAC	GCTTTCCTGTAGGCTTTATT	143	60	0.951
Gastrin	AGGGACTCGGCTCACAGA	TTGGTCATAATCTCCCGTTC	101	60	0.923
Pro-VIP	GTCAAGCGTCACTCAGATGC	GGGTCTTCCAGGCTTCTCTT	116	60	0.974

G/T = K; A/C = M; A/G = R; C/T = Y; A/T = W

3. Results

3.1. Concentrations of glucose and insulin in serum

Concentrations of glucose in serum reached the peak (20.06 ± 1.92 mM) at 5 h after the IP injection of glucose, which was about 28 times as high as that at 0 h (0.71 ± 0.25 mM) (Fig. 1A). From the 5 h to 24 h after injection, the blood glucose decreased to 7.40 ± 5.47 mM. There was no significant difference in blood glucose between the time point of 0 h and 24 h (Fig. 1A). After injection of glucose, the insulin concentration in serum was decreased to the lowest value (1.58 ± 0.21 mIU/L) at 3 h (Fig. 1B). After that, it grew gradually to the normal value at 24 h (3.03 ± 0.006 mIU/L) as that at 0 h (3.20 ± 0.18 mIU/L).

After oral administration of glucose, blood glucose concentration had rapidly increased at 3 h (1.90 ± 0.23 mM), and it was

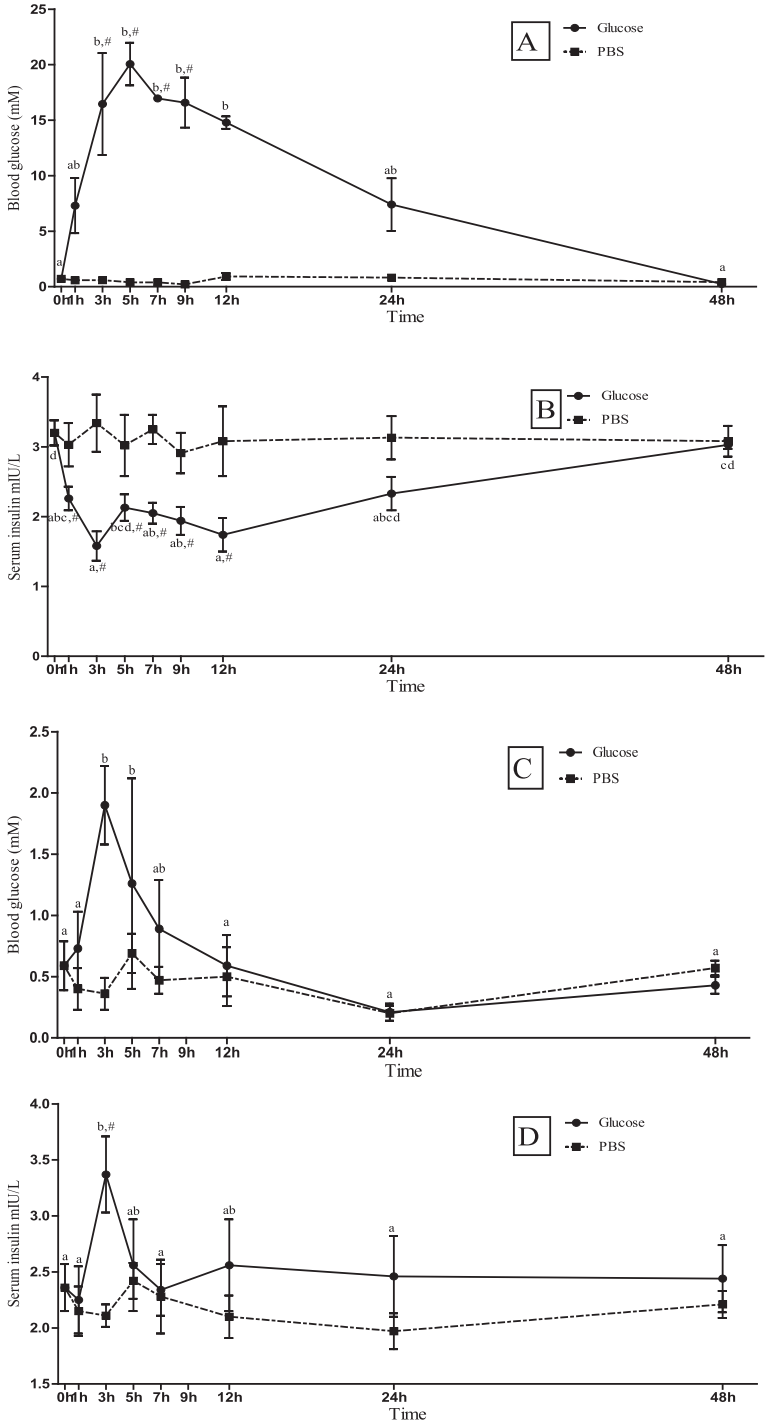

Fig. 1. The concentration of blood glucose and serum insulin in Japanese flounder after intraperitoneal (IP) (A, B) and oral (OR) (C, D) administration of glucose or phosphate buffer solution (PBS). Each value is expressed as the Mean ± SE (n = 9). Different letters indicate significant differences among sampling times within glucose load. There was no significant difference for PBS administration. # means that significantly different from fish administration of glucose and PBS at the same time.

higher than those at the other time points. After the 3rd hour, the blood glucose concentration decreased gradually (Fig. 1C). The serum insulin concentration increased from 2.36 ± 0.21 mIU/L (0h) to 3.37 ± 0.34 mIU/L (3h). It returned to the normal level after 5 h, and then, kept relatively stable (Fig. 1D).

3.2. Tissue distribution of peptides genes

Results of the gene distribution and expression are shown in Table 1. The relative expressions of prosomatostatin (Pro-SS) and orexin precursor (Pro-OX) in brain were relatively higher than that

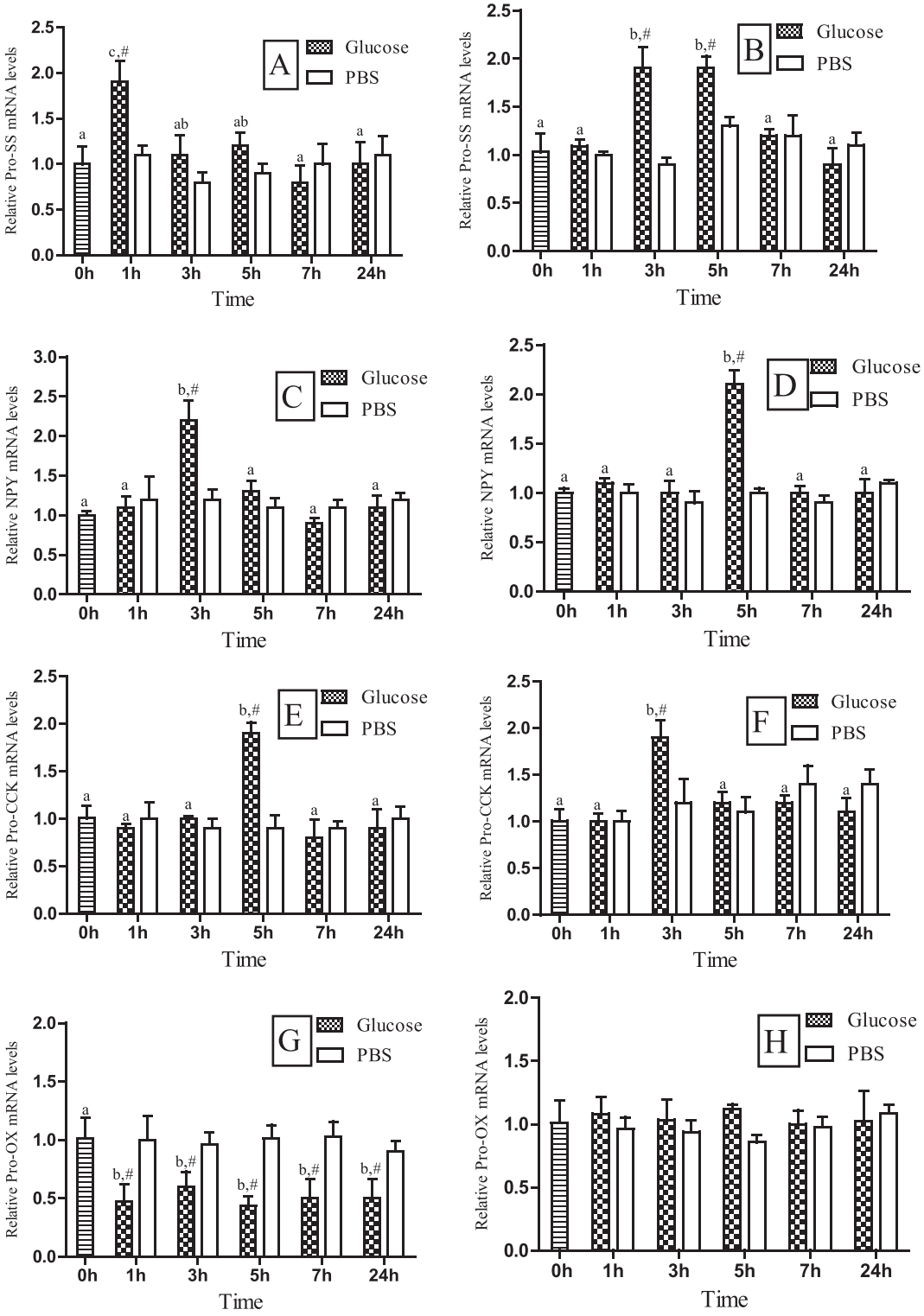

Fig. 2. Gene expressions of prosomatostatin (Pro-SS), neuropeptide Y (NPY), cholecystokinin precursor (Pro-CCK) and orexin precursor (Pro-OX) in the brain of Japanese Flounder after intraperitoneal (IP) (A, C, E, G) and oral (OR) (B, D, F, H) administration of glucose (Glu) or phosphate buffer solution (PBS). Each value is expressed as the Mean ± SE (n = 9). Different letters indicate significant differences among sampling times within glucose load. There was no significant difference for PBS administration. # means that significantly different from fish administration of glucose and PBS at the same time.

in the other analyzed tissues including stomach, gill, spleen, muscle, liver, kidney, intestine and eyes. Gastrin was mainly expressed in intestine. NPY, CCK precursor (Pro-CCK), Preprovasoactive intestinal peptide (Pro-VIP) and PACAP were detected in many tissues. Relative expression of NPY had the highest value in brain, and followed by the eye, gill and spleen. Relative expression of Pro-CCK was up to maximum in brain, followed by the intestine and stomach. The expression of Pro-VIP was relatively high in intestine, but low in brain, stomach, gill, spleen and eyes. The PACAP had the highest expression in intestine, then brain.

3.3. Gene expressions after glucose administration

3.3.1. Gene expression of the Pro-SS, NPY, Pro-CCK and Pro-OX in brain

Results of the gene expression in brain after glucose administration are shown in Fig. 2.

One hour after the intraperitoneal injection of glucose, mRNA levels of the Pro-SS were significantly increased, then it was not significantly differed from that at 0 h at the others time points. In the group of IP PBS, there were no significant different in mRNA levels of Pro-SS among all the time points (Fig. 2A).

After oral glucose administration, Pro-SS mRNA levels were significantly up-regulated at the time point of 3 h and 5 h (Fig. 2B). The NPY mRNA levels were significantly increased only at 3 h after IP and 5 h after OR glucose (Fig. 2C, D). The cholecystokinin precursor (Pro-CCK) mRNA levels were significantly increased only at 5 h after IP and 3 h after OR glucose (Fig. 2E, F). The Pro-OX mRNA levels significantly decreased at all the time points after IP glucose. However, there were no significant differences among all the time points after OR glucose (Fig. 2G, H).

3.3.2. Gene expression of Pro-VIP, PACAP and gastrin in intestine

Results of the gene expression in intestine after glucose administration are shown in Fig. 3. There were no significant differences in gene expressions of preprovasoactive intestinal peptide

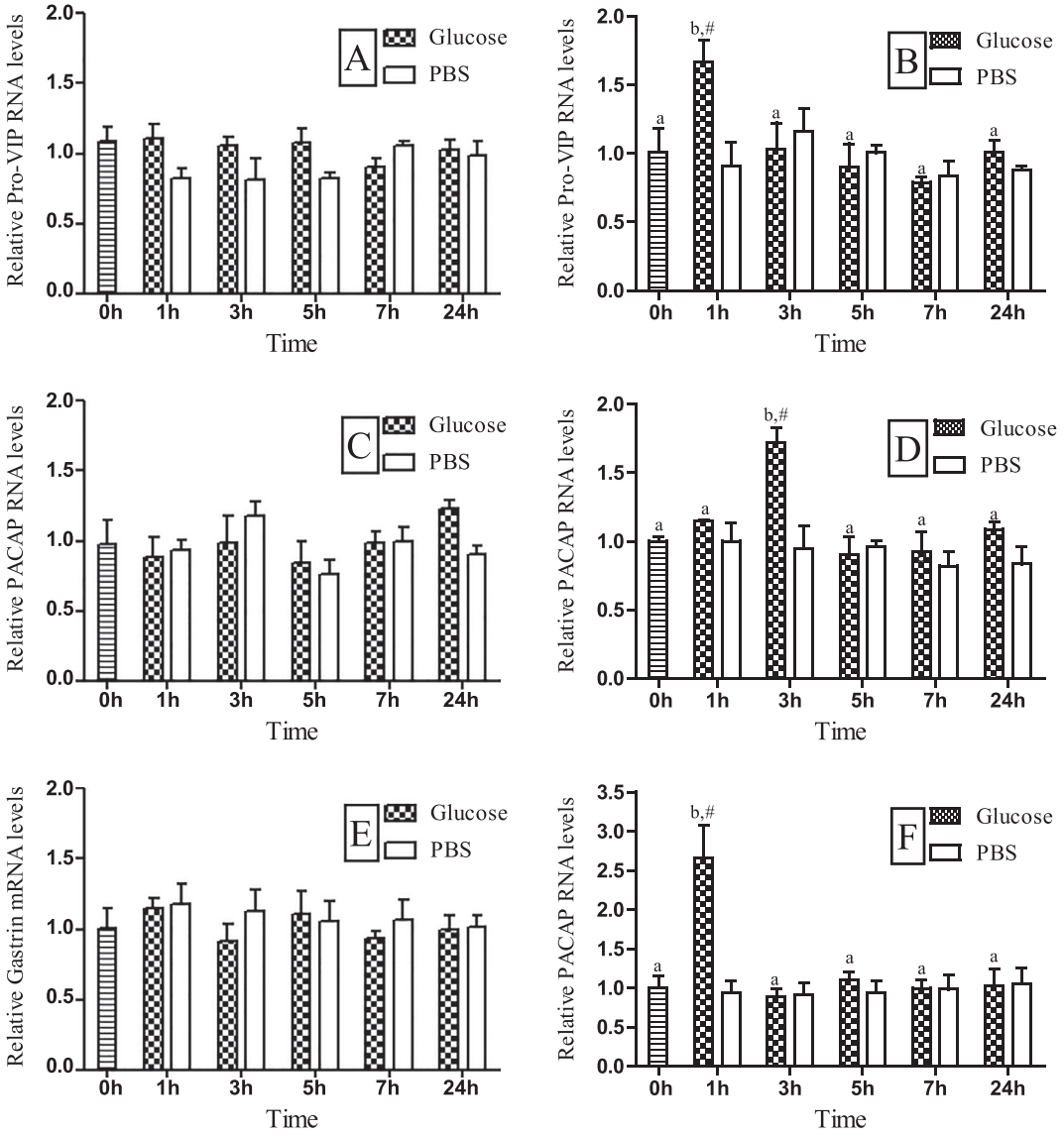

Fig. 3. The gene expression of preprovasoactive intestinal peptide (Pro-VIP), pituitary adenylate cyclase activating polypeptide (PACAP) and gastrin in the gut of Japanese Flounder after intraperitoneal (IP) (A, C, E) and oral (OR) (B, D, F) administration of glucose (Glu) or phosphate buffer solution (PBS). Each value was expressed as the Mean ± SE (n = 9). Different letters indicate significant differences among sampling times within glucose load. There was no significant difference for PBS administration. # means that significantly different from fish administration of glucose and PBS at the same time.

(Pro-VIP), PACAP and gastrin among all the time points after IP glucose (Fig. 3A, C, E). The expressions of these three genes were significantly higher at 1 h, 3 h and 1 h after OR glucose than that at 0 h, respectively (Fig. 3B, D, F). There were no significant differences between the rest time points and 0 h.

3.4. Glycogen in muscle and liver

The maximum muscle glycogen content was found at 5 h both in intraperitoneal and oral administration of glucose group. And then it decreased (Fig. 4A, B). The changes of liver glycogen

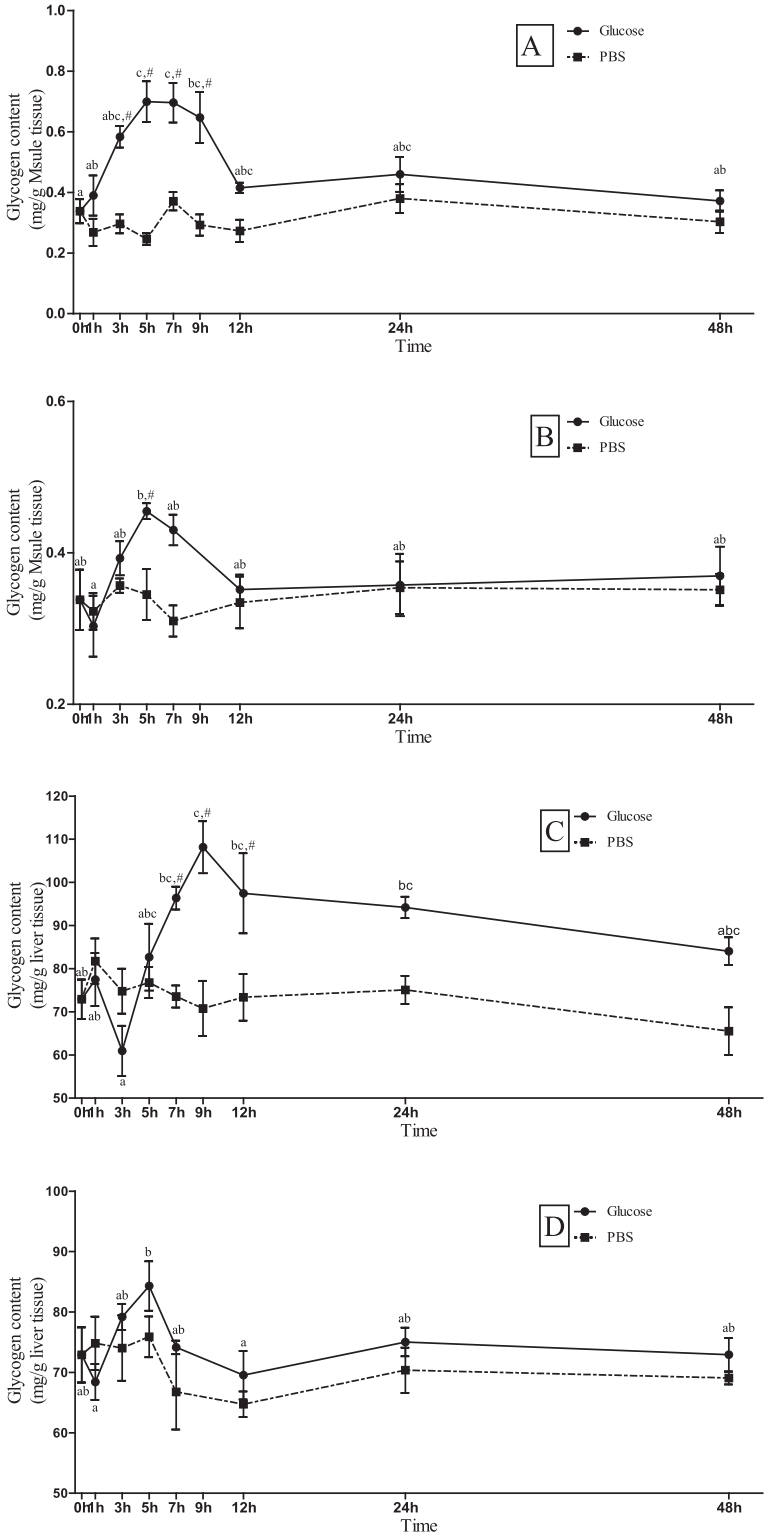

Fig. 4. The glycogen contents in muscle and liver of Japanese flounder after intraperitoneal (IP) (A, C) and oral (OR) (B, D) administration of glucose (Glu) or phosphate buffer solution (PBS). Each value was expressed as the Mean ± SE (n = 9). Different letters indicate significant differences among sampling times within glucose load. There was no significant difference for PBS administration. # means that significantly different from fish administration of glucose and PBS at the same time.

contents are shown in Fig. 4C and 4D. In the IP glucose group, liver glycogen content decreased at 3 h and increased after 5 h. And the significant highest value was found at 9 h (Fig. 4C). In the OR glucose group, the highest value of liver glycogen content was found at 5 h. By contrast, the liver glycogen contents in the PBS groups showed no significant differences among all the time points (Fig. 4D).

3.5. Activities of enzyme in glycolysis and gluconeogenesis in liver

Activity of GK showed a peak at 5 h (10.84 ± 0.68 mU·mg^{-1} protein) after IP glucose, and it was significantly higher than that at 0 h (7.46 ± 0.83 mU·mg^{-1} protein) (Fig. 5A). Activity of GK after OR glucose increased to a peak at 5 h (9.84 ± 0.87 mU·mg^{-1} protein), and it was significantly higher than that at 0 h (Fig. 5B). Meanwhile, the PFK activity was not increased significantly in both IP and OR groups (Fig. 5C, 5D). Pyruvate kinase activity showed a peak at 7 h (112.98 ± 9.75 mU.mg^{-1} protein) after IP glucose, and it was significantly higher than that at 0 h (72.05 ± 7.35 mU.mg^{-1} protein). The activity of pyruvate kinase after OR glucose increased to a peak at 7 h (115.46 ± 7.49 mU.mg-1 protein), and it was significantly higher than that at 0 h (Fig. 5E, F). The activities of FBPase showed a first increasing then decreasing trend in both intraperitoneal and oral glucose administration, peaking at 7 h (Fig. 5G, H).

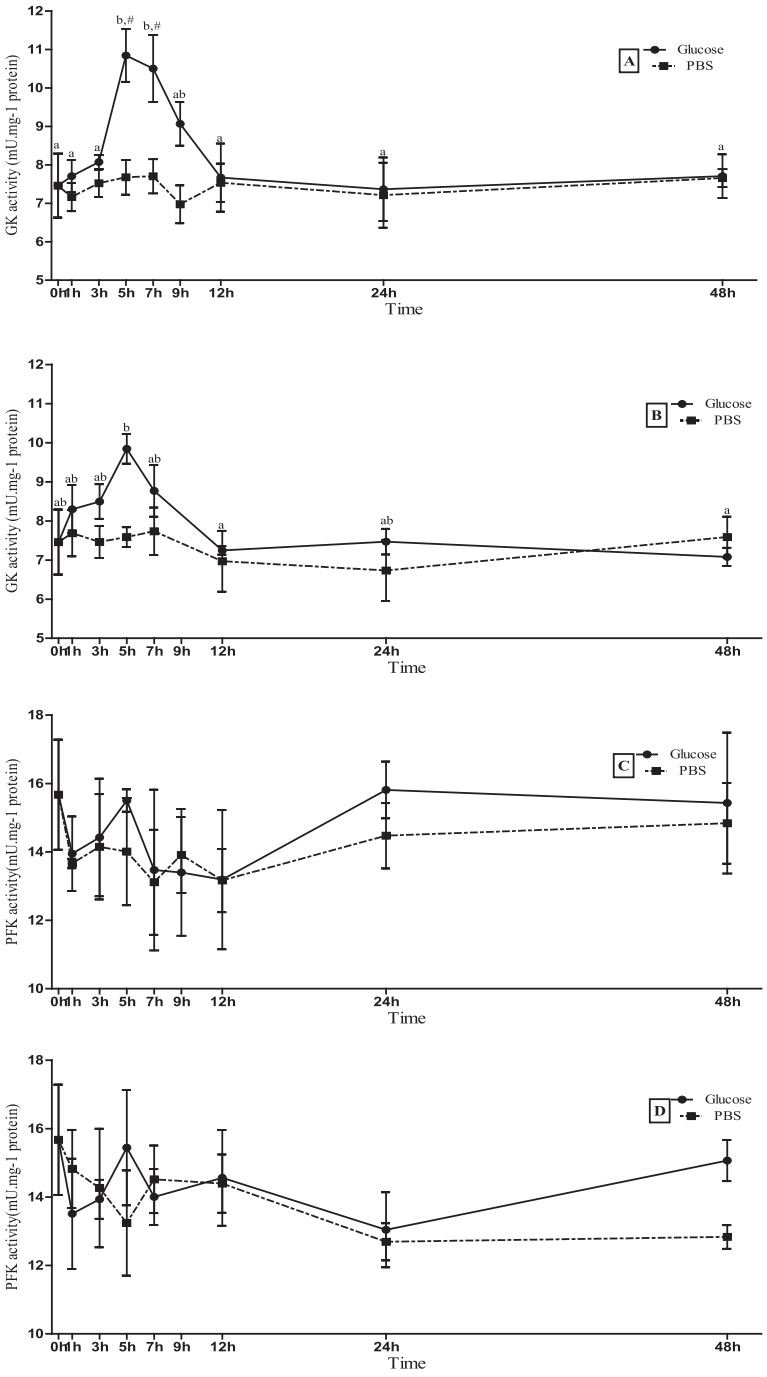

Fig. 5. The glucokinase (GK) (A, B), 6-phosphofructokinase-1 (PFK) (C, D), Pyruvate kinase (PK) (E, F) and fructose-1,6-bisphosphatase (FBPase) (G, H) activities in the liver of Japanese flounder after intraperitoneal administration (A, C, E, G) and oral administration (B, D, F, H) of glucose (Glu) or phosphate buffer solution (PBS). The data were separately analyzed in treatment group or control group. Each value is the mean ± SE (n = 9). Different letters indicate significant differences among sampling times within glucose load. There was no significant difference for PBS administration. # means that significantly different from fish administration of glucose and PBS at the same time.

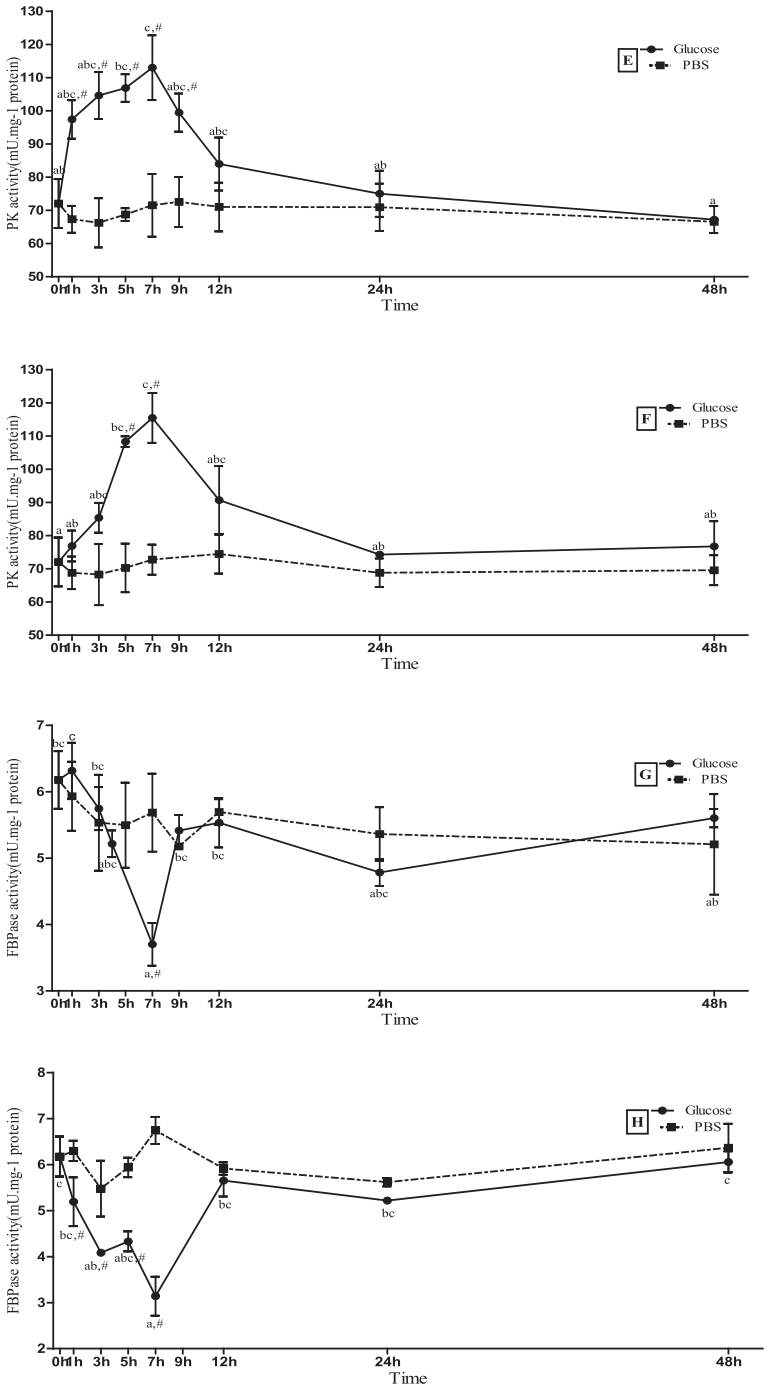

Fig. 5 (*continued*)

4. Discussion

In the present study, the PBS groups were used as the control. Data on the blood glucose and serum insulin concentrations in the PBS groups showed that the operation of injection or oral administration had no significant effects on the experimental animals in the present study.

The present study showed that the blood glucose content of Japanese flounder reached its peak (20.06 ± 1.92 mM) at 5 h after intraperitoneal injection of glucose (1g/kg) and the hyperglycemia lasted about 21 h. This was similar with the previous study on Australian snapper *Pagrus auratus*, in which the blood glucose reached to the peak at 3 h (18.9 mM) and the hyperglycemia lasted for about 18 h (Booth et al., 2006). However, following intraperitoneal injection with the same or even a higher dose of glucose, some omnivorous fish like tilapia and white sea bream, take 1–2 hours before reaching a peak in blood glucose levels and 6–9 hours to recover to basal level (Enes et al., 2012; Wright Jr et al., 1998). It was suggested that the omnivorous fish species had higher ability of blood glucose control than the carnivorous, such as Australian snapper and Japanese flounder. After oral administration of glucose (500 mg/ml, 1.67 g/kg), blood glucose of Japanese flounder peaked (1.90 ± 0.23 mM) at 3 h, and it returned to normal level (0.89 ± 0.04 mM) at 12 h, and duration of hyperglycemia is about 10 h. This is similar with the results in previous studies in carnivorous fish species, such as the black carp *Mylopharyngodon piceus* (Huang

et al., 2005) and grouper (Yang et al., 2012). In omnivorous fish, however, the blood glucose concentration reached the peak value (26.04 mM) at 3 h, and the hyperglycemia lasted about 7 h in carp after oral administration of glucose (1.67 g/kg) (Cai et al., 2003). After oral administration of glucose (1.67 g/kg) in grass carp *Ctenopharyngodon idellus*, a herbivorous fish, the highest blood glucose concentration reached at 3 h, and the hyperglycemia lasted about 6 h (Huang et al., 2005). It was suggested that the carnivorous fish had lower ability of blood glucose control than the omnivorous and herbivorous fish.

In the present study, the hyperglycemia lasted for about 10 h in oral administration of glucose group, and the highest concentration of blood glucose was 1.90 ± 0.32 mM. While in the injection of glucose group, the hyperglycemia lasted for about 21 h, and the peak value of blood glucose was 20.06 ± 2.72 mM. It was suggested that the Japanese flounder has stronger capacity in eliminating glycemia caused by oral administration of glucose than by the IP injection of glucose. This could be partly due to the following reasons. In OR glucose group, a portion of glucose could be absorbed by cells to provide energy when glucose enters gastrointestinal tract before it was absorbed into the bloodstream (Krogdahl et al., 2005). The unabsorbed portion was excreted with faeces. Glucose by the way of intraperitoneal injection was mostly absorbed into the bloodstream directly by the peritoneal capillary not intestine (Mackmull and Michels, 1932; Olson, 1996). In mammals, the intestine can be stimulated by high glucose, which in turn triggers the secretion of intestinal hormones activating a hypoglycemic mechanism, such as the incretin (Hayes et al., 2014). In the present study, serum insulin levels sharply declined in IP group, and were significantly increased in OR group at 3 h. This could be one of the reasons why the hyperglycemia lasted for a longer time in IP group (about 21 h) than in OR group (about 10 h). Glucose comes to the brain through blood circulation. Due to the high blood-brain barrier permeability of arcuate nucleus in the hypothalamus (Xu et al., 2016), after the stimulating of glycemia to brain, the gene expression of Pro-SS, NPY, Pro-CCK and Pro-OX was significantly different at 1 h, 3 h, 5 h and 1 h compared to 0 h, respectively. It has been suggested that the sensitivity of these hormones in the brain was different. This also shows that in the period of blood glucose raised by IP injection glucose, the hormones play different roles. Previous studies in mammals showed that SS and NPY can inhibit insulin secretion, and orexin can promote insulin secretion and enhance the capacity of glucose-stimulated insulin. In consideration of the function of these hormones, it was suggested that insulin declined at 3 h after IP injection glucose administration could be relevant to these hormones.

In mammals, SS, NPY, CCK, OX, VIP, PACAP and gastrin belong to the brain-gut peptide hormones (Dockray, 2009; Kirchgessner, 2002; Lazarczyk et al., 2003; Naito et al., 2001; Tatemoto et al., 1982) and they can be found in brain and intestines. The function of CCK in the gut is to control the release of pancreatic enzyme and gallbladder contraction. It acts as a neurotransmitter, which can control feeding, analgesia, blood pressure, memory, insulin release in the nervous system (Du et al., 2007). Pro-VIP and PACAP are mainly as neurotransmitter in brain. In the digestive system, they mainly act as gastrointestinal hormones, and promote insulin secretion (Filipsson et al., 1999; Xu et al., 2002; Zhang and Li, 2009).

In the present study, only the Pro-CCK, Pro-VIP and PACAP were found both in the brain and intestine. The expressions of Pro-SS and Pro-OX were only detected in the brain. And the expression of gastrin was only found in the gut. In a word, it was confirmed that the brain-gut peptides and their gene expression were detected in brain and gut. This could support the hypothesis of brain-intestine connection in Japanese flounder. The gene transcript of Pro-SS and NPY was up-regulated at 1 h and 3 h, respectively, after IP glucose. And the gene expression of Pro-OX was down-regulated at 1 h. That is to say the secretion of insulin was depressed in the first 3 h after IP glucose. After 3 h, the gene expression of Pro-CCK was significantly elevated, and the blood insulin level increased.

In the IP group, injected glucose did not enter into intestine but directly into abdominal cavity capillary, so there was no significant

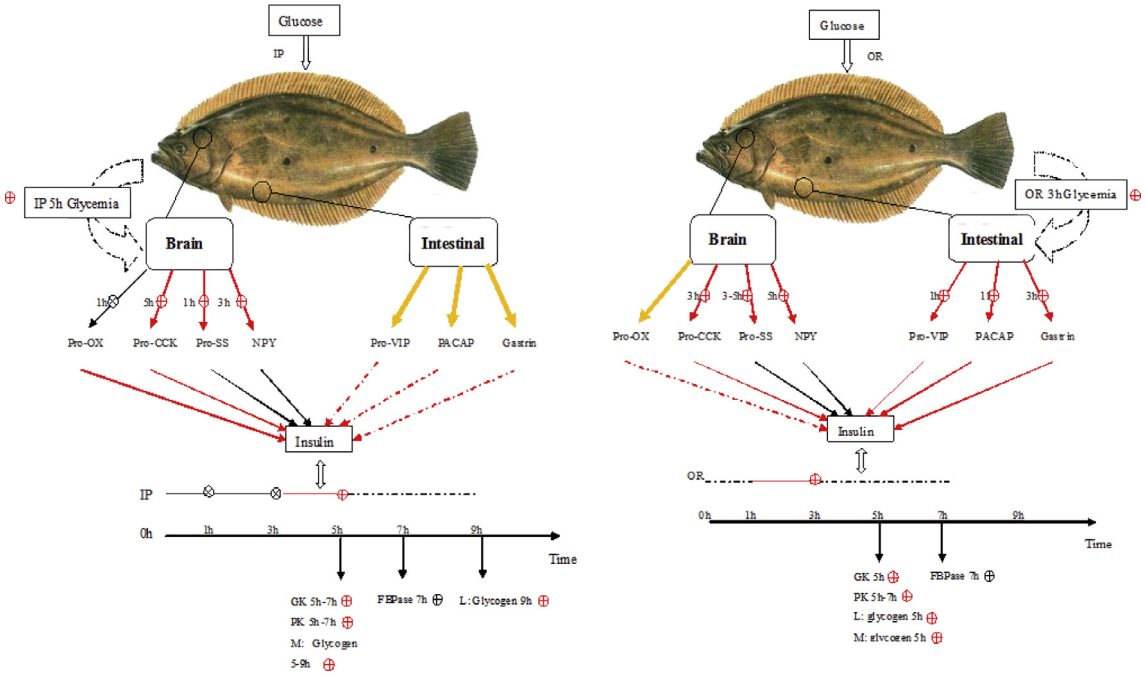

Fig. 6. Summary on the results of the present study. Regulations of glucose metabolism in brain and intestine were affected by intraperitoneal (IP) (A) and oral (OR) (B) administration of glucose. Red line stands for stimulation, and black line means inhibition. The red circle represents the significant promotion effects. The black circle represents the significant inhibition effects. Black dotted line means inhibition, but did not show significant difference. The yellow line stands for there is no significant difference in all time points. Point in time after glucose loads with red and black symbol means significant difference was found at that time point. L: liver; M: muscle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

difference in expression of gut hormone genes, such as Pro-VIP, PACAP and gastrin. However, in the OR group, glucose enters into gastrointestinal tract. After oral glucose administration, blood insulin increased at 1–3 h, meanwhile it begun to drop after 3 h. Pro-CCK expression runs up at 3 h. The differences in expression quantity of Pro-SS occurred at 3–5 h, while for NPY the difference appeared at 5 h. Before hyperglycemia appears, hormones in gut which can stimulate insulin secretion like VIP (Ahren and Lundquist, 1981), PACAP (Filipsson et al., 1999) and gastrin (Ahren and Lundquist, 1981) had high expression levels at 1 h, 1 h and 3 h, respectively. This suggests that the stimulation of gene expression of gastrointestinal hormones have an important effect on the secretion of insulin, which can control the blood glucose level. In mammals, these hormones include glucose-dependent insulinotropic polypeptides, glucagon-like peptide-1, gastrin, PACAP, VIP etc.

In the present study, there are different patterns of blood insulin levels changing with intraperitoneal injection and oral administration of glucose. Moreover, gene expressions of brain-gut peptides showed a temporal and spatial difference, and the peptides have the function of regulating insulin secretion in mammals. Therefore, it is further speculated that the oral administration of glucose could stimulate the brain-gut peptides (VIP, PACAP and gastrin) expression, which have effects on the insulin secretion and then regulate the blood glucose levels. However, in IP group, there is little chance to stimulate brain-gut peptide expression to influence the insulin secretion, which leads to a longer hyperglycemia.

In the present study, muscle glycogen content peaked at 5 h after IP or OR administration of glucose. Meanwhile, the liver glycogen peaked at 7 h in IP group, and 5 h in Org group. The blood glucose level peaked at 3 h in OR group and 5 h in IP group. That is to say the glycogen content peaked as the blood glucose level decreased. This suggests that the glycogen synthesis is a kind of strategy in Japanese flounder to reduce the hyperglycemia.

Glycolysis and gluconeogenesis mainly participate in the catabolism and synthesis of glucose, coordinating with each other to ensure the glucose homeostasis. As one of the hexokinase (HK) isoenzymes, GK is the initial enzyme of the glycolysis pathway. The present study showed that the high glucose load significantly increased the activity of GK. This is in agreement with previous findings in European sea bass (Enes et al., 2006), rainbow trout (Polakof et al., 2008) and turbot (Nie et al., 2013). Furthermore, the changing of GK activity was consistent with or relatively delayed to blood glucose level after IP injection glucose and oral glucose. The PK activity was significantly induced by IP and OR glucose. It peaked at 7 h, delayed to the time of blood glucose peak, which is at 5 h in IP group and 3 h in OR group. With regard to the FBPase activity, it continuously decreased during the first 7 h after IP or OR glucose, suggesting that before the blood glucose peaking and just after the peak the gluconeogenesis is decreased. This helps to lower the blood glucose level. This suggested that Japanese flounder has a definite adaptability to high loaded glucose. All the results are summarized in a Fig. 6.

Acknowledgments

This study was financially supported by the National Natural Science Foundation of China (No. 31572628), the Fundamental Research Funds for the Central Universities of Ocean University of China (No. 201562017), and the Key R&D Program of Shandong Province, China (2016CYJS04A01, 2017CXGC0105).

References

Aguilar, A.J., 2010. Central leptin treatment modulates brain glucosensing function and peripheral energy metabolism of rainbow trout. Peptides 31, 1044–1054.
Ahren, B., Lundquist, I., 1981. Effects of vasoactive intestinal polypeptide (VIP), secretin and gastrin on insulin secretion in the mouse. Diabetologia 20, 54–59.
Booth, M.A., Anderson, A.J., Allan, G.L., 2006. Investigation of the nutritional requirements of Australian snapper Pagrus auratus (Bloch & Schneider 1801): digestibility of gelatinized wheat starch and clearance of an intra-peritoneal injection of d-glucose. Aquac. Res. 37, 975–985.
Borrebaek, B., Christophersen, B., 2000. Hepatic glucose phosphorylating activities in perch (Perca fluviatilis) after different dietary treatments. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 125, 387–393.
Bredkjoer, H., Palle, C., Ekblad, E., Fahrenkrug, J., Ottesen, B., 1997. PreproVIP-derived peptides in the human female genital tract: expression and biological function. Neuropeptides. 31, 209–215.
Cai, C., Liu, Y., Chen, L., Song, X., Wu, P., 2003. Metabolic responses of allogynogenetic gbel carp after oral administration of different doses of glucose. Acta Hydrobiol. Sin. 27, 602–606.
Cerdá-Reverter, J.M., Sorbera, L.A., Carrillo, M., Zanuy, S., 1999. Energetic dependence of NPY-induced LH secretion in a teleost fish (Dicentrarchus labrax). Am. J. Physiol.-Regul. Integr. Compar. Physiol. 277, R1627–R1634.
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.
Dockray, G.J., 2009. Cholecystokinin and gut–brain signalling. Regul. Peptides 155, 6–10.
Du, J., Zhang, L.F., Qin, C., 2007. Progress on the Function of Cholecystokinin. Chin. J. Compar. Med. 17, 233–236.
Eilertson, C.D., O'connor, P.K., Sheridan, M.A., 1991. Somatostatin-14 and somatostatin-25 stimulate glycogenolysis in rainbow trout, Oncorhynchus mykiss, liver incubated in vitro: a systemic role for somatostatins. Gen. Comp. Endocrinol. 82, 192–196.
Eilertson, C.D., Sheridan, M.A., 1993. Differential effects of somatostatin-14 and somatostatin-25 on carbohydrate and lipid metabolism in rainbow trout Oncorhynchus mykiss. Gen. Comp. Endocrinol. 92, 62–70.
Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, 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., Peres, H., Pousao-Ferreira, P., Sanchez-Gurmaches, J., Navarro, I., Gutiérrez, J., Oliva-Teles, A., 2012. Glycemic and insulin responses in white sea bream Diplodus sargus, after intraperitoneal administration of glucose. Fish Physiol. Biochem. 38, 645–652.
Filipsson, K., Sundler, F., Ahrén, B., 1999. PACAP is an islet neuropeptide which contributes to glucose-stimulated insulin secretion. Biochem. Biophys. Res. Commun. 256, 664–667.
Hayes, M.R., Mietlicki-Baase, E.G., Kanoski, S.E., De Jonghe, B.C., 2014. Incretins and amylin: neuroendocrine communication between the gut, pancreas, and brain in control of food intake and blood glucose. Annu. Rev. Nutr. 34, 237–260.
Huang, H.Z., Ding, L., Song, X.H., Wang, Y., Yang, C., 2005. Comparative research on glucose tolerance between black carp Mylopharyngodon piceus and grass carp Ctenopharyngodon idellus. J. Fishery Sci. China 12, 496–500.
Kirchgessner, A.L., 2002. Orexins in the brain-gut axis. Endocr. Rev. 23, 1–15.
Krogdahl, Å., Hemre, G.I., Mommsen, T., 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. Aquac. Nutr. 11, 103–122.
Lazarczyk, M.A., Lazarczyk, M., Grzela, T., 2003. Ghrelin: a recently discovered gut-brain peptide. Int. J. Mol. Med. 12, 279–287.
Lee, S.-M., Kim, K.-D., Lall, S.P., 2003. Utilization of glucose, maltose, dextrin and cellulose by juvenile flounder (Paralichthys olivaceus). Aquaculture 221, 427–438.
Lin, J.H., Ho, L.T., Shiau, S.Y., 1995. Plasma glucose and insulin concentration in tilapia after oral administration of glucose and starch. Fisheries Sci. 61, 986–988.
Liu, H.Y., Mao, Y.B., Tan, B.P., Dong, X.H., Yang, Q.H., Chi, S.Y., Chen, L.Q., 2015. Effects of different dietary carbohydrate levels on growth and glucose tolerance ability in fishes of different feeding habits. J. Fisheries China 39, 1852–1862.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.
Luo, Y.P., Xie, X., 2010. Progress of carbohydrate utilization in fish. J. Fishery Sci. China 17, 381–390.
Mackmull, G., Michels, N.A., 1932. Absorption of colloidal carbon from the peritoneal cavity in the teleost. Tautogolabrus adspersus. Dev. Dyn. 51, 3–47.
May, A.A., Liu, M., Woods, S.C., Begg, D.P., 2016. CCK increases the transport of insulin into the brain. Physiol. Behav. 165, 392–397.
Naito, T., Itoh, H., Nagano, T., TAKEYAMA, M., 2001. Effects of Ninjin-to on levels of brain-gut peptides (motilin, vasoactive intestinal peptide, gastrin, and somatostatin) in human plasma. Biol. Pharm. Bull. 24, 194–196.
Nie, Q., Miao, M.H., Chen, S.Y., Li, C.Y., Zhang, W.B., Mai, K.S., 2013. Effects of dietary carbohydrate sources and levels on the activities of carbohydrate metabolic enzymes in turbot Scophthalmus maximus L. Acta Hydrobiol Sin. 37, 425–433.
Nie, Q., Miao, H., Miao, S., Zhou, H.H., Zhang, Y., Zhang, W.B., Mai, K.S., 2015. Effects of dietary glucose and dextrin on activity and gene expression of glucokinase and fructose-1, 6-bisphosphatase in liver of turbot Scophthalmus maximus. Fish Physiol. Biochem. 41, 819–832.
Olson, K.R., 1996. Secondary circulation in fish: anatomical organization and physiological significance. J. Exp. Zool. Part A: Ecol. Genet. Physiol. 275, 172–185.

Olsvik, P.A., Lie, K.K., Jordal, A.E.O., Nilsen, T.O., Hordvik, I., 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol. Biol. 6, 1.

Panserat, S., Médale, F., Blin, C., Breque, J., Vachot, C., Plagnes-Juan, E., Gomes, E., Krishnamoorthy, R., Kaushik, S., 2000. Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. Am. J. Physiol.-Regul. Integr. Compar. Physiol. 278, R1164–R1170.

Park, J.H., Shim, H.M., Na, A.Y., Bae, J.H., Im, S.S., Song, D.K., 2015. Orexin A regulates plasma insulin and leptin levels in a time-dependent manner following a glucose load in mice. Diabetologia 58, 1542–1550.

Pearse, A., Takor, T., 1979. Embryology of the diffuse neuroendocrine system and its relationship to the common peptides. Federation Proc. 38, 2288–2294.

Pereira, R., Costa, L., Oliveira, I., Araújo, J., Aerts, M., Vigliano, F., Rosa, P., 2015. Relative distribution of gastrin-, CCK-8-, NPY-and CGRP-immunoreactive cells in the digestive tract of dorado (Salminus brasiliensis). Tissue Cell 47, 123–131.

Peres, H., Goncalves, P., Oliva-Teles, A., 1999. Glucose tolerance in gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax). Aquaculture 179, 415–423.

Polakof, S., Míguez, J.M., Soengas, J.L., 2008. Dietary carbohydrates induce changes in glucosensing capacity and food intake of rainbow trout. Am. J. Physiol.-Regul. Integr. Compar. Physiol. 295, R478–R489.

Polakof, S., Miguez, J.M., Soengas, J.L., 2011. Cholecystokinin impact on rainbow trout glucose homeostasis: possible involvement of central glucosensors. Regul. Pept. 172, 23–29.

Romijn, J.A., Corssmit, E.P., Havekes, L.M., Pijl, H., 2008. Gut–brain axis. Curr. Opin. Clin. Nutr. Metabolic Care 11, 518–521.

Sheridan, M.A., Plisetskaya, E.M., Bern, H.A., Gorbman, A., 1987. Effects of somatostatin-25 and urotensin II on lipid and carbohydrate metabolism of coho salmon, Oncorhynchus kisutch. . Gen. Compar. Endocrinol. 66, 405–414.

Soengas, J.L., Aldegunde, M., 2002. Energy metabolism of fish brain. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 131, 271–296.

Tatemoto, K., Carlquist, M., Mutt, V., 1982. Neuropeptide Y—a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 296, 659–660.

Vaudry, D., Gonzalez, B.J., Basille, M., Yon, L., Fournier, A., Vaudry, H., 2000. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacological reviews. 52, 269–324.

Very, N., Knutson, D., Kittilson, J., Sheridan, M., 2001. Somatostatin inhibits growth of rainbow trout. J. Fish Biol. 59, 157–165.

Washburn, B.S., Bruss, M.L., Avery, E.H., Freedland, R.A., 1992. Effects of estrogen on whole animal and tissue glucose use in female and male rainbow trout. Am. J. Physiol.-Regul. Integr. Compar. Physiol. 263, R1241–R1247.

Wood, J., 1996. Enteric nervous system in pathogenesis of gastrointestinal motility disorders. Chin. Natl. J. New. Gastroenterol. 2, 26–31.

Wright Jr, J.R., O'Hali, W., Yang, H., Han, X.-X., Bonen, A., 1998. GLUT-4 deficiency and severe peripheral resistance to insulin in the teleost fish tilapia. Gen. Comp. Endocrinol. 111, 20–27.

Xu, B., Zhang, Y., Du, J., 2016. Progress in the study of the blood-brain barrier. Acta physiologica Sinic. 68, 306–322.

Xu, W., Zhao, Y., Kong, J., 2002. Progress in Vasoactive Intestinal Peptide. Pharm. Biotechnol. 9, 364–368.

Yang, W., Ye, J., Wang, K., Sun, Y., Zhang, C., Zhai, S., Song, K., 2012. Glucose tolerance in grouper (*Epinphelus coioides*). Acta Hydrobiol. Sin. 36, 563–568.

Yue, S.M., Zhou, A., Wang, A., 2007. Research advance of pituitary adenylate cyclase activating polypeptide. Feed Ind. 28, 12–16.

Zhang, S.C., 2001. Hypothesis and Implication of Brain Iitestine Connection. Chin. J. Basic Med. Traditional Chin. Med. 8, 6–7.

Zhang, Y., Li, P., 2009. The Research Progress of Vasoactive Intestinal Peptide. Med. Recapitulate 15, 3235–3238.