A negative feedback mechanism in the insulin-regulated glucose homeostasis in Japanese flounder *Paralichthys olivaceus* by two ways of glucose administration

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List of symbols and abbreviations	
IP	intraperitoneal
OR	oral
Pro-SS	prosomatostatin
Pro-CCK	cholecystokinin precursor
Pro-OX	orexin precursor
OX	orexin
NPY	neuropeptide Y
Pro-VIP	preprovasoactive intestinal peptide
VIP	vasoactive intestinal peptide
PACAP	pituitary adenylate cyclase activating polypeptide
GK	glucokinase
FBPase	fructose-1,6-bisphosphatase
PBS	phosphate buffer solution
qPCR	quantitative RT-PCR

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 (IP) injection and oral (OR) administration of glucose. Samples were collected at 0, 1, 3, 5, 7, 9, 12, 24 and 48h after IP and OR, respectively. Results showed that the hyperglycemia lasted 5 hours and 21 hours in OR and IP group, respectively. The serum insulin concentration significantly decreased (1.58±0.21mIU/L) at 3h after IP glucose. However, it significantly increased at 3h (3.37±0.34mIU/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 the intestine. When the blood glucose concentration peaked in both IP and OR group, the glucokinase expression in liver was stimulated, but the expression of fructose-1,6-bisphosphatase was depressed. 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. A negative feedback mechanism in the insulin-regulated glucose homeostasis was suggested in Japanese flounder. Furthermore, this regulation could be conducted by activating PI_3k-Akt, and then lead to the pathway downstream changes in glycolysis and gluconeogenesis.

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

50 day due to their limited ability in using glucose efficiently (Aguilar, Conde-Sieira et al.

51 2010). They rely more on lipid and protein for energy purposes. Fish has an oxygen

52 consumption rate one-tenth that of mammals. It was also found that the ability of

53 insulin secretion stimulating by dietary amino acids is stronger than that of glucose

54 (Mommsen and Plisetskaya 1991). Meanwhile, the ability of glucose phosphorylation

55 and glucose transport is weak in fish (Cowey and Walton 1989).

56 Although glucose is not the main energy substrate for carnivorous fish species,

57 glucose metabolism is important for the function of specific tissues in fish, such as

58 brain (Soengas and Aldegunde 2002). The brain has the highest glucose utilization

59 rates per unit mass in all tissues examined in rainbow trout (Washburn, Bruss et al.

60 1992). In mammals, the brain is responsible for neuroregulation, meanwhile, gut cells

61 perceive nutrients. The brain and gut have complicated connections in nutrients,

62 hormone as well as nerve, thus a gut–brain axis has been generated (Romijn, Corssmit

63 et al. 2008). Pearse and Takor (Pearse and Takor 1979) pointed out that gastrointestinal

64 peptide secreting cells and peptidergic neurons in the brain is common in

65 embryogenesis originated from the neuroectoderm. Some researches put forward the

66 concept of enteric nervous system, and it has close ties to brain systems (Wood 1996).

67 As the discovery of brain-gut peptides, researchers come up with the hypothesis of

68 brain-intestinal connection (Zhang 2001). Although the function of the gut–brain axis

69 is not completely understood yet in fish, the brain-peptides like gastrin (Pereira, Costa

70 et al. 2015), cholecystokinin (Polakof, Mǵuez et al. 2011) and somatostatin (SS)

71 (Sheridan, Plisetskaya et al. 1987) has been demonstrated to play a crucial role in

72 glucose homeostasis.

73 Somatostatin promotes glycogen breakdown and the release of glucose from liver

74 to plasma (Eilertson, O'connor et al. 1991). And it can also inhibit hormones secretion,

75 including insulin (Sheridan, Plisetskaya et al. 1987, Eilertson and Sheridan 1993, Very,

76 Knutson et al. 2001). Neuropeptide Y (NPY) is also involved in regulating insulin and

77 glucose metabolism in fish. It was found that plasma insulin levels decreased in fasted

78 European sea bass co-injected with NPY plus glucose, but remained stable when NPY

79 was administrated alone to fed and fasted animals (Cerdá-Reverter, Sorbera et al.

3

80   1999). Mammalian studies showed that cholecystokinin (CCK) can stimulate insulin

81   secretion and it can also make influence on β cell proliferation (May, Liu et al. 2016).

82   The CCK promotes the transport of insulin into the central nervous system (CNS) of

83   rats (Hermansen 1984). The Orexin (OX) is involved in glucose and lipid metabolism

84   in mammals. Oerxin-A can promote insulin secretion, and it also enhances the capacity

85   of glucose stimulated insulin (Park, Shim et al. 2015). In addition, the function of

86   vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating

87   polypeptide (PACAP) are very similar. It mainly manifested in animal reproduction,

88   nutrient digestion and energy balance. It deserves to be mentioned that these two

89   hormones can stimulate insulin secretion (Bredkjoer, Palle et al. 1997, Vaudry,

90   Gonzalez et al. 2000). Gastrin has the similar function to VIP/PACAP in glucose

91   metabolism, and it can also stimulate insulin production (ShuangmingYue 2007). In

92   regard to the glucose metabolism in mammalians, the relationship between the

93   hormones mentioned above was reviewed in Figure 1. However, little related

94   information was reported in fish.

95   Japanese flounder *Paralichthys olivaceus* is a typical carnivorous fish species.

96   The aim of the present study is to preliminarily investigate the brain-gut connection,

97   and the roles of insulin and the related hormones in regulation of the glucose

98   homeostasis in Japanese flounder loaded glucose by oral administration and

99   intraperitoneal injection.

100

101   **2. Materials and Methods**

102   2.1. Experimental animals

103   Japanese flounders (body weight: 225±50 g) were provided by a local fish farm in

104   Haiyang, Shangdong Province, China. They were randomly distributed into cylindrical

105   fiberglass tanks (300-L) in a re-circulating water system. During the 4-week

106   acclimation, Japanese flounders were fed with commercial feeds (Qingdao Great

107   Bio-tech Co., Ltd) to satiation twice daily. All animal care and handling procedures

108   were approved by the Animal Care Committee of Ocean University of China.

109   2.2. Intraperitoneal injection

110   There were two intraperitoneal injection (IP) groups. The one was IP injected with

111   phosphate buffer solution (PBS), and the other one was with glucose. There were eight

112   tanks per injection group, and eight fish per tank. Before injection, Japanese flounders

113   were fasted for 48 hours to eliminate the effect of residual food in the digestive tract,

114   and then were anaesthetized with MS-222 (50mg l^{-1}). After being weighed, fish were

115   IP injected with 2 ml glucose/kg body weight. The glucose was purchased from Sigma

116   (500mg/ml, Sigma, USA). Meanwhile, the other group of animal was injected with the

117   same volume PBS (0.01mol/L) as administration glucose group. The operations of

118   injection for each tank were completed within 5 minutes. After injection, Japanese

119   flounders were put back into the tanks. Samples of blood, brain, intestinal and liver

120   were collected just before (time 0h) and 1 h, 3 h, 5 h, 7 h, 9 h, 12 h, 24 h and 48 h after

121   injection. Blood was collected by 1ml syringes from caudal vein. Serum was obtained

122   after centrifugation (10,000g for 10 min at 4 ℃) of blood that allow to stay overnight

123   at 4 ℃. All samples were frozen in liquid nitrogen immediately and stored at -80 ℃.

124   2.3. Oral administration

125   There were two oral administration groups, and eight tanks per group, eight fish

126   per tank. The one group received glucose (500mg/ml, Sigma, USA) with 3.34ml/Kg

127   body mass. The other group received the same volume of PBS (0.01mol/L) and was

128   used as the control. After oral (OR) administration, Japanese flounders were put back

129   into the tanks. Samples of blood, brain, intestinal and liver were collected just before

130   (time 0h), and 1 h, 3 h, 5 h, 7 h, 9 h, 12 h, 24 h and 48 h after OR. These samples were

131   collected and stored as described above.

132   2.4. Analysis of glucose and insulin concentrations in serum

133   Concentration of glucose in serum was determined with the automatic

134   biochemical analyzer (Hitachi, 7600-210, Japan). Concentration of insulin in serum

135   was analyzed with commercial kits (Nanjing Jiancheng Bioengineering Institute,

136   Nanjing, China).

137   2.5. Analysis of glycogen contents

138   The glycogen concentrations in liver were determined using the anthrone

139   chromogenic method with commercial kits (Nanjing Jiancheng Bioengineering

140 Institute, Nanjing, China). They were measured with the UV spectrophotometer

141 (UV-2401PC, Shimadzu, Kyoto, Japan).

142 2.6. Tissue distribution of the selected brain-gut peptides

143   Tissue distributions of the selected brain gut peptides were detected in stomach,

144 gill, spleen, muscle, brain, liver, kidney, intestine and eye by semi-quantitative

145 RT-PCR. Specific primers for peptides and β-actin (reference gene) were shown in

146 Table 1. Isolation of the total RNA and synthesis of the first strand cDNA were carried

147 out following the instructions of the kit (Trizol and PrimeScript Reverse Transcriptase,

148 Takara, Japan). The PCR reaction system consisted of 1μl of cDNA, 12.5μl 2×

149 EsTaqMsterMix (CWbiotech, China), 9.5μl of EDPC water and 1μl of each primer

150 (10μM). The PCR amplification program is pre-denatured at 94 ℃ for 5 min, followed

151 by 30 cycles of denaturation at 94 ℃ for 30s, annealing at 58 ℃ for 30 s, extension at

152 72 ℃ for 1 min 30 s and post-extension at 72 ℃ for 5 min. PCR products were

153 selected by 1.2% agarose gel electrophoresis.

154 2.7. Gene expression analysis by real-time quantitative RT-PCR

155   Gene expression levels were determined by real-time quantitative RT-PCR

156 (q-PCR) using the iCycler iQ™ (Bio-Rad,Hercules, CA, USA). Analyses were using

157 SYBR Green I (CWbiotech, China) according to the manufacturer's instructions. The

158 total reaction volume was 25μl, including 1.0μl cDNA, 12.5μl 2× UltraSYBR Mixture,

159 1μl each of gene-specific primer (10μM, Table2) and 9.5μl DEPC water. Thermal

160 cycling was initiated with incubation at 95 ℃ for 10min using hot-start iTaq™ DNA

161 polymerase activation, 40steps of PCR were performed, each one consisting of heating

162 at 95 ℃, 10s for denaturing, and at specific annealing and extension temperatures is

163 Tm for 30s, 72℃ for 32s. Following the final PCR cycle, melting curves were

164 systematically monitored (58 ℃ temperature gradient at 0.5 ℃/s from 58 to 95 ℃) to

165 ensure that only one fragment was amplified. Relative quantification of the target gene

166 transcript was done using β-actin gene expression as reference (Olsvik, Lie et al. 2005)

167 which was stably expressed in this experiment. Samples without reverse transcriptase

168 and samples without RNA were run for each reaction as negative controls. Relative

169 quantification of the genes were calculated using "$2^{-\Delta\Delta Ct}$" meth (Livak and Schmittgen

6

170 2001) with β-actin as reference gene. All real-time Q-PCR was performed in triplicate

171 biological replicates.

172   The effects of glucose administration on the expression of hormone (peptides)

173 genes were detected by comparing the 0, 1, 3, 5, 7, and 24 h after intraperitoneal

174 injection and oral glucose administration, respectively. Hepatic glucose

175 metabolism-related genes were analyzed by comparing the 0 h, 5h and 24 h in the

176 treatment of intraperitoneal injection of glucose, and 0 h, 3 h and 12 h in the oral

177 glucose administration.

178 2.8. Statistics analysis

179   All data were expressed as means ± standard error and performed using SPSS

180 17.0. A one-way analysis of variance (ANOVA) was used to compare the differences

181 in relative brain-gut peptides gene expression over time. When overall differences were

182 considered statistically significant at $P < 0.05$, Tukey's test was used to compare the

183 means among individual treatments.

184

185 **3. Results**

186 3.1. Concentrations of glucose and insulin in serum

187   Concentrations of glucose in serum reached the peak (20.06±1.92mM) at 5h after

188 the IP injection of glucose, which was about 28 times as high as that at 0h

189 (0.71±0.25mM) (Figure 2A). From the 5h to 24h after injection, the blood glucose

190 decreased to 7.40±5.47mM. There was no significant difference in blood glucose

191 between the time point of 0h and 24h (Figure 2A). After injection of glucose, the

192 insulin concentration in serum was decreased to the lowest value (1.58±0.21mIU/L) at

193 3h (Figure 2B). After that, it grew gradually to the normal value at 24h

194 (3.03±0.006mIU/L) as that at 0h (3.20±0.18 mIU/L).

195   After oral administration of glucose, blood glucose concentration had rapidly

196 increased at 3h (1.90±0.23mM), and it was higher than those at the other time points.

197 After the 3rd hour, the blood glucose concentration decreased gradually (Figure 2C).

198 The serum insulin concentration increased from 2.36±0.21mIU/L (0h) to

199 3.37±0.34mIU/L (3h). It returned to the normal level after 5h, and then, kept relatively

200 stable (Figure 2D).

201 3.2. Tissue distribution of peptides genes

202 Results of the gene distribution and expression are shown in Figure 3 and Table 1.

203 The relative expressions of prosomatostatin (Pro-SS) and orexin precursor (Pro-OX) in

204 brain were relatively higher than that in the other analyzed tissues including stomach,

205 gill, spleen, muscle, liver, kidney, intestine and eyes. Gastrin was mainly expressed in

206 intestine (Figure 3). NPY, CCK precursor (Pro-CCK), Preprovasoactive intestinal

207 peptide (Pro-VIP) and PACAP were detected in many tissues (Figure 3). Relative

208 expression of NPY had the highest value in brain, and followed by the eye, gill and

209 spleen. Relative expression of Pro-CCK was up to maximum in brain, followed by the

210 intestine and stomach. The expression of Pro-VIP was relatively high in intestine, but

211 low in brain, stomach, gill, spleen and eyes. The PACAP had the highest expression in

212 intestine, then brain.

213 3.3. Gene expressions after glucose administration

214 3.3.1. Gene expression of the Pro-SS, NPY, Pro-CCK and Pro-OX in brain

215 Results of the gene expression in brain after glucose administration are shown in

216 Figure 4.

217 One hour after the intraperitoneal injection of glucose, mRNA levels of the

218 Pro-SS were significantly increased, then it was not significantly differed from that at

219 0h at the others time points. In the group of IP PBS, there were no significant different

220 in mRNA levels of Pro-SS among all the time points (Fig.4A).

221 After oral glucose administration, Pro-SS mRNA levels were significantly

222 up-regulated at the time point of 3h and 5h (Fig.4B). The NPY mRNA levels were

223 significantly increased only at 3h after IP and 5h after OR glucose (Fig.4C, 4D). The

224 cholecystokinin precursor (Pro-CCK) mRNA levels were significantly increased only

225 at 5h after IP and 3h after OR glucose (Fig. 4E, 4F). The Pro-OX mRNA levels

226 significantly decreased at all the time points after IP glucose. However, there were no

227 significant differences among all the time points after OR glucose (Fig.4G, 4H).

228 3.3.2. Gene expression of Pro-VIP, PACAP and gastrin in intestine

229 Results of the gene expression in intestine after glucose administration are shown

8

230   in Figure 5.

231   There were no significant differences in gene expressions of preprovasoactive

232   intestinal peptide (Pro-VIP), PACAP and gastrin among all the time points after IP

233   glucose.

234   There was only one time point after OR glucose at which that the expressions of

235   these three genes were significantly higher than that at 0h. The time point was 1h, 3h

236   and 1h, respectively. There were no significant differences between the rest time points

237   and 0h.

238   3.4. Heatmap of glucose affected brain-gut peptides gene expression

239   The expression of brain-gut peptides gene in brain and intestines of Japanese

240   flounder by different ways of glucose administration was analyzed on heatmap (Fig.6).

241   As shown in Figure 6, it consists of two treatment groups: intraperitoneal injection

242   glucose (1g/Kg) and oral glucose administration (1.67/Kg). The results show that in

243   intraperitoneal injection glucose (1g/Kg), the gene expressions of Pro-SS, NPY,

244   Pro-CCK were increased at 1h, 3h, 5h compared to 0h respectively, and Pro-OX

245   expression was declined after 1h. In oral glucose group (1.67g/Kg), Pro-SS, NPY,

246   Pro-CCK expression increased at 3-5h, 5h and 3h respectively, and the mRNA levels of

247   Pro-VIP, PACAP, Gastrin were promoted at 1h, 3h and 1h, respectively.

248   3.5. Gene expression of glucokinase (GK) and fructose-1,6-bisphosphatase (FBPase)

249   in liver

250   Results of the gene expression of GK and FBPase in liver after glucose

251   administration are shown in Figure 7. The fish showed the highest GK mRNA level

252   after IP and OR glucose at 5h and 3h, respectively (Fig.7A, 7B). The FBPase mRNA

253   level significantly decreased at 5h and 3h after IP and OR glucose, respectively. And

254   then increased at 24h and 12h, respectively, and had no significant differences with that

255   at 0h.

256   3.6. Glycogen in liver

257   The changes of liver glycogen contents are shown in Figure8. In the IP glucose

258   group, liver glycogen content decreased at 3h and increased after 5h. And the

259   significant highest value was found at 9h. In the OR glucose group, the highest value

9

260 of liver glycogen content was found at 5 h. By contrast, the liver glycogen contents in

261 the PBS groups showed no significant differences among all the time points.

262

263 **4. Discussion**

264 *4.1 Blood glucose levels changed by different ways of glucose administration*

265 The present study showed that the blood glucose content of Japanese flounder

266 reached its peak (20.06±1.92mM) at 5h after intraperitoneal injection of glucose

267 (500mg/ml, 1g/Kg) and hyperglycemia lasted about 21 hours. This was similar with the

268 previous study on Australian snapper *Pagrus auratus*, in which the blood glucose

269 reached to the peak at 3h（18.9mM）and the hyperglycemia lasted for about 18 hours

270 (Booth, Anderson et al. 2006). However, after intraperitoneal injected same or even a

271 higher dose of glucose, some omnivorous fish like tilapia, white sea bream, spends

272 from 1-2 hours reaching to the peak in the concentration of blood glucose and 6-9

273 hours recovering as usual (Wright Jr, O'Hali et al. 1998, Enes, Peres et al. 2012). It was

274 suggested that the omnivorous fish species had higher ability of blood glucose control

275 than the carnivorous. After oral administration of glucose (500mg/ml，1.67g/kg), blood

276 glucose of Japanese flounder peaked (1.90±0.23mM) at 3h, and it returned to normal

277 level (0.89±0.04mM) at 7h. Duration of hyperglycemia is about 5 hours. These results

278 are similar to those previous researches in carnivorous grouper (1.67g/Kg) (Yang, Ye et

279 al. 2012). As carnivorous fish, however, the duration of hyperglycemia is about 10

280 hours and 36 hours in black carp *Mylopharyngodon piceus* and Chinook salmon

281 *Oncorhynchus tshawytscha* after oral administration of glucose (1.67g/Kg),

282 respectively. It was suggested that in carnivorous fishes, glucose tolerance significantly

283 differed in species. In addition to the species difference, the culture environment and

284 conditions could also be part of the reasons. As an omnivorous fish, after oral

285 administration of glucose (1.67g/Kg) in allognogenetic gibel carp, the blood glucose

286 concentration reached the peak value (26.04mM) at 3h, and the hyperglycemia lasted

287 about 7 hours (Ying 2003). As a herbivorous fish, after oral administration of glucose

288 (1.67g/Kg) in grass carp *Ctenopharyngodon idellus*, the highest blood glucose

289 concentration reached at 3h, and the hyperglycemia lasted about 6 hours (Huang, Ding

290   et al. 2005). It was suggested that the carnivorous fish had lower ability of blood

291   glucose control than the omnivorous and herbivorous.

292   In the present study, the hyperglycemia lasted 5 hours in oral administration of

293   glucose group, and the highest concentration of blood glucose was 1.90 ± 0.32mM.

294   While in the IP injection of glucose group, the hyperglycemia lasted 21 hours, and the

295   peak value of blood glucose was 20.06 ± 2.72mM. It was suggested that the Japanese

296   flounder has stronger capacity in eliminating glycaemia caused by oral administration

297   of glucose than by the IP injection of glucose. This could be partly due to the following

298   reasons. In OR glucose group, a portion of glucose could be consumed when glucose

299   enters gastrointestinal tract. While the left portion of glucose was absorbed into

300   bloodstream. If high glucose loads in this experiment caused pathology, oral glucose

301   may likely discharge to the vitro through digestive tract. Glucose by the way of

302   intraperitoneal injection was mostly absorbed into the bloodstream directly by the

303   peritoneal capillary.

304   In IP injection glucose, the content of insulin declined as blood glucose rose

305   (3h-24h), from 0h to 3h. After the third hour, the insulin did not recover at the original

306   value at all. However, in oral glucose administration, as the blood glucose going up

307   (1h- 7h), the insulin was growing as well (1h-5h). Therefore, the different tendency of

308   insulin is also the sound reason of short last of hyperglycemia in oral glucose

309   administration.

310

311   *4.2 Serum insulin changed by different ways of glucose administration*

312   In the present study, serum insulin level sharply declined in intraperitoneal

313   injection of glucose group, and then increased. The results are similar to the previous

314   study in rainbow trout with IP injection of glucose (Harmon, Eilertson et al. 1991) and

315   grouper *Epinphelus Coioides* by OR administration of glucose (Yang, Ye et al. 2012).

316   In Japanese flounder, IP glucose was mostly absorbed in the bloodstream through

317   the peritoneal capillary. This leads to the continuous rise of blood glucose. Glucose

318   comes to the brain through blood circulation. Due to the arcuate nucleus in the

319   hypothalamus of the blood-brain barrier permeability being high, after the stimulating

11

320 of glycemia to brain, the gene expression of Pro-SS, NPY, Pro-CCK and Pro-OX was

321 significantly different at 1h, 3h, 5h and 1h compared to 0h, respectively. It was

322 suggested that the sensitivity of these hormones in the brain was different. This also

323 shows that in the period of blood glucose raised by IP injection glucose, the hormones

324 play different roles. Combining the function of these hormones in mammal, it was

325 suggested that insulin declined at 3h after IP injection glucose administration could be

326 relevant to these hormones.

327 The gene transcript of Pro-SS and NPY was up-regulated at 1h and 3h,

328 respectively after IP glucose. The function of the SS and NPY is to inhibit the secretion

329 of insulin. Pro-OX expression was down-regulated at 1h. The function of OX is to

330 enhance insulin production which is stimulated by glucose (Nowak, Maćkowiak et al.

331 1999). That is to say the secretion of insulin was depressed in the first 3 hours after IP

332 glucose administration. After 3h of IP glucose, insulin levels rose. Simultaneously, the

333 gene expression of Pro-CCK significantly elevated. The CCK function is to stimulate

334 insulin secretion (Hermansen 1984). After intraperitoneal injection, glucose didn't

335 enter into intestinal but directly into abdominal cavity capillary, so there was no

336 significant difference in expression of gut hormone genes, such as Pro-VIP, PACAP

337 and gastrin (Fig 9A).

338 In OR glucose group, glucose enters into gastrointestinal tract, while parts of

339 glucose were consumed by cells and other parts of glucose were absorbed into the

340 bloodstream. After oral glucose administration, insulin increased at 1-3h, meanwhile it

341 begun to drop after 3h. Pro-CCK expression which could promote the secretion of

342 insulin runs up at 3h. The differences of expression quantity of Pro-SS occurred at 3-5h,

343 while for NPY the difference appeared at 5h. Both SS and NPY can inhibit the

344 secretion of insulin in mammals. Before high glycemia appears, hormones in gut which

345 can stimulate insulin secretion like VIP (Ahren and Lundquist 1981), PACAP

346 (Filipsson, Sundler et al. 1999), gastrin (Ahren and Lundquist 1981) were high in

347 expression at 1h, 1h and 3h, respectively. In mammals, when food enters into intestine,

348 intestinal mucosal cells can secrete a variety of gastrointestinal hormones, which have

349 an important effect on the secretion of insulin. These hormones include

12

350 glucose-dependent insulinotropic polypeptides, glucagon-like peptide-1, gastrin, etc.

351 As the intestinal signals, it can stimulate insulin secretion before glycemia appears to

352 align the body absorbed nutrient.

353 In mammals, SS，NPY，CCK，OX，VIP，PACAP and gastrin belong to the

354 brain-gut peptide hormones, and they can be found in the brain and intestines. In the

355 present study, only the Pro-CCK, Pro-VIP and PACAP were found both in the brain

356 and intestine. The expressions of Pro-SS and Pro-OX were only detected in the brain.

357 And the expression of gastrin was only found in the gut. This could be due to the less

358 secretion of these hormones. Anyway, it was confirmed that the brain-gut peptides and

359 their gene expression were detected in brain and gut. This could support the hypothesis

360 of brain-intestine connection in Japanese flounder. In mammals, the function of CCK

361 in the gut is to control the release of pancreatic enzyme and gallbladder contraction.

362 It acts as a neurotransmitter, which can control feeding, analgesia, blood pressure,

363 memory, insulin release in the nervous system (Du Jing 2007). Pro-VIP and PACAP

364 are mainly as neurotransmitter in brain. In the digestive system, they mainly act as

365 gastrointestinal hormones, and promote insulin secretion (Filipsson, Sundler et al. 1999,

366 Xu Wei 2002, Zhang Yang 2009).

367 The reason could be that in the early stage of glucose administration, the blood

368 glucose increased sharply. It could cost large number of insulin original in the body. At

369 the same time, the insulin secretion stimulating by glucose administration could be less

370 than the costing. So the serum insulin concentration declined sharply.

371 The insulin content in group of IP glucose as a whole tends to increase after 3h,

372 but it does not exceed value at 0h. The result, with the value higher than that at 0h, is

373 different from that in OR group. This may in that the apoptosis of β-cell leads to the

374 decrease of the function of insulin

375 In my experiments, serum insulin will increase with blood glucose rise. Therefore,

376 we can get a conclusion that insulin changes are the result of a combination of blood

377 glucose and brain-gut peptides.

378

379 *4.3 Metabolism responses of the glycolysis and gluconeogenesis in liver*

13

380   Glycolysis and gluconeogenesis mainly participate in the catabolism and synthesis
381   of carbohydrate, coordinating with each other to ensure the glucose homeostasis of fish
382   (Nie Q, 2013). As one of the hexokinase (HK) isoenzymes, GK is the initial enzyme of
383   the glycolytic pathway, and the FBPase is one of the key enzymes in the
384   gluconeogenesis pathway. In the present study, the gene expression of GK was
385   significantly enhanced both after IP injection and OR glucose. Similar results were
386   found in turbot (Nie, Miao et al. 2015), gilthead sea bream (Metón, Caseras et al. 2004)
387   and common carp (Panserat, Rideau et al. 2014). Compared to the GK, the gene
388   expression of FBPase was significantly decreased in the treatment of intraperitoneal
389   injection at 5 h. Meanwhile, the similar results were found in the group of oral glucose.
390   Researches in European sea bass found that dietary starch (10% to 30%) could not
391   affect the FBPase activity and gene expression (Moreira, Peres et al. 2008). In
392   omnivores like common carp, Panserat, Plagnes-Juan et al. (2002) also reported that
393   there are no significant differences in FBPase gene expression between the fish fed
394   with carbohydrates or not. The result suggested that the gene expression was
395   irrespective to the carbohydrate intake. Furthermore, (Fernández, Miquel et al. 2007) it
396   also found that dietary cornstarch (5% to 26%) could not affect the FBPase gene
397   expression in gilthead sea bream, whereas Panserat, Plagnes-Juan et al. (2002) found
398   that the gene expression of FBPase significantly decreased after fed dietary
399   carbohydrate (20%).

400   Researches in rainbow trout, tilapia, European sea bass and gilthead sea bream
401   showed that the liver glycogen content significantly increased when fed dietary
402   carbohydrates (Mazur, Higgs et al. 1992, Shiau and Liang 1995, Couto, Enes et al.
403   2008, Moreira, Peres et al. 2008). In the present study, the reduction in liver glycogen
404   content during the first 3 hours after injection or 1 hour after oral glucose might be
405   explained by the effect of glycogenolysis in fish. To some degree, this was similar to
406   the previous finding in turbot (Garcia-Riera and Hemer 1996). Peres, Goncalves et al.
407   (1999) found that liver glycogen content of seabream significantly decreased during
408   the first hour after glucose injection, while the liver glycogen content of seabass
409   increased significantly. In present study, IP glucose group, liver glycogen content

14

410 increased after 5h. And the significant highest value was found at 9h. In the OR

411 glucose group, the highest value of liver glycogen content was found at 5 h. The liver

412 glycogen content reached their maximum behind the occurrence of blood glucose peak.

413 This is an evidence for Japanese flounder to turn glucose into glycogen. It could be a

414 strategy to ease the stress of high blood glucose in Japanese flounder.

415 In the present study, when the blood glucose concentration peaked in both IP and

416 OR group, the GK was stimulated and the FBPase was depressed. This could be the

417 responses to decrease the hyperglycemia. In the IP glucose group, liver glycogen

418 content decreased at the 3h, then went up at the 5h, peaked at the 9h. In the OR glucose

419 group, the highest value of liver glycogen content was found at the 5h. It is obviously

420 that the time of glycogen synthesis and reaching peak was later than those of

421 hyperglycemia. It is suggested that organism reduces blood glucose by glycogen

422 synthesis.

423 The function of insulin in glucose metabolize is through insulin receptor

424 substrate-1 (IRS-1), activation of PI_3K, and consequent Akt phosphorylation, further

425 phosphorylate downstream signaling protein, such as AS160，mTORC1、FoxO1 and

426 GSK3. phosphorylation of AS160 is required for GLUT4 translocation and it can

427 promote tissue uptake glucose (Sano, Kane et al. 2003). mTORC1 activation is

428 sufficient to stimulate glycolysis, the oxidative arm of the pentose phosphate pathway,

429 and promote the decomposition of glucose (Düvel, Yecies et al. 2010). Insulin levels

430 increase upon feeding and signal through Akt to suppress Foxo1 by phosphorylation

431 and exclusion from the nucleus. gluconeogenesis genes are regulated by Foxo1 (Gross,

432 Wan et al. 2009). Akt phosphorylates and inactivates GSK-3 increased GLUT1 levels

433 and in enhanced glucose uptake through these high-affinity transporters. (Buller,

434 Loberg et al. 2008) Insulin signal pathway also promote glycogen synthesis by

435 inactivates GSK-3 though phosphorylates Akt (Lochhead, Coghlan et al. 2001).

436 In conclusion, in the present study, brain-gut peptides were confirmed in the

437 present study. And the serum insulin and the brain-gut peptides have different

438 responses between the IP and OR administration of glucose. A negative feedback

439 mechanism in the insulin-regulated glucose homeostasis was suggested in Japanese

440 flounder. Furthermore, this regulation could be conducted by activating PI3k-Akt, and

441 then lead to the pathway downstream changes in glycolysis and gluconeogenesis. This

442 supposition was expressed in Figure 10.

443

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449

450 **Competing interests**

451 The authors declare no competing or financial interests.

452

453 **Author contributions**

454 W.B.Z. and K.S.M. designed the experiment and revised the manuscript. D.L.,

455 D.D.H., B.Y.G, K.Y.D., Z.X.G., M.X.Y. and W.X. completed the experiment and

456 analyzed the data. D.L. and D.D.H. prepared the manuscript. All authors were involved

457 in the discussion of experimental data.

458

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663

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

	Pro-CCK	NPY	Pro-VIP	PACAP
Eye	0.06 ± 0.02^a	24.42 ± 0.33^e	1.01 ± 0.19^b	1.04 ± 0.39^b
Stomach	1.08 ± 0.33^b	0.32 ± 0.03^{ab}	4.19 ± 0.61^c	0.02 ± 0.00^a
Gill	0.01 ± 0.00^a	4.87 ± 1.72^{cd}	1.19 ± 0.03^b	0.01 ± 0.00^a
Spleen	0.00 ± 0.00^a	5.90 ± 1.20^d	1.03 ± 0.01^b	0.01 ± 0.00^a
Muscle	0.00 ± 0.00^a	1.13 ± 0.08^{ab}	0.02 ± 0.01^a	0.00 ± 0.00^a
Brain	24.17 ± 5.65^d	36.82 ± 3.32^f	8.38 ± 1.01^c	8.47 ± 0.77^c
Liver	0.03 ± 0.02^a	1.54 ± 1.02^{ab}	0.22 ± 0.24^a	0.01 ± 0.01^a
Kidney	0.00 ± 0.00^a	0.04 ± 0.01^a	$0.03 \pm 0.01a$	0.00 ± 0.00^a
Intestine	5.01 ± 0.69^c	2.18 ± 0.72^{bc}	$14.54 \pm 1.44d$	17.81 ± 2.77^d

24

668 **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
GK	CTCGGAGCTGCTGAAGACA	CAGAACACCCAGGGATGAC	115	60	1.002
FBPase	CGGTGAGTTCATCTTGGTG	TCCTCTGGGTATTTCTTCTT	135	60	0.970
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

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

670

671 **Figure legends**

672

673   **Figure 1** The relationship between different hormones in mammalians. Red line

674 stands for promotion, and black line means inhibition. Somatostatin (SS), neuropeptide

675 Y (NPY), cholecystokinin (CCK), vasoactive intestinal peptide (VIP), pituitary

676 adenylate cyclase activating polypeptide (PACAP)

677   References: 1(Lloyd, Maxwell et al. 1994), 2 (Deavall, Raychowdhury et al.

678 2000), 3(Li, Grinevich et al. 1996), 4(Voisin, Rouet-Benzineb et al. 2003), 5(May and

679 Braas 1995), 6(Hermansen 1984), 7(Hermansen 1984), 8(Fu, Acuna-Goycolea et al.

680 2004), 9(Alberti, Christensen et al. 1973), 10(Björkqvist, Bernsand et al. 2005),

681 11(Nowak, Maćkowiak et al. 1999), 12(Wang and Leibowitz 1997), 13(Moltz and

682 McDonald 1985), 14(Ahren and Lundquist 1981), 15(Filipsson, Sundler et al. 1999),

683 16(Ahren and Lundquist 1981).

684   **Figure 2** The concentration of blood glucose and serum insulin in Japanese

685 flounder after intraperitoneal (IP) (A, B) and oral (OR) (C, D) administrationof glucose

686 or phosphate buffer solution (PBS). Each value is expressed as the Mean $\pm$ SE (n=3).

687 Values sharing a common superscript letter were not significantly different.

688   **Figure 3** Tissue distributions of brain-gut peptides genes. L: ladder, 1: stomach, 2:

689 gill, 3: spleen, 4: muscle, 5: brain, 6: liver, 7: kidney, 8: intestine, 9: eyes, N: negative

690 control. Expression of housekeeping gene β-actin was observed to ensure the integrity

691 of the cDNA template of each tissue sample. Prosomatostatin (Pro-SS),

692 cholecystokinin precursor (Pro-CCK), neuropeptide Y (NPY), orexin precursor

693 (Pro-OX), pituitary adenylate cyclase activating polypeptide (PACAP),

694 preprovasoactive intestinal peptide (Pro-VIP)

695   **Figure 4** Gene expressions of prosomatostatin (Pro-SS), neuropeptide Y (NPY),

696 cholecystokinin precursor (Pro-CCK) and orexin precursor (Pro-OX) in the brain of

697 Japanese Flounder after intraperitoneal (IP) (A, C, E, G) and oral (OR) (B, D, F, H)

698 administration of glucose (Glu) or phosphate buffer solution (PBS). Each value is

699 expressed as the Mean $\pm$ SE (n=3). Values sharing a common superscript letter were

700 not significantly different.

701   **Figure 5** The gene expression of preprovasoactive intestinal peptide (Pro-VIP),

702 pituitary adenylate cyclase activating polypeptide (PACAP) and gastrin in the gut of

703 Japanese Flounder after intraperitoneal (IP) (A, C, E) and oral (OR) (B, D, F)

704 administration of glucose (Glu) or phosphate buffer solution (PBS). Each value was

705 expressed as the Mean ± SE (n=3). Values sharing a common superscript letter were

706 not significantly different.

707   **Figure 6** Visualizing gene expression of prosomatostatin (Pro-SS), neuropeptide

708 Y (NPY), cholecystokinin precursor (Pro-CCK), orexin precursor (Pro-OX),

709 preprovasoactive intestinal peptide (Pro-VIP), pituitary adenylate cyclase activating

710 polypeptide (PACAP) and gastrin on heatmap

711   Note: It consists of two treatment groups: intraperitoneal injection glucose (1g/Kg)

712 and oral glucose administration (1.67/Kg). In this figure, the graphical presentation of

713 data, numerical values are displayed by colors.

714   **Figure 7** The gene expression of glucokinase (GK) and

715 fructose-1,6-bisphosphatase (FBPase) in the liver of Japanese Flounder after

716 intraperitoneal (IP) (A, C) and oral (OR) (B, D) administration of glucose (Glu) or

717 phosphate buffer solution (PBS). Each value was expressed as the Mean ± SE (n=3).

718 Values sharing a common superscript letter were not significantly different.

719   **Figure 8** The glycogen contents in liver of Japanese flounder after intraperitoneal

720 (IP) (A) and oral (OR) (B) administration of glucose or phosphate buffer solution

721 (PBS). Each value was expressed as the Mean ± SE (n=3). Values sharing a common

722 superscript letter were not significantly different.

723   **Figure 9** Summary on the results of the present study. Regulations of glucose

724 metabolism in brain and intestine were affected by intraperitoneal (IP) (A) and oral

725 (OR) (B) administration of glucose. Red line stands for stimulation, and black line

726 means inhibition. The red circle represents the significant promotion effects. The black

727 circle represents the significant inhibition effects. Black dotted line means inhibition,

728 but did not show significant difference. The yellow line stands for there is no

729 significant difference in all time points. Point in time after glucose loads with red and

730 black symbol means significant difference ($P<0.05$) was found at that time point.

27

731   **Figure 10** A supposed negative feedback regulation mechanism of glucose

732   homeostasis controlled by insulin and brain-gut peptides. The insulin could decrease

733   the blood glucose level of Japanese flounder by activating PI3k-Akt, and then it could

734   also cause the pathway downstream changes in glycolysis, gluconeogenesis and

735   glycogenesis. The dashed and dashed boxes represent the relationships that exist in

736   mammals, which may exist in Japanese flounder.

737

Figure 1

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747 **Figure 2**

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Pro-SS — 117bp

Pro-CCK — 105bp

NPY — 99 bp

Pro-Orexin — 127bp

Gastrin — 101bp

PACAP — 143bp

Pro-VIP — 116bp

β -Actin — 155bp

1 2 3 4 5 6 7 8 9 L N
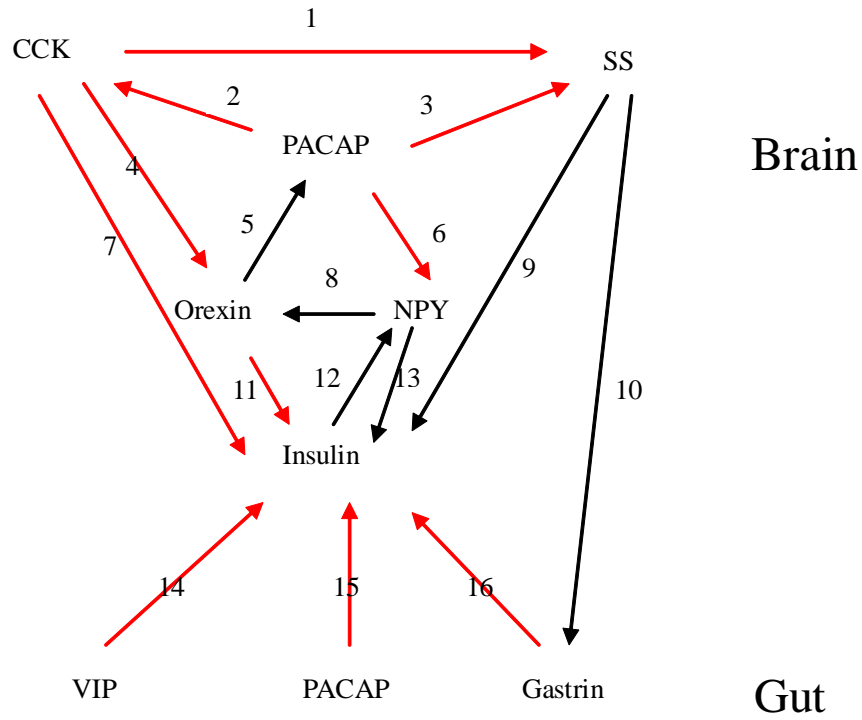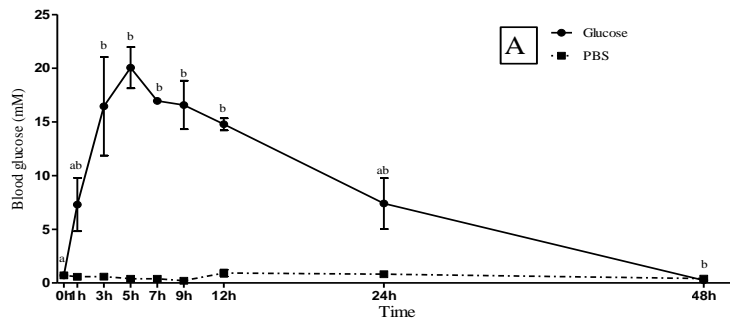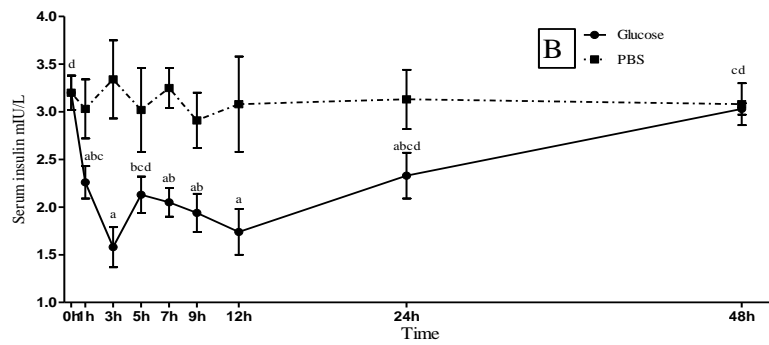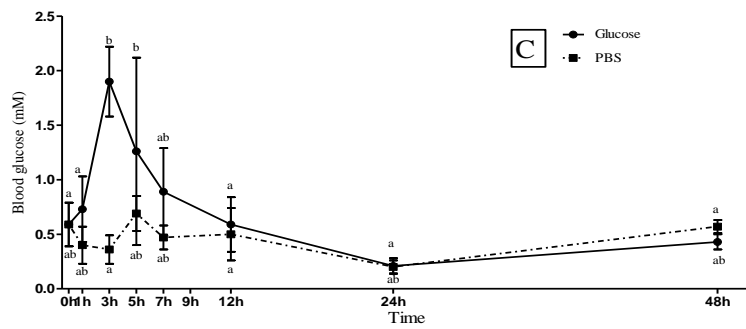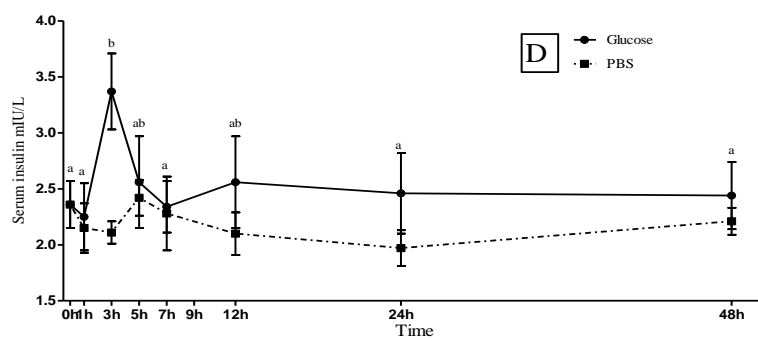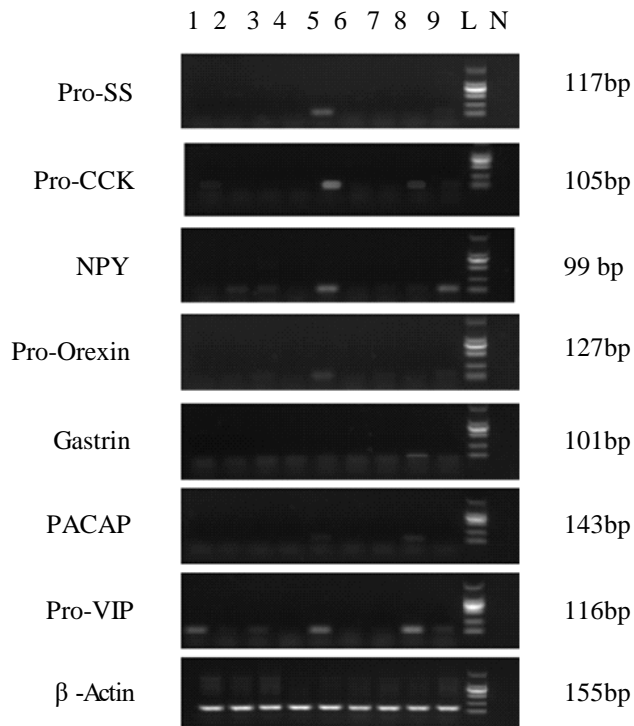
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752 **Figure 3**

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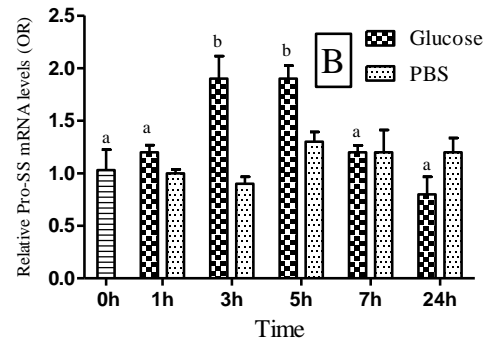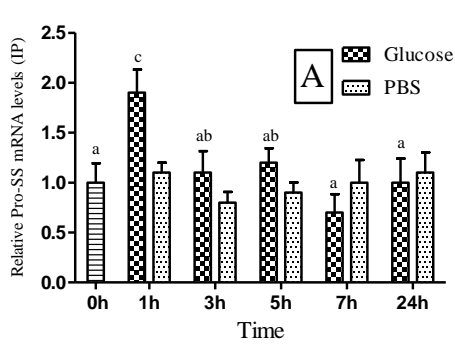
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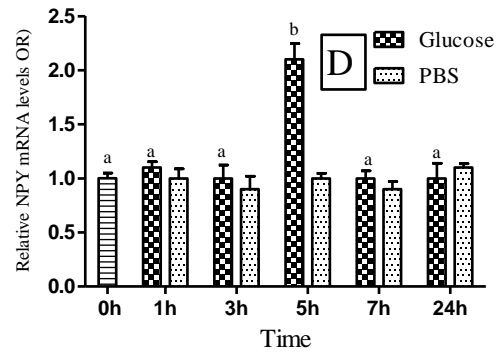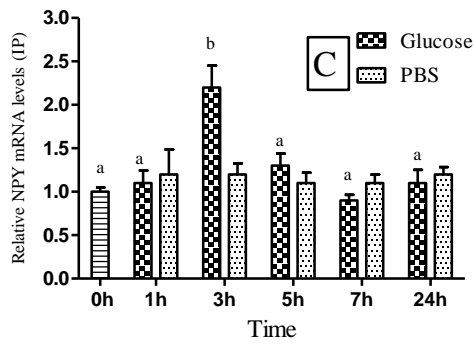
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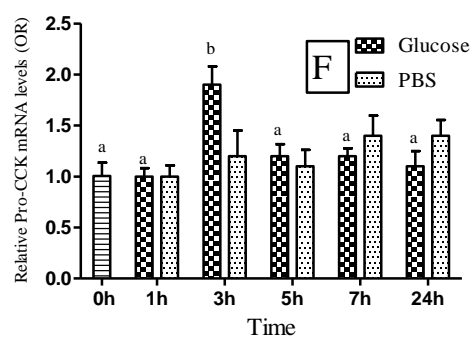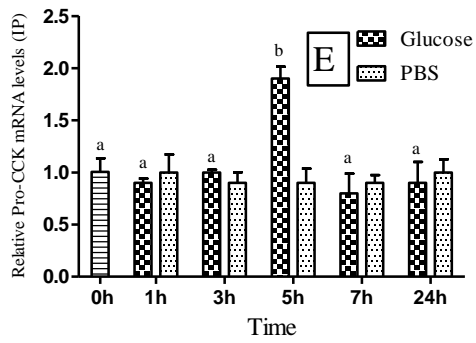
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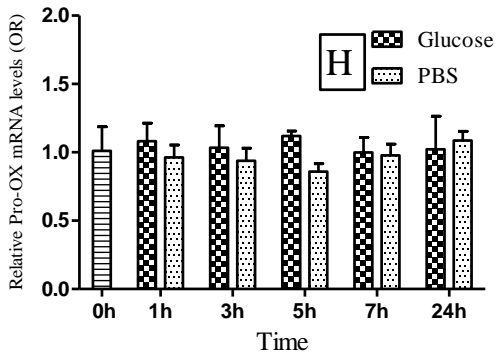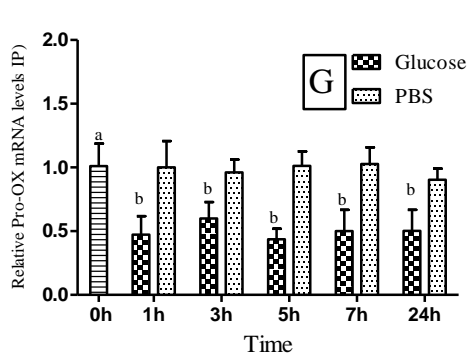
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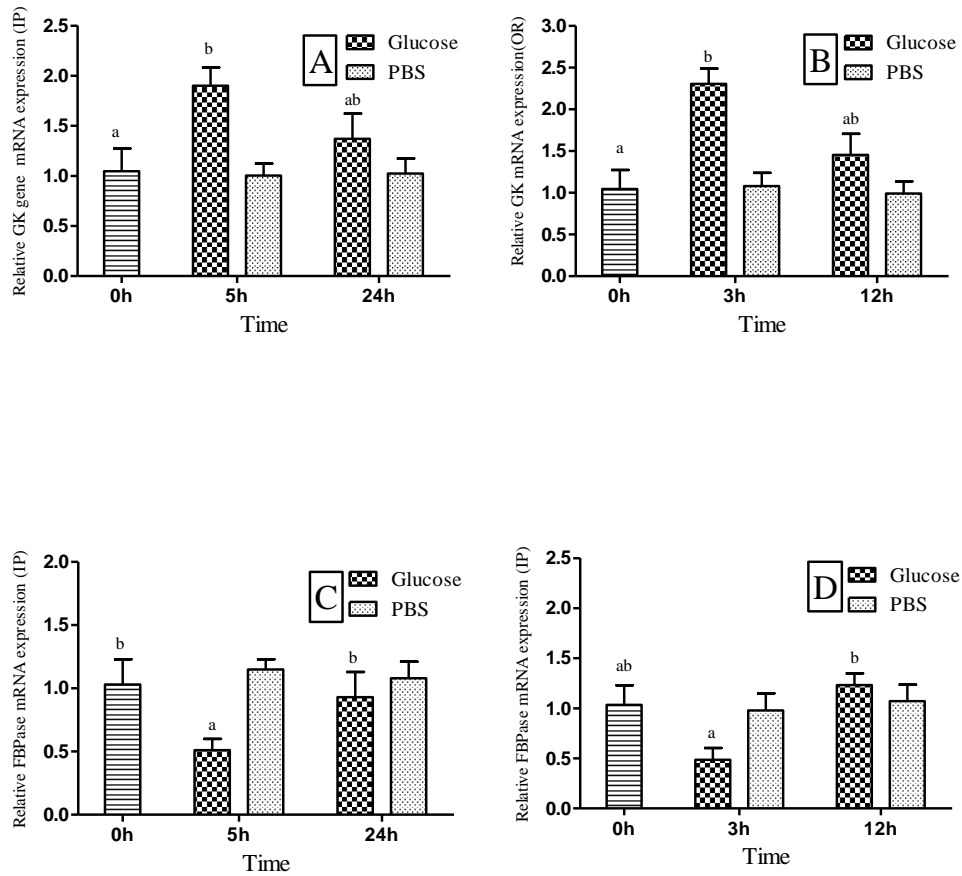
759 **Figure 4**

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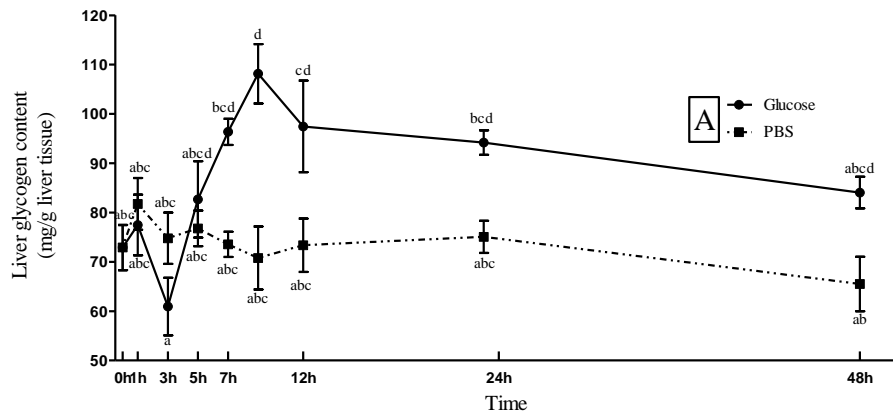762   Figure 5

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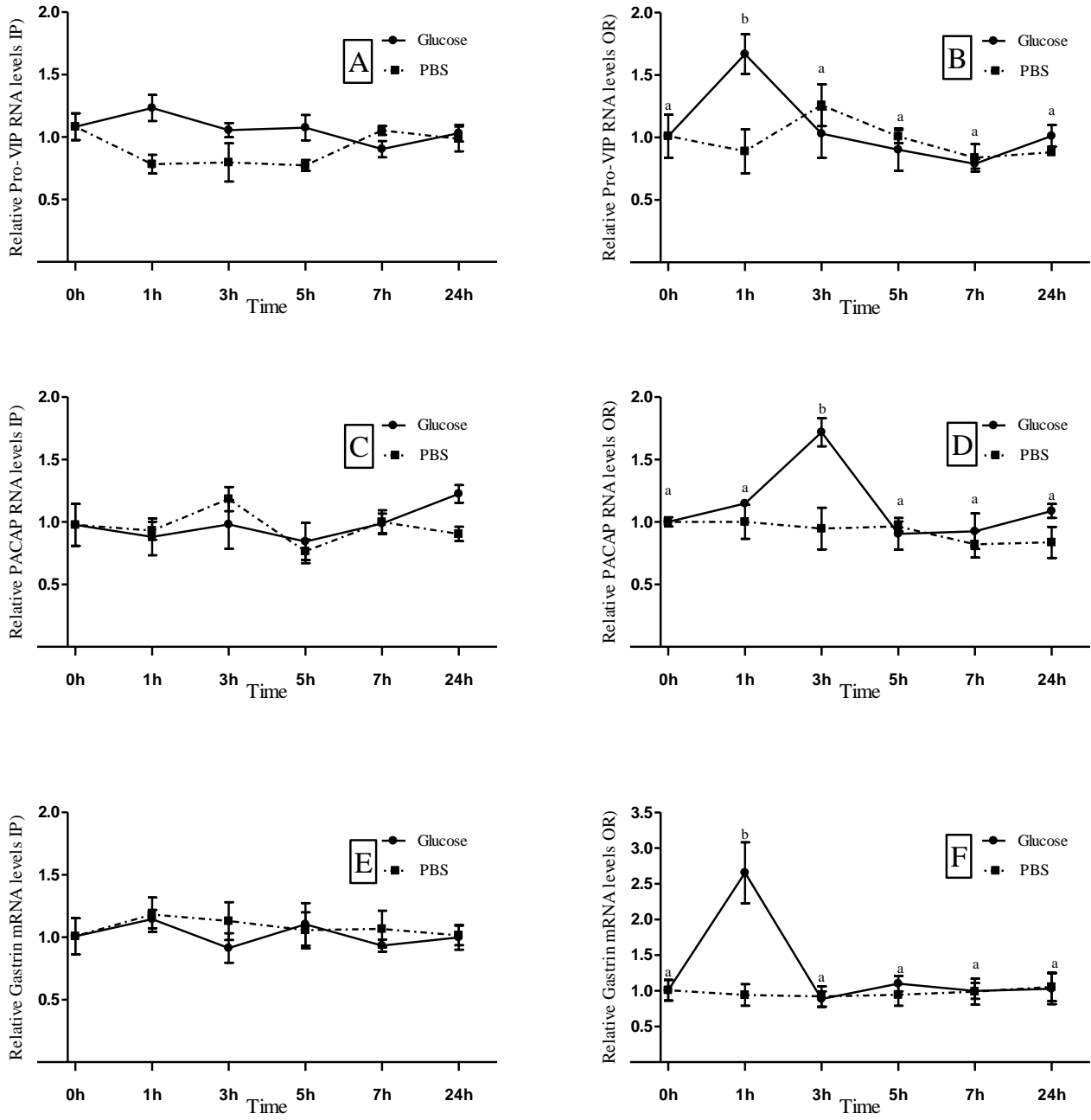
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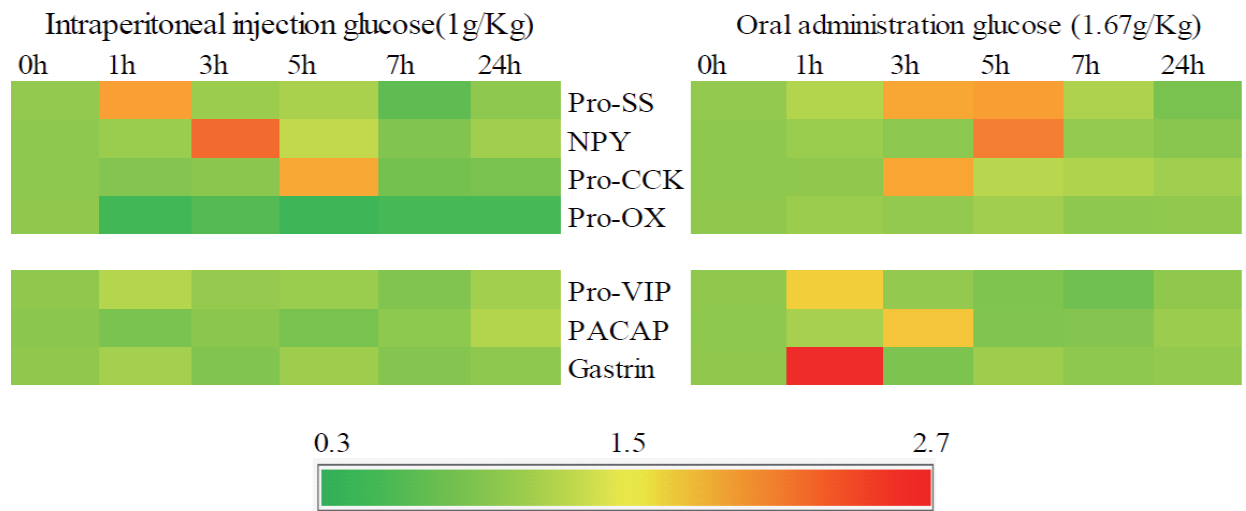
766   **Figure 6**

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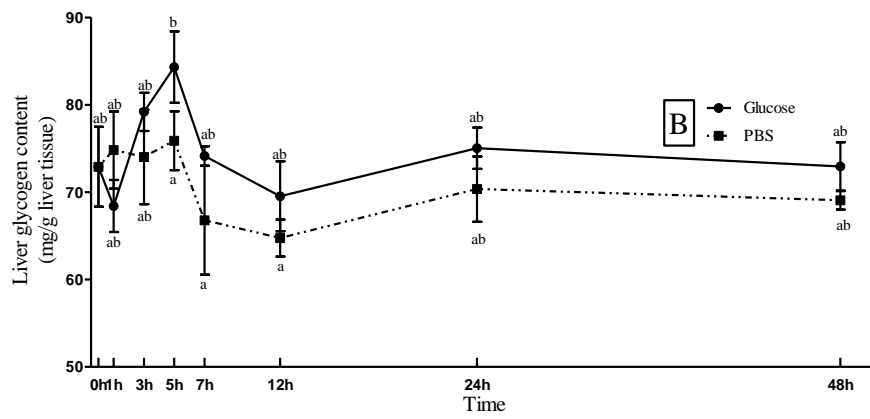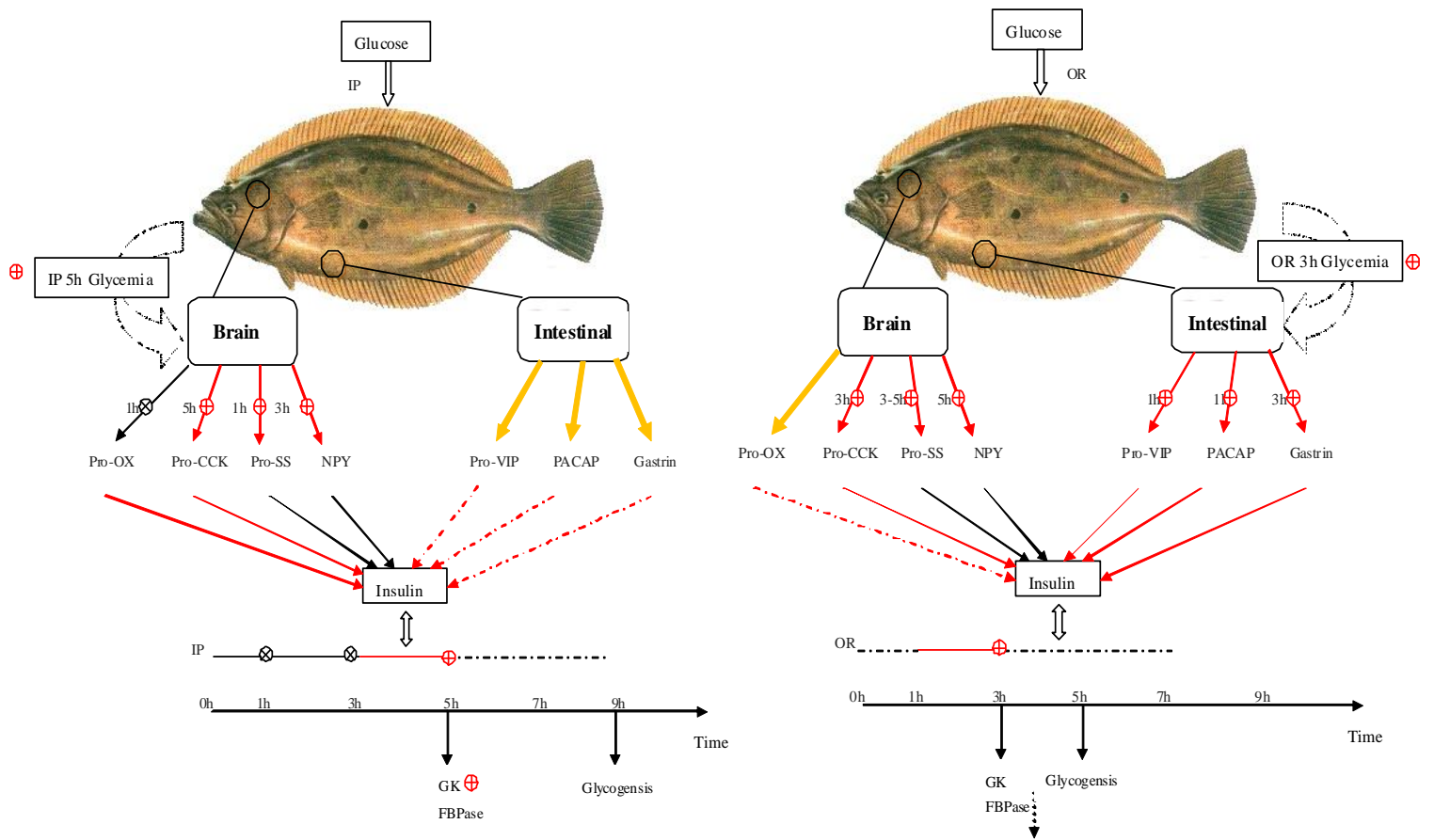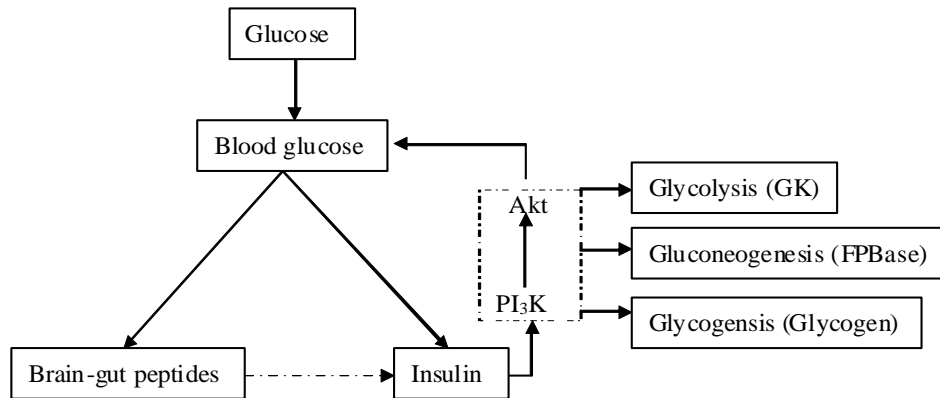769 **Figure 7**

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774 **Figure 8**

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777   **Figure 9**

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780 **Figure 10**