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RESEARCH PAPER

Molecular Response of Carbohydrate Metabolism to Dietary Carbohydrate and Acute Low Salinity Stress in Pacific White Shrimp *Litopenaeus vannamei*

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Abstract

Molecular response of carbohydrate metabolism in Litopenaeus vannamei was evaluated at transcriptional level through quantitative real-time PCR analysis. The mRNA expressions of six genes hexose-6-phosphotransferase, pyruvate kinase, phosphoenolpyruvate carboxykinase, crustacean hyperglycemic hormone, glucose transporter and insulin-like growth factors binding protein in the hepatopancreas, muscle, gill and eyestalk were analyzed in shrimp fed different levels of carbohydrate and challenged with acute salinity stress. Among these genes, the relative expression of GLUT1, CHH and IGF-BP in the muscle was lower or nearly the same as the control group regardless of the dietary carbohydrate level. The relative expressions of HK and PK were much higher in the hepatopancreas, muscle, gill and eyestalk at 12 h after salinity stress. The relative expression of PEPCK showed a down-up-down tendency and the expression was much higher in different tissues (except in eyestalk) than in the control from 12 to 24 h after stress. This study indicates that there was no significant dietary carbohydrate regulation in muscle. The glycogen and amino acid in muscle were the main source energy for osmoregulation in L. vannamei when the animals were challenged with acute salinity stress. The muscle and gills were the main osmoregulation organ in shrimp under hypo-saline stress.

Keywords: *Litopenaeus vannamei*, carbohydrate metabolism, dietary carbohydrate, acute salinity stress, molecular response.

Introduction

Carbohydrate is the least expensive energy supply in the diet for aquatic animals. However, dietary carbohydrate cannot be efficiently used by most aquatic animals compared with poultry and mammals (Wilson and Poe, 1987) and a high level of dietary carbohydrate may cause slow growth and other negative effects (Shiau *et al.*, 1991). Nevertheless, carbohydrate is often included in the diet of aquatic animals as an energy source to spare protein and reduce feed cost in aquaculture (Cruz-Suarez *et al.*, 1994; Cuzon *et al.*, 2004).

The white shrimp *Litopenaeus vannamei* is a euryhaline crustacean and is able to tolerate a wide range of salinity from 1 to 50 psu (Pante, 1990). Because of this, *L. vannamei* has been cultured worldwide in the past decades (Li *et al.*, 2007). Despite high production volume in inland saline water, *L. vannamei* display slow growth and low survival (Diaz and Farfan, 2001; Li *et al.*, 2007) and poor tolerance to stress at low salinity <5 psu (Li *et al.*, 2007; Li *et al.*, 2008; Wang *et al.*, 2013). Therefore, there is a need to further explore the physiological adaptation to hypo-osmoregulation and to identify a practical way to improve growth performance and anti-stress ability of *L. vannamei* at low salinity. Previous studies have shown that dietary carbohydrate can meet the high energy requirement of aquatic animals under stress (Tseng and Hwang, 2008; Wang *et al.*, 2014b; Wang *et al.*, 2012) because carbohydrate is a readily available source of energy for most crustacean species (Lehninger, 1978). So far, most research on the utilization of dietary carbohydrate in shrimp and crab has focused on enzymes relevant to carbohydrate metabolism (Marqueze *et al.*, 2006; Niu. *et al.*, 2012; Wang *et al.*, 2014a; Wang *et al.*, 2014b) and immunity (Arasta *et al.*, 1996; Tseng and Hwang, 2008; Wang *et al.*, 2012; Welcomme and Devos, 1991). However, the molecular mechanism underlying the impact of dietary carbohydrate utilization on growth and physiological performance of shrimp at low salinity is not clear.

The pathways of carbohydrate metabolism differ among tissues in crustacean species. The energy stored in the hepatopancreas can be transferred to other tissues for growth, molting and reproduction

(Cuartas *et al.*, 2003; Johnston *et al.*, 1998; Wen *et al.*, 2006). Hepatopancreas is the main tissue for lipid storage (Schmitt and Santos, 1993) while the muscle is the main place for protein storage in crustaceans (Buckup *et al.*, 2008; Claybrook, 1983). In *L. vannamei*, protein can be converted to glycogen in the hepatopancreas and muscle through gluconeogenesis and glycogenesis (Rosas *et al.*, 2001). The gills are the main site for ion transportation in decapod crustaceans and they are also a reservoir for lipid storage and a site for gluconeogenesis (Antunes *et al.*, 2010; Vinagre and Dasilva, 1992). The eyestalk is closely related to carbohydrate metabolism because crustacean hyperglycemic hormone (CHH) is a polypeptide hormone secreted by the X-organ/sinus gland in the eyestalk (Kung *et al.*, 2013). This hormone participates in many physiological processes including stress response in crustaceans, and is responsible for the regulation of glucose in hemolymph (Bocking, 2002; Kim *et al.*, 2013).

Dietary polysaccharide can be hydrolyzed to oligosaccharides, branched chain α-dextrin or maltose in the digestive gland of crustaceans (van Wormhoudt and Favrel, 1988), and then is completely hydrolyzed to monosaccharide, glucose before absorption in hemolymph (Kumlu, 1999; Sousa and Petriella, 2006; Speck and Urich, 1970; Urich *et al.*, 1973). The first step of carbohydrate utilization in cells is phosphorylation which is catalyzed by hexokinase (ATP: hexose-6-phosphotransferase, E.C. 2.7.1.1; HK) (Tsai and Wilson, 1997). The HKs are a family of glycolysis related enzymes in organisms from yeasts to mammals (Gaxiola *et al.*, 2005; Iynedjian, 1993). Pyruvate kinase (PK) is another key enzyme in glycolysis (Fraenkel, 1996). The pentose phosphate pathway is involved in carbohydrate metabolism in decapods during ecdysis (McWhinnie, 1962) and phosphoenolpyruvate carboxykinase (E.C. 4.1.1.32; PEPCK) is a key enzyme for gluconeogenesis (Schein *et al.*, 2005). Crustacean hyperglycemic hormone (CHH), insulin-like peptides and insulin-like growth factors (IGF-I and IGF-II) are the main hormone in the crustacean hemolymph for glucose regulation (Verri *et al.*, 2001). In recent years, insulin-like peptides and insulin-like growth factor have been discovered in invertebrates, and these polypeptides have similarity in sequence and function with insulin and insulin-like growth factors (Gricourt *et al.*, 2003; Lebel *et al.*, 1996; Masumura *et al.*, 2000; Sevala *et al.*, 1993; Wu and Brown, 2006). Most studies on these key factors in crustaceans have focused on gene cloning, gene function and enzyme activity (Chang *et al.*, 2010; Hsiao *et al.*, 2002; Jeon *et al.*, 2012; Wiwegweaw *et al.*, 2004). There were also some studies on mRNA expression of these genes (Martinez-Quintana *et al.*, 2014; Schein *et al.*, 2005), but most of them focused on carbohydrate digestion, transportation or regulation. There was little research that simultaneously explores the carbohydrate digestion, transportation and regulation.

Therefore, this study aims to investigated the effect of acute salinity stress and dietary carbohydrate levels on the molecular response of the carbohydrate metabolism of *L. vannamei* by identifying the key metabolism pathways including glycolysis, gluconeogenesis, glucose transportation and hormone regulation processes in the hepatopancreas, muscle, gills and eyestalk. The results of study will provide an insight into the understanding on carbohydrate metabolism in shrimp and nutritional physiology in crustacean.

Materials and Methods

Animal Source and Salinity Acclimation

Juvenile shrimp were obtained from a local company in Hainan, China. Shrimp were cultured in fiberglass tanks (80 × 60 × 50 cm) at a salinity of 32 psu for one week, and were acclimated to the three target salinities (30, 20 and 3) by changing 2 psu per day. During acclimation, shrimp were fed with a commercial feed. Seawater and tap water were thoroughly aerated before being added to the tanks to adjust the salinity level.

Manipulation of Dietary Carbohydrate and Salinity

Three iso-nitrogenous (40.29%) and iso-lipid (11.31%) diets, using fish meal as protein, fish oil and soybean oil (1:1) as lipid sources, were formulated to contain 50, 200 and 300 g kg^{-1} of carbohydrate and the compositions of experimental basal diets are shown in the (Tables S1, S2, S3). Shrimp (1.83 ± 0.02 g) were stocked at the salinities of 3 and 30 psu in triplicate tanks (40 × 50 × 50 cm) and fed twice daily (0800 h and 1700 h) for 8 weeks. Based on feed leftover from the previous day, daily rations were adjusted to slight over satiation. Uneaten feed was daily removed with a siphon tube. Daily water exchange was 1/3 of the tank volume. Water quality parameters including pH, salinity, temperature, dissolved oxygen, and ammonia were monitored 2 to 3 times a week and were maintained at 26-28°C, pH 7-8, 4.8-6.4 mg dissolved oxygen L^{-1}, and <0.02 mg total ammonia nitrogen L^{-1}. At the end of the experiment, shrimp were deprived of feed for 24 h, and only those at intermolt (Stage C) were sampled. The muscle, hepatopancreas, gill and eyestalk from 10 shrimp at stage C in each tank were sampled to measure gene expression. All shrimp specimens were collected, put into the RNAlater and then kept at -80°C for RNA extraction.

Acute Salinity Challenge

After a week of acclimation of at 20 psu, 300 shrimp were divided into two salinity groups (3 and

Supplement 1. The compositions of experimental basal diets (different carbohydrate levels).

Ingredients	Diets g kg-1		
	1	2	3
Fish meal	550	550	550
corn starch	50	200	300
Fish oil	20	20	20
Soybean oil	20	20	20
Lecithin	10	10	10
Cholesterol	5	5	5
Vitamin Premix[a]	20	20	20
Mineral Premix[b]	5	5	5
Carboxymethyl cellulose	20	20	20
Vitamin C	1	1	1
Cellulose	279	129	29
Calcium carbonate	20	20	20
Crude protein	350.1	349.9	353.4
Crude lipid	111.4	115.6	112.1

a Vitamin premix, diluted in cellulose, provided the following vitamins (g kg-1 premix): thiamin HCl 0.5, riboflavin 3.0, pyrodoxine HCl 1.0, DL Ca-Pantothenate 5.0, nicotinic acid 5.0, biotin 0.05, folic acid 0.18, vitaminB12 0.002, choline chloride 100.0, inositol 5.0, menadione 2.0, vitamin A acetate (20,000 IU g^{-1}) 5.0, vitamin D3 (400,000 IU g^{-1}) 0.002, dl-alphatocopherol acetate (250 IU g^{-1}) 8.0, Alpha-cellulose 865.266.
b Trace mineral premix provided the following minerals (g $100g^{-1}$ premix): cobalt chloride 0.004, cupric sulfate pentahydrate 0.250, ferrous sulfate 4.0, magnesium sulfate heptahydrate 28.398, manganous sulfate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, sodium dihydrogen phosphate 15, filler 38.428

Supplement 2. Growth performance and morphological parameter of shrimp fed different levels of carbohydrate (at 30psu).

CBH level (g kg-1)	Weight gain (%)	Survival (%)	Hepatosomatic index (%)
50	167.06	96.67a	3.67
200	180.99	98.89ab	4.03
300	178.37	90.00b	3.96
Pooled SEM	16.19	2.03	0.41

* Values with different superscripts in the same row are significantly different (P<0.05).

Supplement 3. Growth performance and morphological parameter of shrimp fed different levels of carbohydrate (at 3 psu).

CBH level (g kg^{-1})	Weight gain (%)	Survival (%)	Hepatosomatic index (%)
50	56.03	77.78	4.59
200	47.69	77.78	5.42
300	68.98	88.89	4.66
Pooled SEM	10.75	5.56	0.39

* Values with different superscripts in the same row are significantly different (P<0.05).

20 psu) in fiberglass tanks (80×60×50 cm) with three replicates of each group. A total of 50 shrimp (1.60±0.12 g) were randomly assigned to each tank. The muscle, hepatopancreas, gills and eyestalk from 10 shrimp at intermolt (stage C) in each group were sampled at 0, 6, 12, 24, 48, 72 and 96 h to study the gene expressions over time. All shrimp specimens were collected for RNA extraction using the same protocol above.

Total RNA Extraction

Total RNA was extracted from the target tissues using a Unizol reagent kit (Biostar, Shanghai, China) according to the manufacturer's protocol, whereas RNA quantity and quality were measured at 260 nm and 280 nm on a NanoDrop (Hach, America) and agarose-gel electrophoresis, respectively.

cDNA Reverse Transcription

Total RNA was reverse transcribed using the PrimeScript™ RT reagent kit (Takara, Japan) for real-time quantitative RT-PCR (qRT-PCR) analysis. The reactions were carried out in a total volume of 10 µl, containing 2 µl of 5× PrimeScript™ buffer, 0.5 µl of random 6 mers (100 mM), <500 ng of total RNA and up to 10°C µl of RNase free dH_2O. The reverse transcription was conducted at 37°C for 15 min and 85°C for 5 s.

Quantitative Real-time PCR (qPCR) Analysis

The expressions of the HK, PK, PEPCK, CHH, IGF-BP and GLUT1 genes in shrimp tissues were detected by qRT-PCR according to these gene sequences. Six pairs of gene-specific primers (Table 1) were designed, and the primers against β-actin RT-F and β-actin RT-R were used as the internal standard gene control. Samples were run in triplicate and normalized to the control gene, β-actin. qPCR was carried out in the CFX96TM Real-Time system (Bio-Rad). The cycle time (Ct) in different tissues and the values of different tissues at different time points after salinity stress were compared and converted to fold differences with 20 psu as the salinity control by the relative quantification method using the Relative Expression Software Tool 384 v.1 (REST) (Pfaffl *et*

al., 2002). The amplifications were performed on a 96-well plate in a reaction volume of 20 µl, containing 10 µl of SYBR Green Premix Ex Taq™ (2×) (TaKaRa, Japan), 0.4 µl of 10 mM gene-specific forward and reverse primers, 2 µl of diluted cDNA template (200 ng/µl) synthesized by a NanoDrop (Hach, America) and 7.2 µl of H_2O. The PCR conditions were as follows: 95°C for 30 s; 40 cycles of 94°C for 15 s, 58°C for 20 s, 72°C for 20 s, and a 0.5°C per 5 s incremental increase from 60 to 95°C. The results and data were analyzed using the CFX Manager TM sºftware (version1.0).

Statistical Analysis

The fold-change method was used in data analysis as it could more effectively identify differentially-expressed genes than a T-statistic method. The treatments difference of gene expression analyzed by fold change are more reproducible (Shi *et al.*, 2006; Shi *et al.*, 2005). The 20 psu, which was thought to be the optical salinity for *L. vannamei* (Li *et al.*, 2007), was used as the control to measure the fold change of gene expression relative to the salinity at 3 psu to examine the differential metabolism under the acute low salinity stress. The 30 psu, which was near the salinity of seawater, was used as the control relative to 3 psu in the growth trial where shrimp were fed different carbohydrate diets. The growth trial was designed to explore the response of carbohydrate metabolism in shrimp fed different dietary carbohydrate under a chronic hypo-salinity stress. The calculation was conducted using the relative expression software tool 384 v.1 (Pfaffl *et al*. 2002). Differences were considered significant at $P<0.05$.

Results

Transcriptional mRNA Expression in Shrimp Fed Different Carbohydrate Diets

The mRNA expressions of HK, PK, PEPCK, GLUT1, CHH and IGF-BP in the hepatopancreas, muscle, gills and eyestalk tissues were analyzed using quantitative real time RT-PCR after 8-week culture. Fold change was expressed as the ratio of the gene expression of shrimp in the 3 psu to the 30 psu control group. The relative fold changes of the genes in the transcriptional levels on the enzymes relevant to carbohydrate metabolism in the hepatopancreas, muscle, gills and eyestalk are shown in Figure 1, Figure 2, Figure 3 and Figure 4.

In the hepatopancreas (Figure 1), the expression of these six genes was lower or nearly the same in the shrimp fed 5 g kg^{-1} carbohydrate in 3 psu when compared with those fed the same diet in 30 psu. Among these six genes, the mRNA levels of shrimp fed 20 g kg^{-1} carbohydrate (3 psu) were higher than the control group except for the HK and IGF-BP genes. The relative expression of these genes was much higher in the shrimp fed 30 g kg^{-1} carbohydrate.

In the muscle (Figure 2), the relative expressions of GLUT1, CHH and IGF-BP were similar to the control regardless of the level of dietary carbohydrate. The mRNA levels of PK and PEPECK in 3 psu were higher than those in 30 psu for all the three carbohydrate diets. For PK, the relative expression was much higher in shrimp fed 5 g kg^{-1} carbohydrate while the relative of PEPCK was much higher in those fed the 30 g kg^{-1} carbohydrate.

In gills (Figure 3), the fold changes increased with the increase of dietary carbohydrate for almost all these six genes. In shrimp fed 5 g kg^{-1} carbohydrate, the expression of all genes in 3 psu was lower than that in 30 psu (fold change value was lower than 1). In the eyestalk, the mRNA levels of these genes in shrimp at 3 psu was higher than that at 30 psu (fold change value >1). The fold changes initially decreased and then increased in the shrimp fed 5 g kg^{-1}, 20 g kg^{-1} and 30 g kg^{-1} carbohydrate for all genes except for HK.

Transcriptional Response to Acute Salinity Stress

The mRNA expressions of HK, PK, PEPCK, GLUT1, CHH and IGF-BP in the hepatopancreas,

Table 1. Primers used for quantitative real time RT-PCR

Primer name	Sequence (5'- 3')	Length (bp)	GenBank No.
HK- RT- F	ATCGGCAAGTTAGATACGC		
HK- RT- R	AAGGATGGCACCACAGGA	185	EF102106.1
PK- RT- F	ATCCTTGATGGTGCTGAC		
PK- RT- R	CCGTGTTCGTTGAGAAGT	133	EF102105.1
PEPCK- RT- F	AAGACCAGTGATGGAGGAGTG		
PEPCK- RT- R	GACGAGTAGGGTTGAGGG	133	FJ441189.1
CHH- RT- F	TCTGCGAGGACTGCTACA		
CHH- RT- R	CCTTCAACCATTAGGGATAG	168	AY167044.1
IGF-BP- RT- F	GTGGGCAGGGACCAAATC		
IGF-BP- RT- R	TCAGTTACCACCAGCGATT	123	KP420228
GLUT1-RT-F	CTTCGCTGCTGTGCTTGG		
GLUT1-RT-R	ATCCTGCTTGCTGCCTTC	139	KM201335
β-actin RT-F	CGCGACCTCACAGACTACCT		
β-actin RT-R	GTGGTCATCTCCTGCTCGAA	140	AF300705

* hexokinase (HK); pyruvate kinase (PK); phosphoenolpyruvate carboxykinase (PEPCK); Crustacean Hyperglycemic Hormone (CHH); Insulinlike growth factors binding protein (IGF-BP); facilitative glucose transporter 1 (GLUT1)

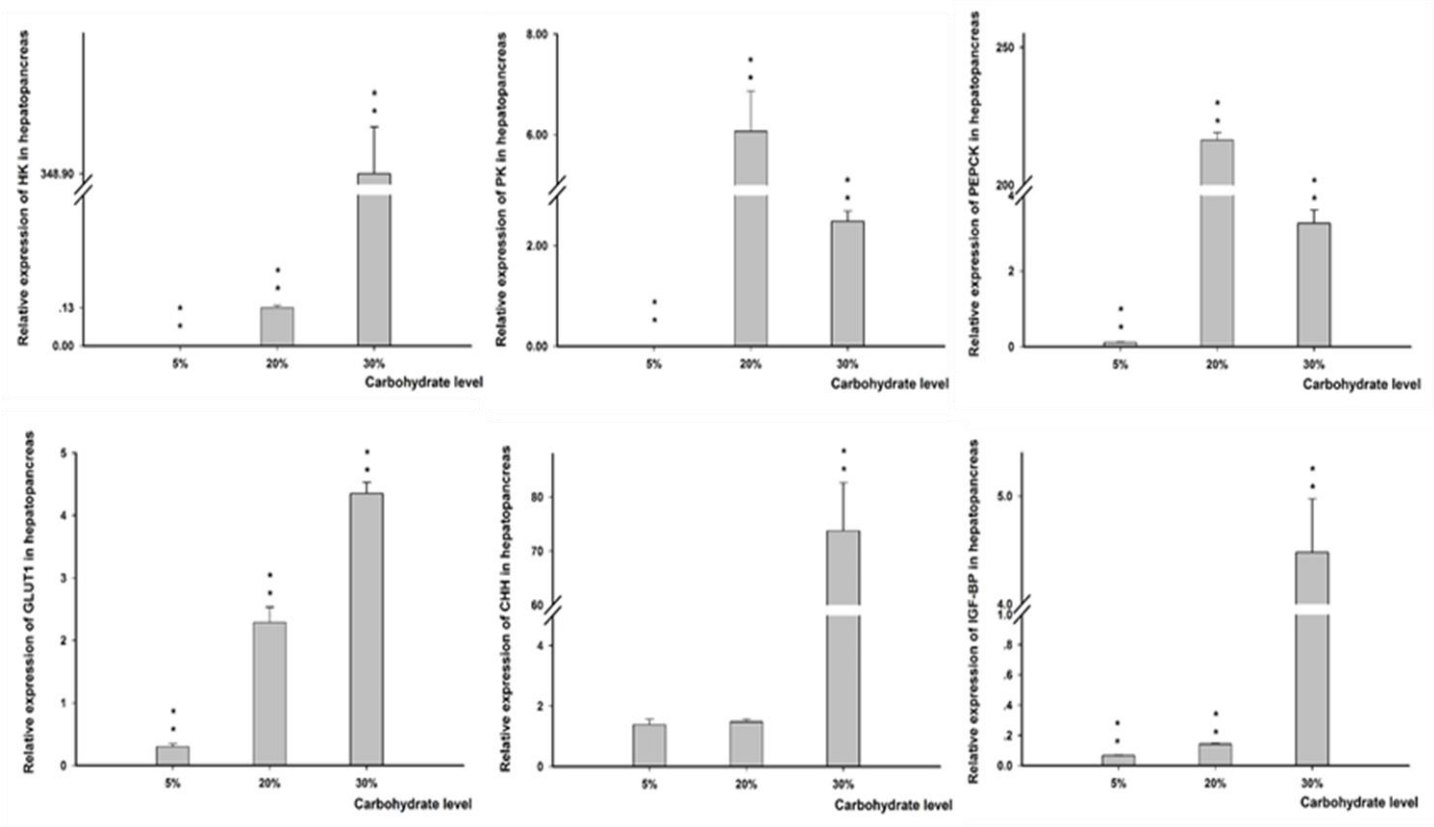

Figure 1. Analysis of expression of HK, PK, PEPCK, GLUT1, CHH and IGF-BP using quantitative real time RT-PCR in the hepatopancreas of shrimp fed different dietary carbohydrate levels. The relative expression value stands for the fold change, which is expressed as the ratio of gene expression in the 3 psu to the 30 psu control group as normalized with the β-actin gene. Error bars indicate standard error and asterisks indicate statistical significance of relative expression to the control group ($P<0.05$).

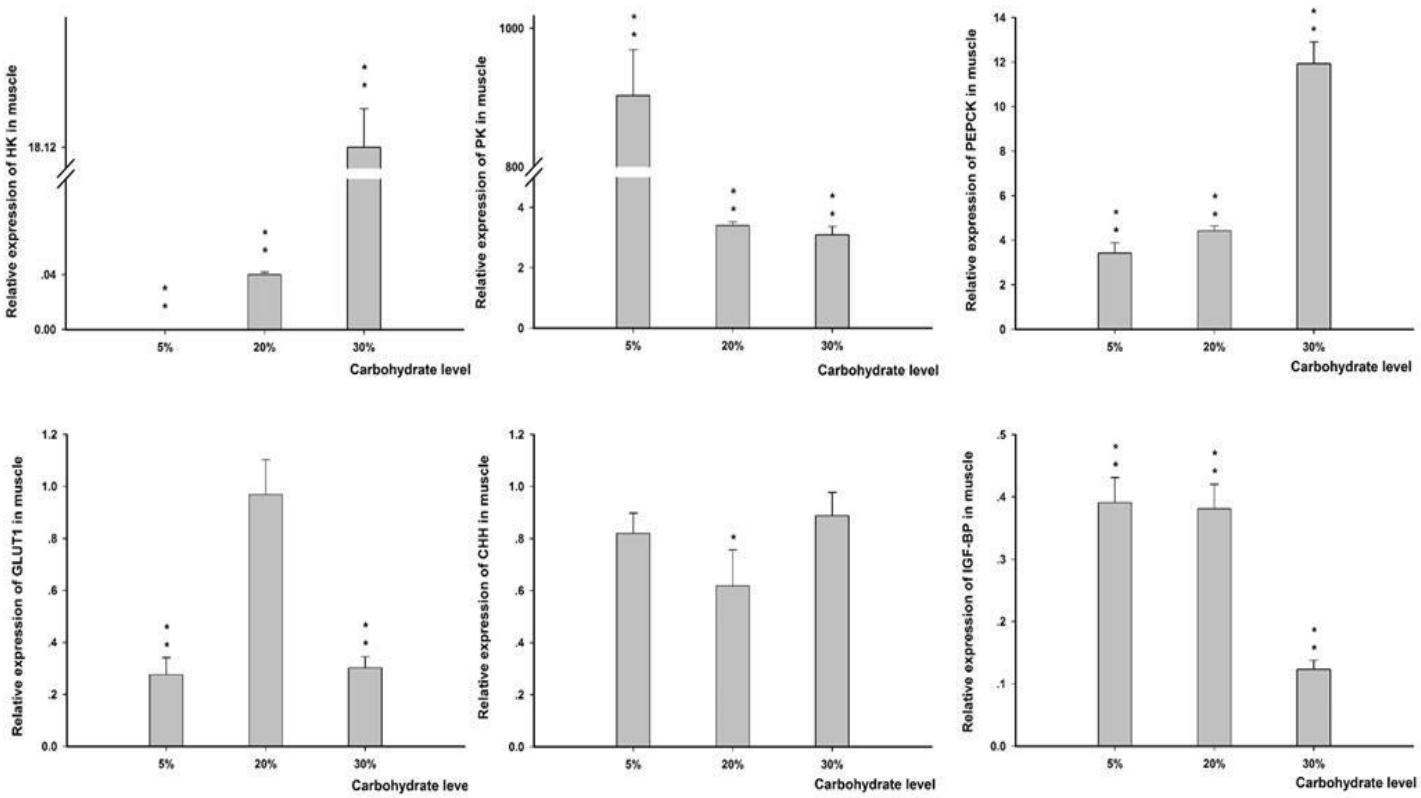

Figure 2. Analysis of expression of HK, PK, PEPCK, GLUT1, CHH and IGF-BP using quantitative real time RT-PCR in the muscle of shrimp fed different dietary carbohydrate levels. The relative expression value stands for the fold change, which is expressed as the ratio of gene expression in the 3 psu to the 30 psu control group as normalized with the β-actin gene. Error bars indicate standard error and asterisks indicate statistical significance of relative expression to the control group ($P<0.05$).

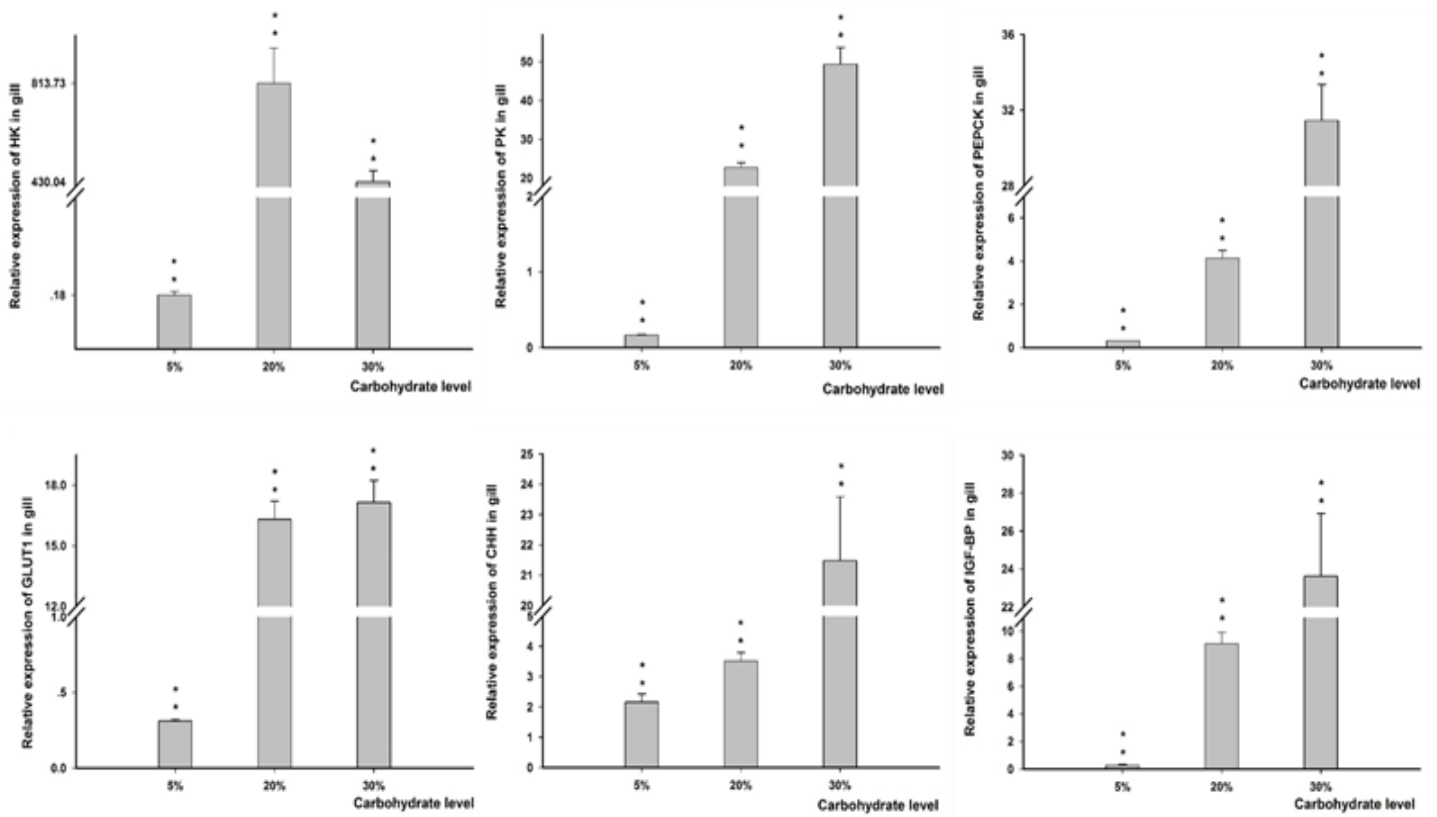

Figure 3. Analysis of expression of HK, PK, PEPCK, GLUT1, CHH and IGF-BP using quantitative real time RT-PCR in the gill of shrimp fed different dietary carbohydrate levels. The relative expression value stands for the fold change, which is expressed as the ratio of gene expression in the 3 psu to the 30 psu control group as normalized with the β-actin gene. Error bars indicate standard error and asterisks indicate statistical significance of relative expression to the control group ($P<0.05$).

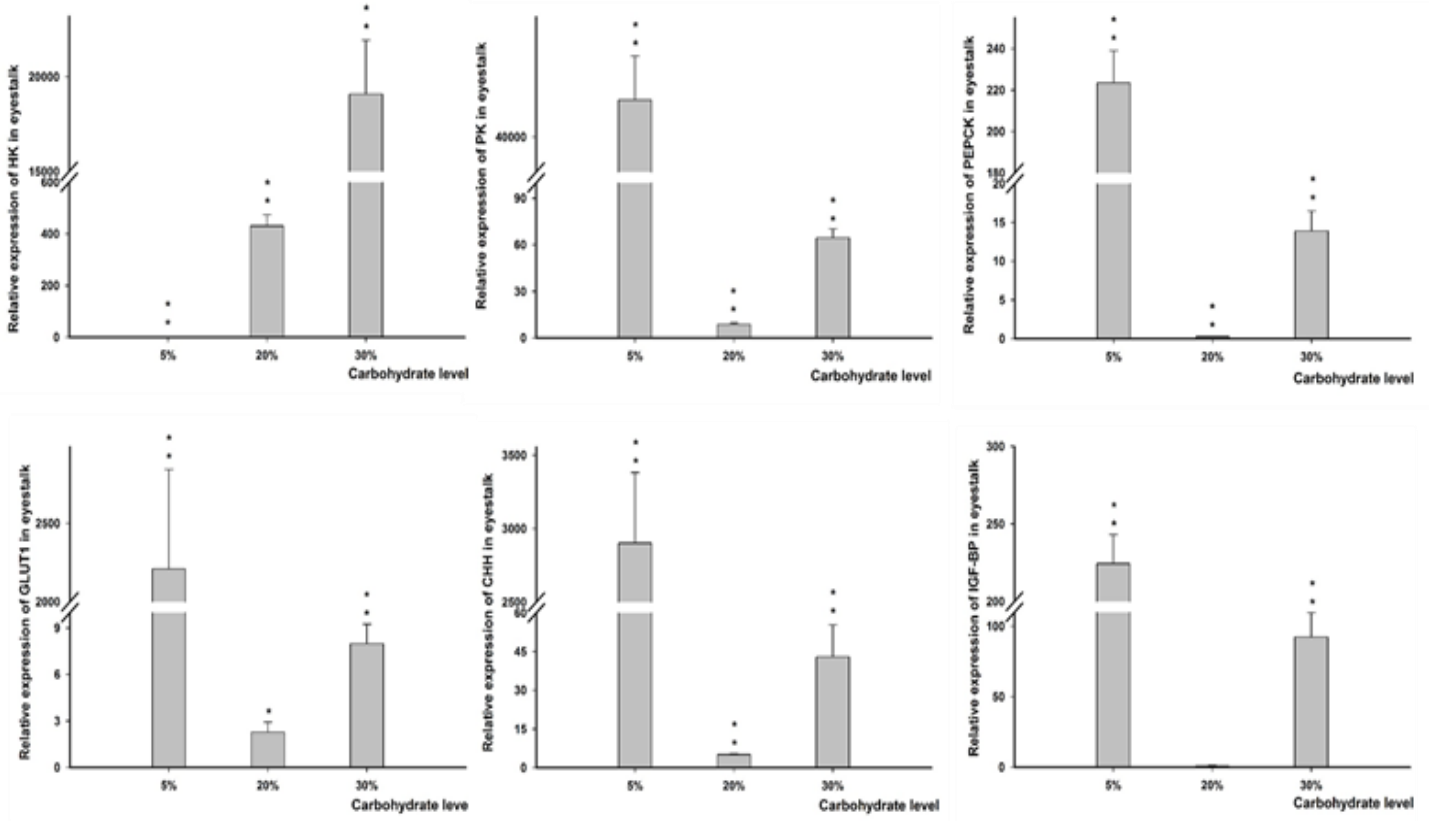

Figure 4. Analysis of expression of HK, PK, PEPCK, GLUT1, CHH and IGF-BP using quantitative real time RT-PCR in the eyestalk of shrimp fed different dietary carbohydrate levels. The relative expression value stands for the fold change, which is expressed as the ratio of gene expression in the 3 psu to the 30 psu control group as normalized with the β-actin gene. Error bars indicate standard error and asterisks indicate statistical significance of relative expression to the control group ($P<0.05$).

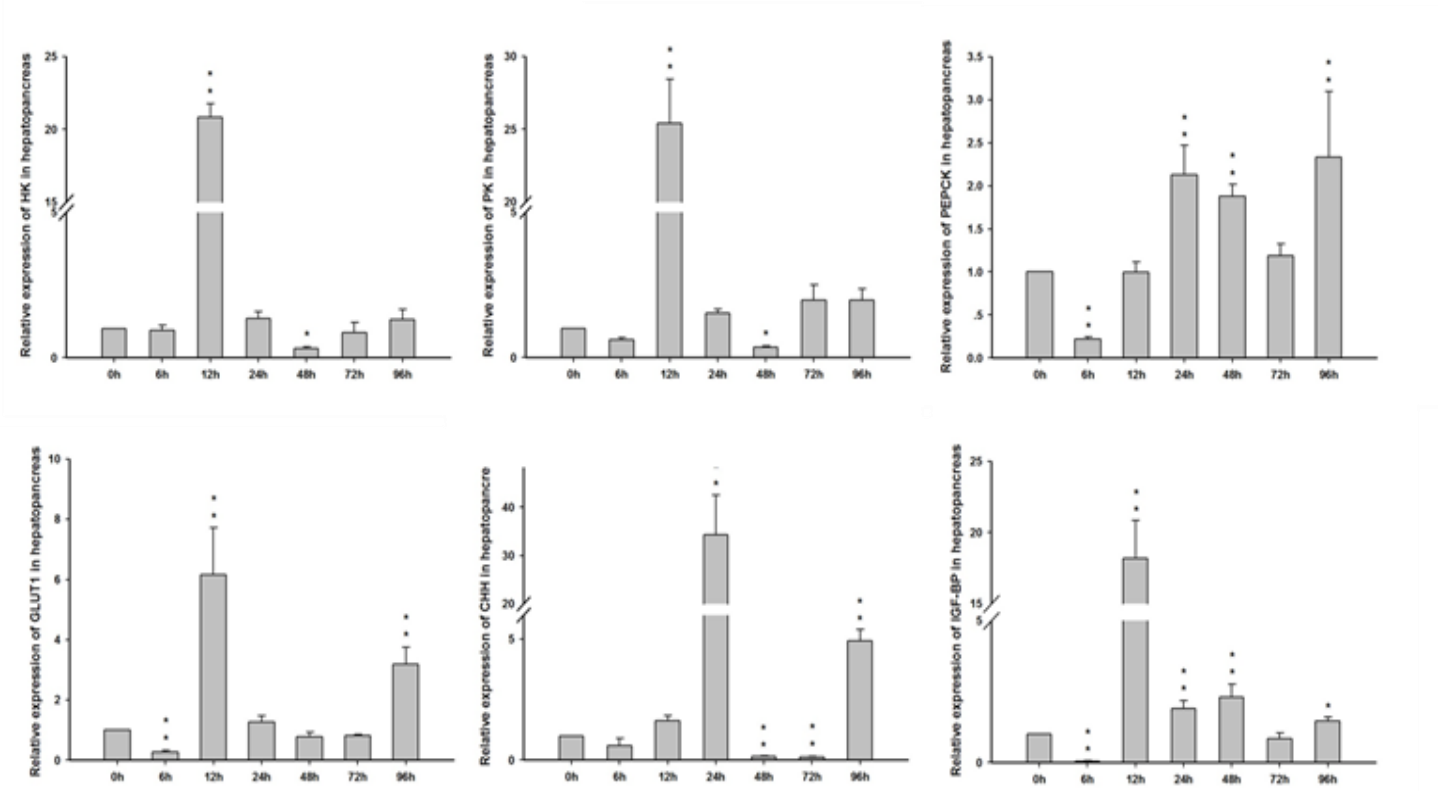

Figure 5. Analysis of expression of HK, PK, PEPCK, GLUT1, CHH and IGF-BP using quantitative real time RT-PCR in hepatopancreas after 96 h salinity stress at various time points (0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h). The relative expression value stands for the fold change, which is expressed as the ratio of gene expression in the 3 psu to the 20 psu control group as normalized with the β-actin gene. Error bars indicate standard error and asterisks indicate statistical significance of relative expression to the control group ($P<0.05$).

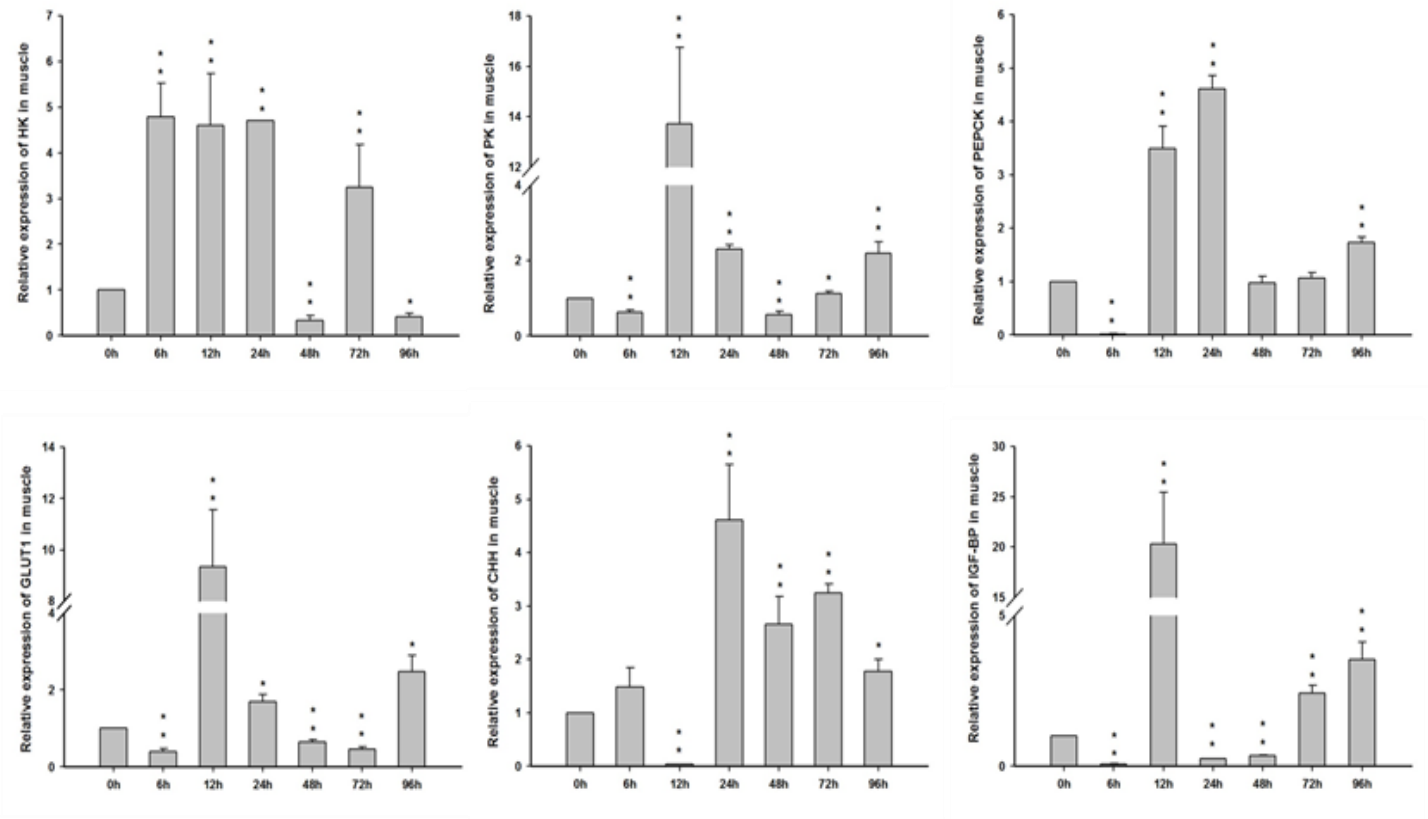

Figure 6. Analysis of expression of HK, PK, PEPCK, GLUT1, CHH and IGF-BP using quantitative real time RT-PCR in muscle after 96 h salinity stress at various time points (0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h). The relative expression value stands for the fold change, which is expressed as the ratio of gene expression in the 3 psu to the 20 psu control group as normalized with the β-actin gene. Error bars indicate standard error and asterisks indicate statistical significance of relative expression to the control group ($P<0.05$).

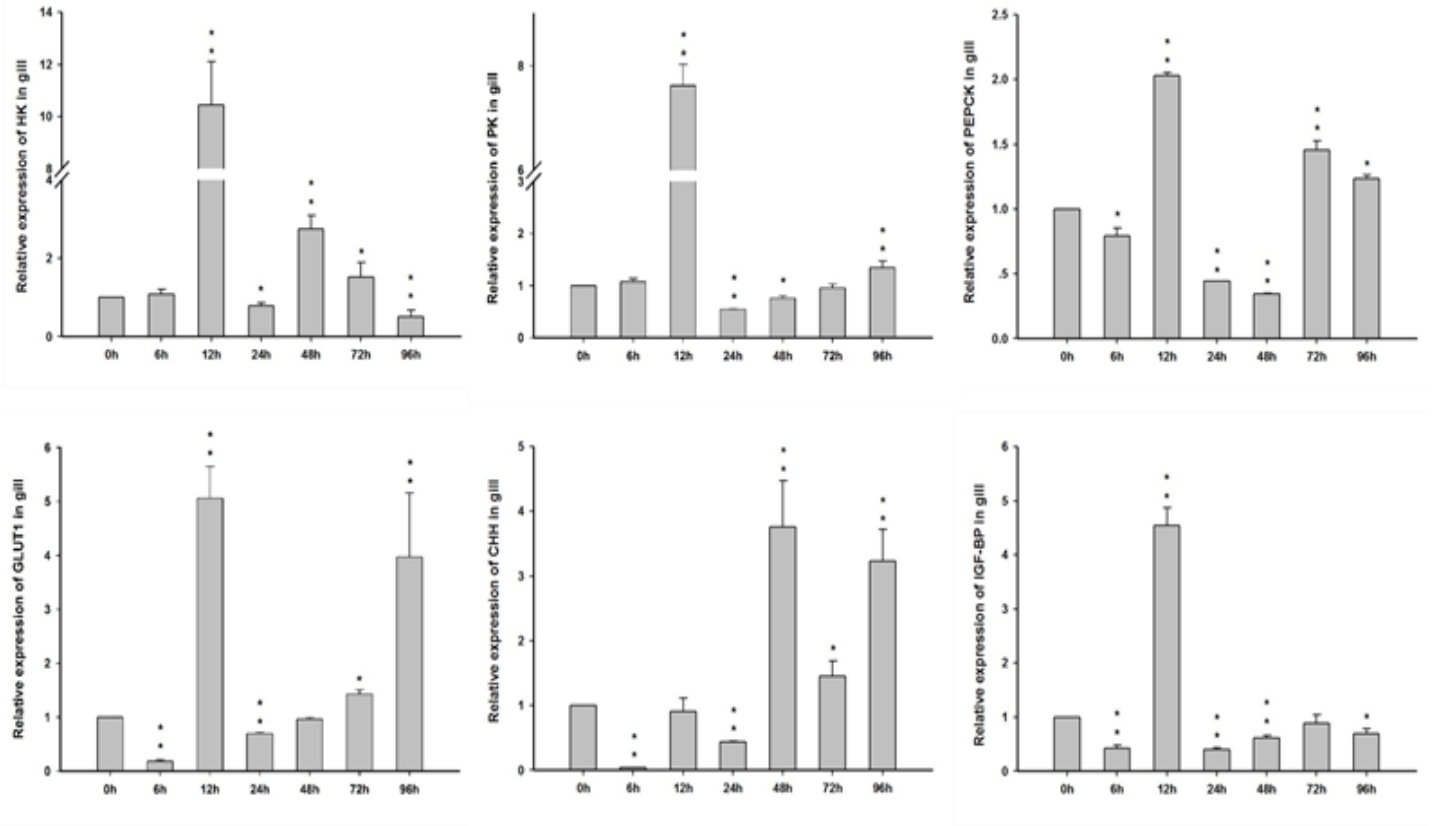

Figure 7. Analysis of expression of HK, PK, PEPCK, GLUT1, CHH and IGF-BP using quantitative real time RT-PCR in gill after 96 h salinity stress at various time points (0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h). The relative expression value stands for the fold change, which is expressed as the ratio of gene expression in the 3 psu to the 20 psu control group as normalized with the β-actin gene. Error bars indicate standard error and asterisks indicate statistical significance of relative expression to the control group ($P<0.05$).

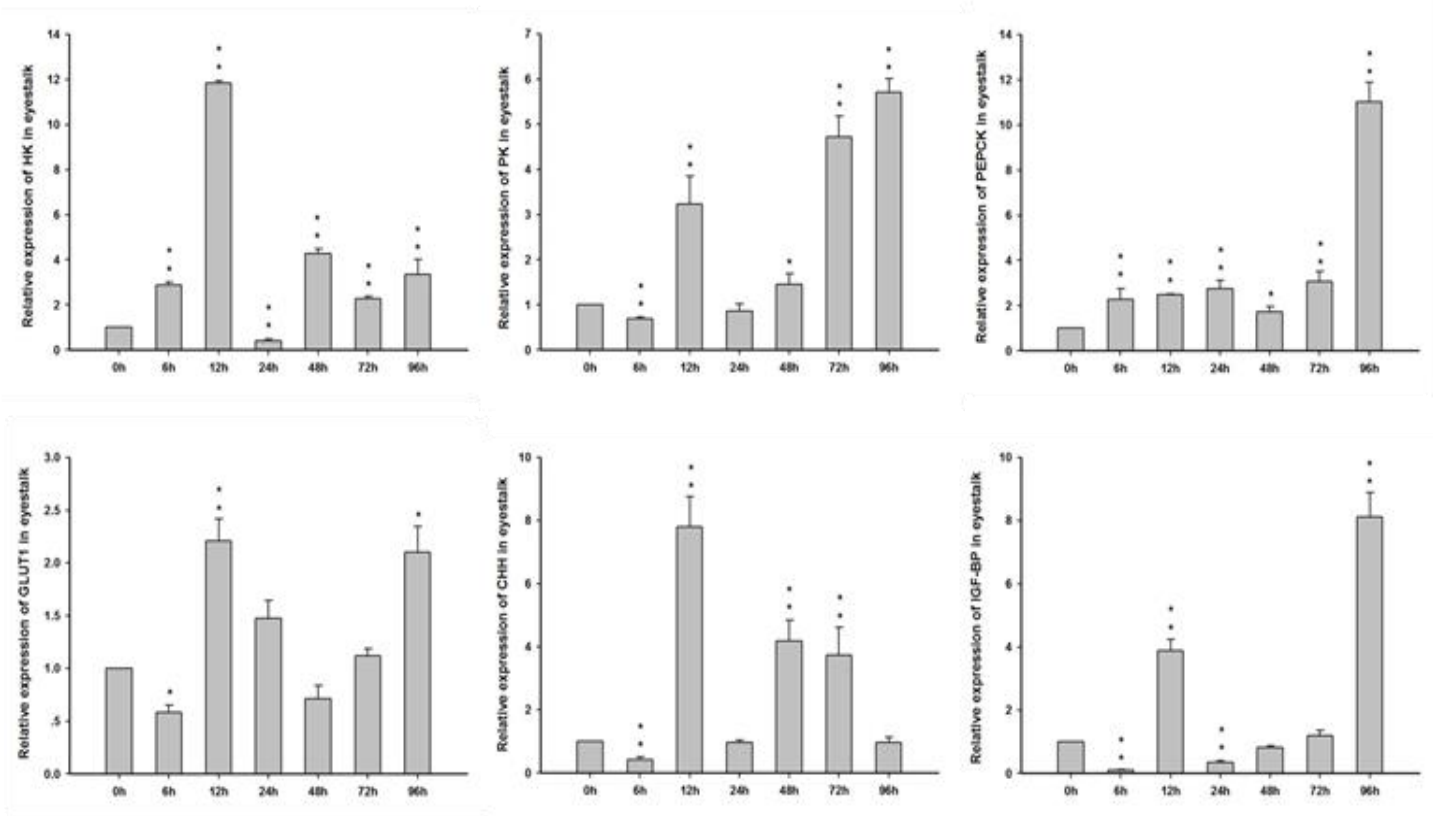

Figure 8. Analysis of expression of HK, PK, PEPCK, GLUT1, CHH and IGF-BP using quantitative real time RT-PCR in eyestalk after 96 h salinity stress at various time points (0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h). The relative expression value stands for the fold change, which is expressed as the ratio of gene expression in the 3 psu to the 20 psu control group as normalized with the β-actin gene. Error bars indicate standard error and asterisks indicate statistical significance of relative expression to the control group ($P<0.05$).

muscle, gills and eyestalk tissues were analyzed using quantitative real time RT-PCR after shrimp were transferred from 20 psu to 3 psu at various time points (0, 6, 12, 24, 48, 72 and 96 h). Fold change was expressed as the ratio of the gene expression after salinity stress to the 20 psu control group at the same time point. Relative fold changes after salinity stress in the enzymes related to carbohydrate metabolism in the hepatopancreas, muscle, gill and eyestalk are shown in Figure 5, Figure 6, Figure 7 and Figure 8.

The relative expression of HK and PK was much higher at 12 h in the hepatopancreas, muscle, gill and eyestalk after salinity stress. In the gill and hepatopancreas, the transcription seemed to have no noticeable changes at other time points. The mRNA expression of HK substantially changed in the muscle during the 96 h after stress. The relative expression of PK was higher in the muscle and both PK and HK expressions in the eyestalk were higher after 96 h.

The relative expression of PEPCK had a trend of down-up-down changes and the expression was much higher during 12-24 h after stress in different tissues except the eyestalk. The transcription of PEPCK was much higher than non-stress after 96 h in the hepatopancreas, muscle, gill and eyestalk tissues.

The expressions of GLUT1 in different tissues were all significantly up-regulated at 12 h and 96 h ($P<0.05$). The expressions in the above four tissues were all down-regulated at 6 h ($P<0.05$) and 48 h. During the 96-h acute stress, every tissue showed a time dependent changing pattern of down (6 h) – up (12 h) – down (48 h) – up (96 h).

For the IGF-BP gene, the mRNA level was much higher than non-stress after 12 h. At most time points after acute salinity stress, the relative expression of this gene was lower than 1. For the eyestalk, it had the highest transcription of IGF-BP after 96 h.

Discussion

Glucose Metabolism Response to Dietary Carbohydrate Levels

In the hepatopancreas of shrimp fed 5 g kg^{-1} carbohydrate, the expression of HK, PK, PEPCK, GLUT1, CHH and IGF-BP did not significantly increase at low salinity when compared with the 30 psu control. It indicates that the carbohydrate metabolism does not depend on the dietary carbohydrate at normal salinity or under low salinity stress in mRNA level. The expression of most carbohydrate metabolism related genes in shrimp fed 20 g kg^{-1} carbohydrate was higher than that in the salinity control, suggesting that at such level of dietary carbohydrate, shrimp in 3 psu have more transcriptional changes. This finding on the level of mRNA expression is consistent with the result in another study by Wang *et al* (2014b), which indicated that that 15-20 g kg^{-1} dietary carbohydrate was optimal for growth and could improve the ability of *L. vannamei* to cope stress in 3 psu.

In muscle, the relative expressions of PK and PEPCK in 3 psu were higher than in the control salinity at all dietary carbohydrate levels. It is possible that shrimp need more energy in 3 psu and the carbohydrate can meet the high energy requirement at such a stress condition (Arasta *et al.*, 1996; Tseng and Hwang, 2008; Wang *et al*., 2012; Welcomme and Devos, 1991). Therefore, mRNA expression of the key enzymes in glycolysis and gluconeogenesis pathway were more active in the muscle of shrimp at 3 psu. The relative expression of GLUT1, CHH and IGF-BP was lower or nearly the same as the control group regardless of the carbohydrate level, indicating that carbohydrate regulation did not significantly change under salinity stress irrespective of dietary carbohydrate level on mRNA level. As the level of enzymes for carbohydrate in muscle is limited (Stone *et al.*, 2003; Wang *et al.*, 2014b), shrimp cannot utilize a lot of extra carbohydrate even the intake of dietary carbohydrate is high. Although the glycolysis and gluconeogenesis activities were high in 3 psu, the amount to the level of carbohydrate may still within the upper threshold that the muscle tissue can tolerate.

Transcriptional Response to Acute Salinity Stress of Different Genes

The expression of key genes related to glycolysis (HK and PK), gluconeogenesis (PEPCK), glucose transportation (GLUT1) and hemolymph glucose regulation (CHH and IGF-BP) was quantified in this study. The mRNA expression of these genes in all tissues (hepatopancreas, muscle, gill and eyestalk) at 3 psu was much higher than in the salinity control (20 psu) at many time points. Moreover, the relative expression was all much higher for all genes in almost all tissues at 12 h after acute salinity stress, indicating that stress can escalate carbohydrate metabolism on mRNA over time. As dietary carbohydrate can provide more energy to aquatic animals in a stress condition (Arasta *et al.*, 1996; Tseng and Hwang, 2008; Wang *et al.*, 2012; Welcomme and Devos, 1991), which may explain the increase of metabolism after stress.

The fold change of HK in muscle increased from 6 h to 24 h after stress, which may be related to glycolysis elevation due to stress. In shrimp, muscle is the major tissue for glycogen storage (Chang and O' Connor, 1983; Vinagre and Dasilva, 1992), and it is possible that acute stress augments glycogen levels as a consequence of glycolysis. The high expression of PEPCK in muscle during 12-24 h could be a result of gluconeogenesis in the muscle during this time period. In a previous study, Wang (*et al*. 2012) found reduction of free amino acids in the muscle of *Eriocheir sinensis* after salinity stress for 12 h. Free amino acid and glycerol can be used to maintain the glucose level in the tissue of crab *Neohelice granulata*

through gluconeogenesis under hypo-osmotic stress (Lauer *et al.*, 2012; Nery and Santos, 1993). As muscle is a pool of amino acids in crustaceans, it can function as a source of amino acid supply to other tissues (Wang *et al.*, 2012) and the amino acid can be further converted to glycogen through gluconeogenesis (Ramamurthi *et al.*, 1968; Rosas *et al.*, 2001; Wang and Scheer, 1963). Therefore, *L. vannamei* may first use glycogen in muscle as the energy for osmoregulation, and then use amino acids in the muscle through gluconeogenesis after saline stress for 12 h, but more confirmation need to done on protein levels later.

The increase of PEPCK expression in hepatopancreas at 24 h could be related to the increase gluconeogenesis activity after stress. This may attribute to the release of amino acids from muscle and their transportation to hepatopancreas for gluconeogenesis (Oliveira and da Silva, 2000). The gluconeogenesis in hepatopancreas seemed to be one of the pathways for osmoregulation during salinity stress in *Chasmagnathus granulatae* (Oliveira & da Silva 2000). The glycogen synthesis in hepatopancreas depended on the utilization of amino acid during osmotic regulation, which can be enhanced by low salinity in shrimp (Rosas *et al.*, 2001). In the present study, salinity stress produced a marked increase of PEPCK mRNA after 48 h in gills, demonstrating the activation of gill gluconeogenesis on mRNA level in response to the stress. In the crab *Neohelice granulata*, the increase of proteolysis-derived amino acids could satisfy gluconeogenesis from amino acids in posterior gills for osmoregulation under stress (Oliveira and da Silva, 2000). In an amphidromous freshwater prawn *Macrobrachium olfersii*, the amount of free amino acid in gills was increased by 110% after 24 h of salinity stress (McNamara *et al.*, 2004), indicating that the amino acid released from other tissues can be used by the gluconeogenic process in gill for acclimation of salinity stress. Therefore, the amino acids stored in the muscle would be transported to hepatopancreas and gills for gluconeogenesis to cope with salinity stress.

The CHH mRNA level increased from 24 h in different tissues, especially in muscle and gills, which indicates that glucose metabolism is enhanced by low salinity stress. The expression of osmoregulation related genes is usually increased in the gills of euryhaline animals after salinity stress (Havird *et al.*, 2013) as the gill is a main site for ion transportation and osmoregulation in decapod crustaceans (Morris, 2001; Towle and Weihrauch, 2001). Abdomen in most shrimp, lobster and crayfish species is a muscular part to support swimming movements (Duffy, 2007) and the muscle is also the main pool of amino acids in crustaceans (Wang *et al.*, 2012).

The relative expression of IGF-BP was higher in all tissues after low salinity stress for 12 h, indicating the increased transcription of insulin-like peptides and insulin-like growth factor (IGF-I and IGF-II). IGF can accelerate the synthesis of glycogen and reduce glucose content (Sanders, 1983). It has been proved that the content of glycogen in the hepatopancreas and gill of *L.vannamei* significantly increased after the injection of IGF-I (Gutiérrez *et al.*, 2007) and the glycogen synthesis in muscle of the redclaw crayfish *Cherax quadricarinatus* was also increased by the injection of IGF-I using the isotope labelling method (Richardson *et al.*, 1997). As the carbohydrate metabolism activity was very active in different tissues at 12 h after stress, the activity of IGF related factors may be improved to balance the glucose level.

In summary, the genes related to the carbohydrate metabolism were more actively expressed at the mRNA level in shrimp fed 20 g kg^{-1} dietary carbohydrate. During chronic salinity stress, there was little carbohydrate regulation in muscle on mRNA level with the change of dietary carbohydrate level. However, *L. vannamei* may first use muscle glycogen as the energy for osmoregulation during acute low salinity stress, and then the shrimp used amino acids in the muscle through gluconeogenesis by 12 h after stress. From 24 h onwards, amino acids would be released from different tissues, mainly from muscle, and the amino acid would be transported to the hepatopancreas and gill for gluconeogenesis to cope with salinity stress. Glucose metabolism was found in muscle and gills after 24 h of the stress to allocate energy for osmoregulation and physical activities in shrimp.

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