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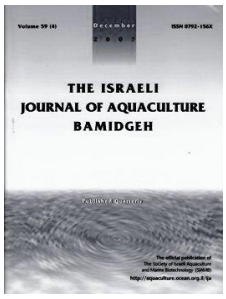

Changes of Biochemical Components Contents and Gene Expression Profiles in Tissues of *Litopenaeus vannamei* During Spermatophore Regeneration

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Keywords: *Litopenaeus vannamei;* spermatophore regeneration; glucose; triglyceride; total cholesterol; gene expression

Abstract

Spermatophore regeneration is an important biological process in *Litopenaeus vannamei.* In this study, changes of biochemical components contents during spermatophore regeneration showed that in hemolymph, the glucose content significantly increased from day 2 to day 8 and then decreased from day 10 to day 12; triglyceride (TG) and total cholesterol (TC) contents were relatively stable. In the hepatopancreas, glucose, and TG contents significantly decreased during regeneration; TC contents significantly increased at day 4 and then decreased. In the testes, glucose content significantly increased while TC content significantly decreased during whole stage; TG content at day 4 was highest. In terminal ampoule, changes of glucose and TC contents were similar, the contents at day 4 were highest; TG content at day 6 was significantly lower than day 2. During spermatophore regeneration, expression level of pyruvate kinase (PK) was significantly higher during day 2 to day 4 than at other times, and expression levels of three lipogenesis-related genes diacylglycerol O-acyltransferase homolog 1 (DGAT1), sterol regulatory element-binding protein 1 (SREBP1) and sterol O-acyltransferase 2 (SOAT2) were significantly higher during day 2 to day 8 than at other times. The results indicated that the hepatopancreas could be the energy source that provides glucose and TG; glucose, TG, and TC play important roles in spermatophore regeneration.

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Introduction

The pacific white shrimp (*Litopenaeus vannamei*) is one of the most important economical aquaculture species in the world. This species is adaptive to a wide range of salinities, it grows fast, and demands low levels of dietary protein (Aranyakananda and Lawrence, 1993). Reproduction in shrimp represents a critical step in shrimp culture and high-quality broodstocks are important for producing healthy shrimp larvae. *L. vannamei* belongs to open thelyca penaeoid shrimps. Most published research about the reproductive system of male *L. vannamei* focuses on aspects such as functional anatomy of the reproductive system, endocrine control of male reproduction, male sexual maturation, male sexual problems, spermatophore renovation, nutrition in male reproduction, acrosome reaction and sperm maturation, and spermatophore preservation. (Alfaro-Montoya, 2010).

The existence of a molt-dependent spermatophore renovation mechanism, in which the shrimp empties the terminal ampoules before molting, and produces a new spermatophore after molting has been demonstrated (Heitzmann et al., 1993); Parnes et al., 2006). However, Alfaro-Montoya (2010) stated that the molt-dependent renovation of spermatophores is operational in the wild but not in ponds. During male reproduction, nutrition such as lipids, total lipids, fatty acids, protein, carbohydrates, and vitamins, are essential (Wouters et al., 2001). The quality and quantity of sperm, weight of spermatophore, and spermatophore maturation time can be significantly influenced by temperature or salinity (Yao, 2001). Basic knowledge on the nutrition metabolism, changes of biochemical components in tissues and gene expression profiles during testes maturation or spermatophore regeneration is relatively scarce. It has been suggested that the baseline values of glucose concentration in the hemolymph are positively correlated to baseline values of sperm count, while baseline values of several lipids in the hemolymph were negatively correlated to several traits of sperm quality at first sampling (baseline values) or at first regeneration (Ceballos-Vázquez (2004). The energy balance of spermatophores and sperm viability during the molt cycle in intact and bilaterally eyestalk ablated male pacific white shrimp *L. vannamei* are quite different (Vázquez-Islas et al., 2013).

The genes involved in glycolysis and lipogenesis are important since the glucose and lipids are active during the male reproduction process. Pyruvate kinase (PK) (ATP-pyruvate 2-O-phosphotranferase, EC 2.7.1.40) is a key enzyme in the glycolytic pathway. In almost every cell type it controls the flux through the pathway, together with phosphofructokinase-1 (PFK) and hexokinase (Allert et al., 1991). This enzyme catalyzes the essentially irreversible transphosphorylation from phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP (Kayne, 1973; Valentini et al., 2000). For TG synthesis, the final and the only committed step in the biosynthesis of TGs is catalyzed by acyl-CoA: diacylglycerol acyltransferase (DGAT) enzymes, and the enzymes are encoded by DGAT1 and DGAT2 genes. For TC synthesis, sterol regulatory element binding proteins (SREBPs) regulate multiple genes involved in cholesterol biosynthesis and uptake and they are first implicated in regulating fatty acid synthesis by Tontonoz et al. (1993). Another enzyme, SOAT2, is a transmembrane-associated enzyme localized to the endoplasmic reticulum of hepatocytes and enterocytes and catalyzes the transfer of fatty acid from long chain acyl CoA to the 3’ hydroxyl group of cholesterol (Chang et al., 1997).

In this study, we analyzed changes of biochemical components not only in hemolymph, but also in the hepatopancreas, testes, and terminal ampoule during spermatophore regeneration; four genes related to glycolysis and lipogenesis in hepatopancreas were also measured.

Materials and Methods

Animals

One hundred healthy adult male *L. vannamei* (30.0 ± 1.5 g) with mature spermatophore at late post-molt stage were used in this study; they were equally divided into control and regeneration groups. In the regeneration group, spermatophores was gently squeezed out but not in the control group. Shrimps in the two groups were separately

cultured in 10 m^2 pond, 30‰ salinity seawater at 28℃, pH 7.8. They were fed with *Perineresis aibuhitensis* three times a day, and 70% seawater was changed every 2 days.

Biochemical components analysis during spermatophore regeneration

Six shrimp tails in each group were sampled every 2 days for 12 d, after hemolymph was drawn; shrimp were dissected into hepatopancreas, testes, and terminal ampoule; hemolymph, part of hepatopancreas, testes and terminal ampoule were weighed, immersed in liquid nitrogen and stored at -80℃ for biochemical components analysis. Part of hepatopancreas was also immersed in liquid nitrogen and stored at -80℃ for RNA isolation. To measure the glucose, TG and TC contents, same-size ratio of 0.86% normal saline was added to hemolymph and 9 times of 0.86% normal saline were added to other tissues and then homogenized. Then the contents were measured by automatic biochemical analyzer (Mindray, BS-390, Shenzhen, China) using kits bought from Mindray company.

RNA isolation and cDNA synthesis

Total RNA of hepatopancreas were isolated using Easyspin Plus kit (Aidlab, Beijing, China). RNA concentration and purity were checked using NanoDrop spectrophotometer (Aoseng, Hangzhou, China) and RNA quality was analyzed by gel electrophoresis analysis. For cDNA synthesis, 1 μg total RNA was used according to protocol of PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Dalian, China), and then cDNAs were diluted and stored at -20℃.

Transcription analysis of genes in hepatopancreas

Primers used for Real-Time RT-PCR analysis were designed using Primer Premier 5.0 and then the primer specificity was confirmed. Primers used in this study are listed in Table 1. The C_T values were determined in a 10 μl reaction volume containing 5 μl SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Takara, Dalian, China), 0.2 μM each of gene-specific primers and 1 μl cDNA (10 ng RNA) by using StepOnePlus system (ABI, America). Relative transcript levels were calculated according the method described by Zhang et al. (2018).

Table 1 Primers used for Real Time RT-PCR.

Gene	*Forward (5′→3′)*	*Reverse (5′→3′)*
18s rRNA	GGAATGATGGAACAGGACC	ATCCTTGGCAGATGCTTTC
pyruvate kinase (PK)	TTGACCACAGATGCCAGTTA	ACTCCCATTTTGACACCAAG
diacylglycerol O-acyltransferase homolog 1 (DGAT1)	TCGTATGCTGGAGGTTGC	GGATATGGTTTGGGATTGC
sterol regulatory element-binding protein 1 (SREBP1)	GCGGCACAGATACAACAAG	TTAGCAGTAGGACGAGACCC
sterol O-acyltransferase 2 (SOAT2)	GTGTATTTGGGGCTGTTCC	CAAGTATCGTTTCTCGGGTAT

Statistical analysis

Data of biochemical components and gene expression levels were processed using SPSS package (version #16.0), analysis of variance (one-way ANOVA) followed by LSD (Least Significant Difference) was performed on the data sets, with the mean value and S.E. Graphical work of Real-Time RT-PCR data was carried out using SigmaPlot (version #10).

Results

Glucose content in different tissues of L. vannamei during spermatophore regeneration

The glucose content in hemolymph, hepatopancreas, testes, and terminal ampoule are shown in Fig. 1. The results showed that, in regeneration group, glucose content in hemolymph increased significantly from day 2 to day 8 (1.27±0.10 to 1.86±0.16 mmol/L) and it significantly decreased at day 10 and day 12; in hepatopancreas, glucose

content significantly decreased from day 2 to day 12 (10.88±1.75 to 5.47±0.42 mmol/L); in the testes results differed from hepatopancreas: the glucose content in testes significantly increased from day 2 to day 12 (1.63±0.23 to 4.33±0.48 mmol/L); in terminal ampoule, glucose content increased first then decreased, the glucose content at day 4 was significantly higher than other times. In control group, glucose contents in different tissues at different times were not significantly different.

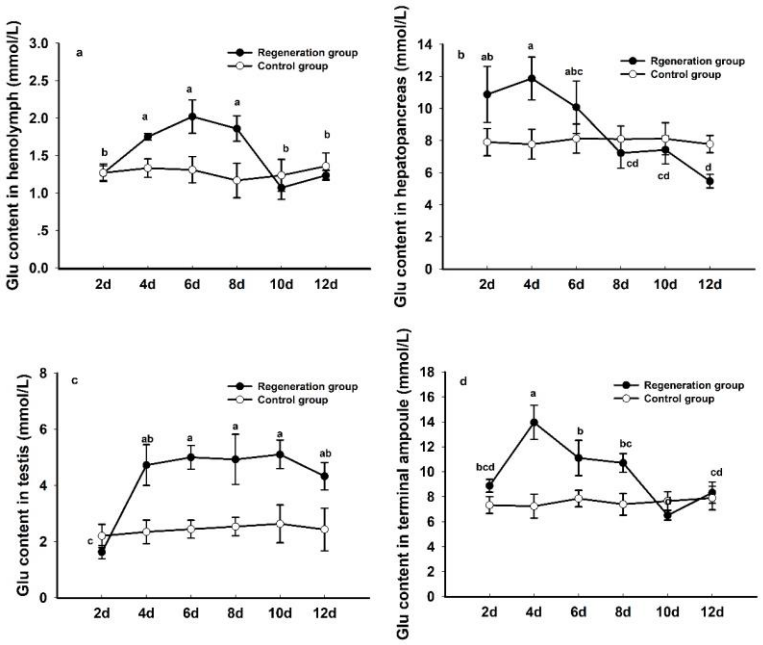

Figure 1 Glucose contents in different tissues of *L. vannamei* during spermatophore regeneration. Shrimps of both control group and regeneration group were sampled every 2 d, and they were dissected into hemolymph, hepatopancreas, testis and terminal ampoule. Results are means ± S.E. (n=6). Different letters indicate the mean values are significantly different between samples harvested at different times by LSD test (p<0.05) in regeneration group.

Triglyceride (TG) content in different tissues of L. vannamei during spermatophore regeneration

As shown in Fig. 2, in regeneration group, TG content in hemolymph did not significantly change; TG content in hepatopancreas significantly decreased from day 2 to day 12 (51.37±6.74 to 25.68±3.93 mmol/L); in testes, TG content significantly increased at day 4 and then decreased to the same level when compared with day 2. In terminal ampoule, the TG content was significantly lower at day 6 when compared with day 2. TG contents in different tissues of control group were not significantly different at different times.

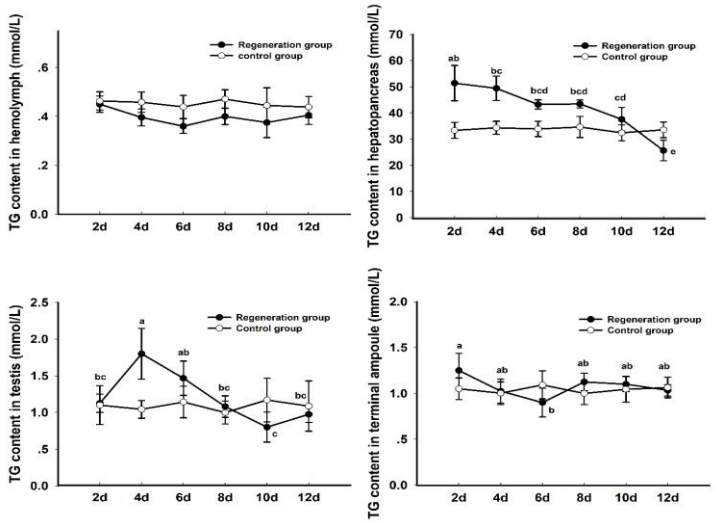

Figure 2 Triglyceride contents in different tissues of *L. vannamei* during spermatophore regeneration. Shrimps of both control group and regeneration group were sampled every 2 d, and they were dissected into hemolymph, hepatopancreas, testis and terminal ampoule. Results are means ± S.E. (n=6). Different letters indicate the mean values are significantly different between samples harvested at different times by LSD test (p<0.05) in regeneration group.

Total cholesterol (TC) content in different tissues of L. vannamei during spermatophore regeneration

TC contents in hemolymph, hepatopancreas, testes and terminal ampoule are listed in Fig. 3. In regeneration group, TC content in hemolymph did not change during the regeneration period; in hepatopancreas, TC contents significantly increased at day 4 and then decreased (2.25±0.21 to 3.36±0.39 to 2.31±0.31 mmol/L); TC content in testes significantly decreased during spermatophore regeneration (2.33±0.33 to 1.40±0.12±); in terminal ampoule, TC content significantly increased from day 2 to day 4 and then kept relative stable level when compared with day 2. TC contents in different tissues of control group were not significantly changed.

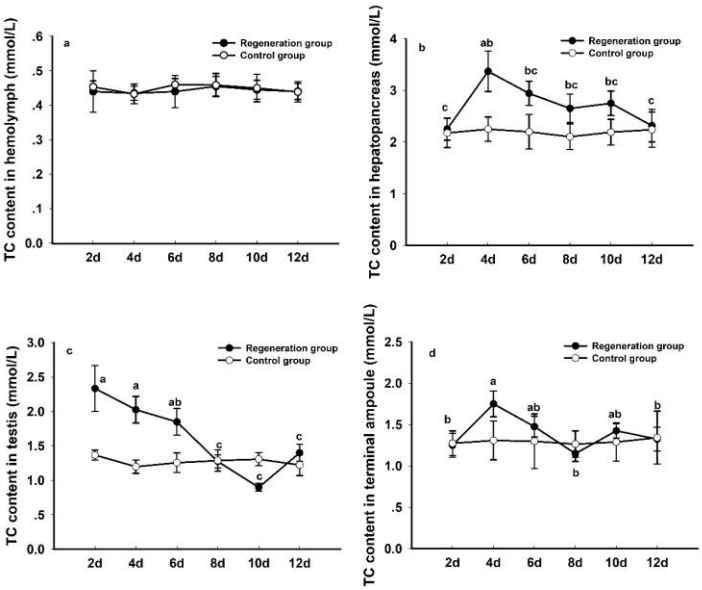

Figure 3 Total cholesterol contents in different tissues of *L. vannamei* during spermatophore regeneration. Shrimps of both control group and regeneration group were sampled every 2 d, and they were dissected into hemolymph, hepatopancreas, testis and terminal ampoule. Results are means ± S.E. (n=6). Different letters indicate the mean values are significantly different between samples harvested at different times by LSD test (p<0.05) in regeneration group.

Expression levels of glycolysis and lipogenesis related genes in hepatopancreas during spermatophore regeneration

Expression profiles of four glycolysis and lipogenesis related genes in hepatopancreas were measured. The expression level of pyruvate kinase (PK) significantly decreased during day 6 to day 12, although it was significantly elevated at day 8 but was significantly lower than expression level at day 2 or day 4 (Fig. 4a). The expression level of TG synthesis related gene DGAT1 during day 2 to day 10 was significantly higher than day 12 and the transcript level at day 8 was highest (Fig. 4b).

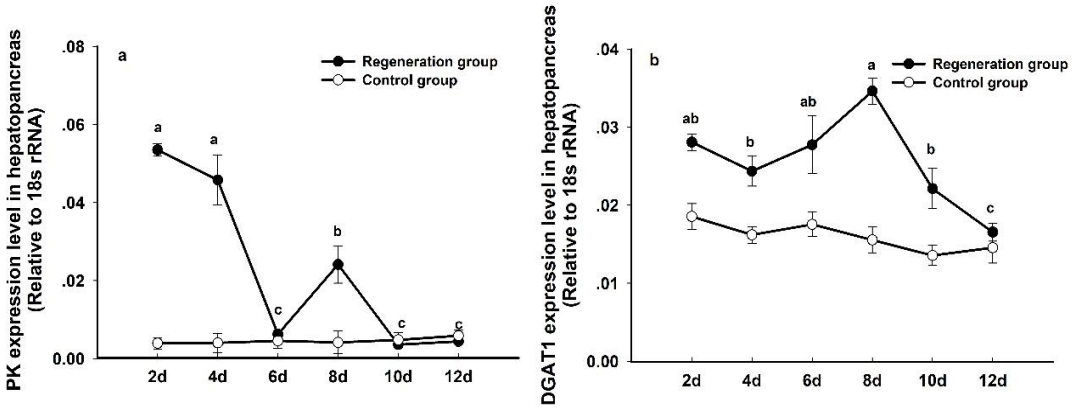

Figure 4 Expression profiles of pyruvate kinase (PK) and diacylglycerol O-acyltransferase homolog 1 (DGAT1) in hepatopancreas during spermatophore regeneration. Shrimps of both control group and regeneration group were sampled every 2 d, and hepatopancreas was sampled for Real-Time RT-PCR analysis. Results are means ±

S.E. (n=6). Different letters indicate the mean values are significantly different between samples at different times by LSD test (p<0.05) in regeneration group.

The expression levels of a transcription factor or sterol regulatory element-binding protein (SREBP1) and a gene named sterol O-acyltransferase 2 (SOAT2) which were involved in cholesterol biosynthesis were also determined. The expression level of SREBP1 was significantly higher during day 2 to day 8 than day 10 to day 12 (Fig. 5a). The expression level of SOAT2 was significantly up-regulated from day 2 to day 8 and then the transcript level was down-regulated from day 10 to day 12 (Fig. 5b). The expression levels of PK, DGAT1, SREBP and SOAT2 of control group were not significantly changed during the regeneration period.

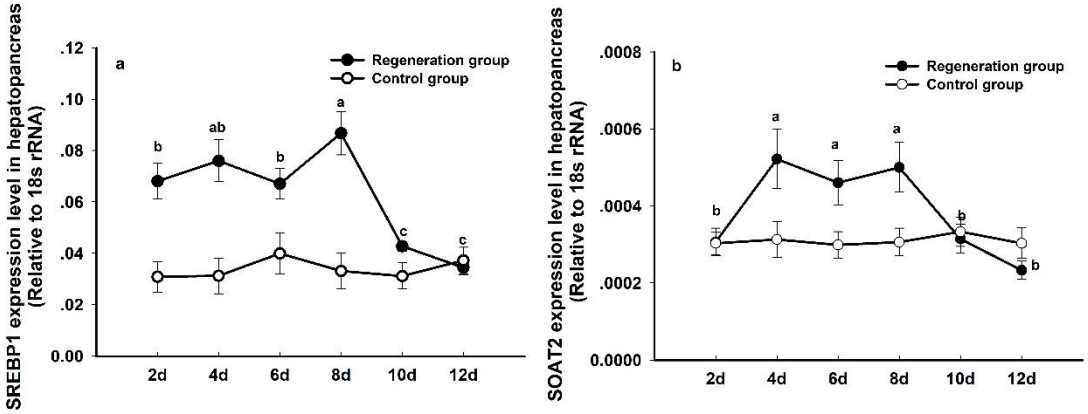

Figure 5 Expression profiles of sterol regulatory element-binding protein 1 (SREBP1) and sterol O-acyltransferase 2 (SOAT2) in hepatopancreas during spermatophore regeneration. Shrimps of both control group and regeneration group were sampled every 2 d, and hepatopancreas was sampled for Real-Time RT-PCR analysis. Results are means ± S.E. (n=6). Different letters indicate the mean values are significantly different between samples isolated from different times by LSD test (p<0.05) in regeneration group.

Discussion

During spermatophore regeneration of shrimp, nutrition and energy are important, the availability of optimal diets and environment condition are identified as crucial factors for sexual maturation and reproduction. Glucose is important for physiological and biochemical processes in all organisms. In spermatophore regeneration group, glucose content in hemolymph, testes, and terminal ampoule increased from day 2 to day 8, while the glucose content in hepatopancreas significantly decreased. This indicates that glucose in hepatopancreas was transported to testes and terminal ampoule via hemolymph. When compared with the control group, glucose content in the hepatopancreas was higher than in the control from day 2 while glucose content in testes or terminal ampoule were higher than in the control from day 4, which suggests that the source of glucose was in the hepatopancreas. Furthermore, glucose content in terminal ampoule was relatively higher than in the testes, which suggests that glucose is the main energy source in this tissue. In hepatopancreas, the expression level of PK was significantly higher during the first 4 d which indicated glucose was consumed to produce energy. From day 6 to day 12, the expression level was significantly reduced which suggests that glycolysis was inhibited to provide glucose for other tissues.

Triglyceride (TG) is the energy source of eggs and nauplii of shrimp, and the energy source for the reproductive process of crustaceans (Ward et al., 1979; Middleditch et al., 1979). Cholesterol is an important component of cell membrane and is also the precursor substance of a variety of bioactive molecules. Cholesterol is already known as the precursor substance of sex hormone of crustaceans (Wang et al., 2004). It regulates shrimp sex maturation and reproductive process (Quackenbush, 1986). When compared with the control group, TG and TC contents were relative higher in regeneration group during early to middle periods which explains why the synthesis of these two components were upregulated. The expression patterns of DGAT1, SREBP, and SOAT2 in regeneration

group was consistent with the content change in hepatopancreas. Furthermore, TG was highest in the hepatopancreas and its decrease during the regeneration period indicated it was consumed for spermatophore regeneration. TC content in testes was reduced during spermatophore regeneration which suggest that TC is the sex hormone in this physiological process.

The results of this study showed that glucose, TG, and TC play different but important roles in spermatophore regeneration process, and the expression patterns of glycolysis and lipogenesis related genes are consistent with the biochemical component changes.

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