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Feasibility of Ripening *Penaeus monodon* by using *Pheretima tschiliensis* as a Bait and its Verification by Vitellogenin Gene Expression

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ABSTRACT

Traditional broodstock diets, being susceptible to virus attacks or carrying the virus to parent shrimps, bring a huge bio-security risk to shrimp breeding. This study was carried out to determine the feasibility of *Pheretima tschiliensis* as a replacement of traditional bait to accelerate the maturity of parent shrimps of *Penaeus monodon*, which was verified by the dynamics of vitellogenin gene expression in hepatopancreas and ovary of *P. monodon*. The parent shrimps fed on four different diets including commercial parent shrimps feed (Diet A), *Pheretima tschiliensis* combination bait (Diet B), *Pheretima tschiliensis* (Diet C) and *Nereis succinea* combination bait (Diet D), and the effect of each diet on the maturity of parent shrimps was evaluated by testing the gonad weight increase rate, eggs amount, average spawning time and levels of VTG mRNA expression. The results showed that *P. tschiliensis*, as a closely related terrestrial species of *N. succinea*, had the best effect on maturation of parent shrimps. The highest gonad weight increase rate of 1845.28% and the largest consecutive spawning rate with average value of 70.66% were both in groups fed on *P. tschiliensis*, with no significant difference from the groups fed on *N. succinea* combination bait. The dynamics of VTG mRNA level were observed in hepatopancreas and ovary of *P. monodon* with the trend of increasing firstly and then decreasing, which reflected reproductive performance of the parent shrimps and was suggested to be an indicator of reproductive performance for selective breeding. As a result, *P. tschiliensis* is a suitable substitute of *N. succinea* based on its effect on the reproductive traits and VTG mRNA expression level in parent shrimps of *P. monodon*.

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Authors' Contribution
SGJ, SJ and FLZ designed the study. QBY and JHH took part in the execution of study. LSY, QBY, and MG Z implemented the study and were involved in sampling as well as testing. FLZ, SJ and SGJ drafted the manuscript.

Key words
Vitellogenin, Gene expression, Feasibility of ripening, *Penaeus monodon*, *Pheretima tschiliensis*

INTRODUCTION

The black tiger shrimp (*Penaeus monodon*, Crustacea, Decapoda, Penaeidae) is an economically and globally important marine species in China (Song *et al*., 2018, 2019), *Noereis succinea*, which is rich in unsaturated fatty acids such as EPA and DHA, is widely used as a kind of ripening food for *P. monodon* (Shi *et al*., 2016). *P. monodon* can also achieve good ripening effect with natural biological baits such as squid and oyster. However, *N. succinea*, squid, oyster and *P. monodon* are all sea water species, which are susceptible to infection or carry viruses that common to shrimp, so there are huge biological safety risks in shrimp breeding and seedling breeding (Shi *et al*., 2016). Therefore, it is urgent to develop safe, high-quality and high-efficiency ripening bait for *P. monodon*.

Pheretima tschiliensis, also known as earthworms, is similar to *N. succinea* in taxonomic status, living habits and organizational structure (Reid *et al*., 2005). Some scholars have shown that the protein and unsaturated fatty acid contents of *P. tschiliensis* were also abundant (Jayanthy *et al*., 2014). The protein content of dried products reached 62% and the fat content was more than 18%, the unsaturated fatty acid generally accounted for more than 58% of the total fat (Hickman *et al*., 2008). Moreover, the composition of amino acid and fatty acid of *P. tschiliensis* was similar to that of *N. succinea* and oysters (Roy *et al*., 2005). *P. tschiliensis* is terrestrial annelid with low probability of pathogenic cross-infection with shrimp cultured in seawater and high biological safety. Therefore, the risk of infecting virus with fresh food could be avoided

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and the cost of parent shrimp breeding could be greatly reduced if the *P. tschiliensis* could replace the *N. succinea* as the prey for the ripening of parent shrimp of *P. monodon*.

The formation and accumulation of yolk substances is an important event in the oogenesis of egg laying animals. During ovarian maturation, oocytes rapidly accumulate a large number of vitellin precursors, Vitellogenin (VTG), and then divide into mature Vitellin (Vt). Vt is the main component of yolk substance, which provides a lot of nutrition for the development of oocyte and embryo, and plays an important biological function (Rodgers *et al*., 1993). Both hepatopancreas and ovary are the sites of yolk proteogen synthesis in shrimp, but the expression level of yolk proteogen changes dynamically at different stages of development (Yano *et al*., 1987; Browdy *et al*., 1990; Fainzilber *et al*., 1992; Chang *et al*., 1995; Vazquez *et al*., 2002). It has been confirmed that VTG content in hepatopancreas of adult shrimp before eyestalk removal reflects ovarian development of female shrimp, so VTG gene is considered as an important gene controlling reproductive trait (Jasmani *et al*., 2000; Arcos *et al*., 2003; Tsutsui *et al*., 2005; Okumura *et al*., 2007; Raviv *et al*., 2006; Ibarra *et al*., 2007). One *VTG* gene has been cloned from *Litopenaeus vannamei* and the VTG content in hepatopancreas has significant genetic correlation with the average egg diameter of shrimp (Ibarra *et al*., 2009). From July to September, 2018, the effects of four diets on the ripening of parent shrimp *P. monodon* were studied and compared. The expression of VTG in the process of intensified cultivation and ripening of parent shrimp with four combinations of diets was tracked by fluorescence quantitative PCR test. The ripening effect of each group was judged by the change of VTG expression.

MATERIALS AND METHODS

Test material

The parent shrimp used in the experiment was cultured in the breeding base of *P. monodon* genetic breeding center in the Ministry of Agriculture and Rural Areas of the People's Republic of China. Four kinds of parent shrimp baits were commercial parent shrimp feed (Diet A, Guangdong Haida Group), *Pheretima tschiliensis* combination bait (Diet B, 50% squid + 50% *Pheretima tschiliensis*), *Pheretima tschiliensis* (Diet C) and *Noereis succinea* combination bait (Diet D, 50% squid + 50% *Noereis succinea*). Samples were taken before nutritional enrichment (sample number A1, B1, C1, D1), 5 days after enrichment (sample number A2, B2, C2, D2), 15 days (sample number A3, B3, C3, D3), 25 days (sample number A4, B4, C4, D4), and 5 days after unilateral eyestalk ripening operation (sample number A5, B5, C5, D5). Each time, 5 ind of well-developed females were selected and their hepatopancreas and ovarian samples were taken in appropriate amounts, and then stored in RNAlater for RNA extraction.

Parent shrimp management

The parent shrimp of *P. monodon* were cultured in a circular cement pond of 20 m^2 with a water depth of 60 cm. Breeding workshop used black opaque plastic film as shading treatment. The water temperature of the shrimp culture pond was kept at 28±0.5℃. The air was continuously aerated to keep the dissolved oxygen in the water not less than 6 $mg \cdot L^{-1}$. A total amount of 50% water was renewed daily at 10:00 and 15:00 every day. Feeding time was 6:30, 10:30, 15:30, 19:30 and 22:30 every day, and the feeding amount was kept within 2 hours to complete the feeding of *P. monodon*.

Total RNA isolation and synthesis of the cDNA

Total RNA was isolated from the examined tissue (pure weight about 50 mg) of the shrimps using Trizol (Invitrogen, Japan) reagent following the protocol of the manufacturer, and resuspended in DEPC-treated water and stored at -80℃.

The cDNA was synthesized by TaKaRa gDNA Eraser kit (TaKaRa code: DRR047A). The synthesis reaction consisted of two steps, 1) RNA 1000ng, 5×gDNA Eraser Buffer2μL, gDNA Eraser1μL and RNase Free dH_2O were added to 10μL system, 2) In the reaction system of 20μL, 5×Prime Script Buffer (for Real Time)4μL, Prime Script RT Enzyme Mix I 1μL, RT Primer Mix1μL, the first step of the reaction solution 10μL, RNase Free dH_2O supplemented to 20μL. The retroviral DNA template was diluted at a ratio of 1:1 and stored at - 20℃.

Real-time PCR

Primer 5.0 was used to design primers (Table I) based on the sequence of VTG and 18S-rRNA of *P. monodon* in Gen Bank. The primers were synthesized by Shanghai Yingjun Biotechnology Co., Ltd.

THUNDERBIRD qPCR Mix containing SYBR Green I Dye fluorescent dye was used for Real-time PCR in ABI 7500-fast instrument. Reaction system: 1.0 μL cDNA, 10 μL 2 × THUNDERBIRD qPCR Mix (contain ROX), 0.5 μL each primer (0.2 μM), replenishing water to total volume 20 μL (repeat 3 tubes per sample). Reaction conditions: pre-denaturation: 94℃ 30 s, 40 cycles: 94℃ 10 s, 56℃ 30 s,72℃ 30 s, 72℃ 10 min.

Table I. Primer sequences and parameters used in RT-PCR.

Gene	Primer name	Primer sequences	Register number
Vitellogenin	Vitellogenin -F Vitellogenin -R	GATGGTTTGGGATCTGAGGAACA GGAAACTTATGGCATTAACAGGGA	EU492542
18S-rRNA	18SrRNA-F 18SrRNA-R	TTGTACGAGGATCGAGTGGA ATGCTTTCGCAGTAGGTCGT	GK26736

Table II. Reproductive performance of parent shrimps.

Group	Gonad weight gain rate/%	Egg production/10 thousands	Average spawning times	Continuous oviposition rate/%
Diet A	820.87±213.43^{b}	75.25±12.33^{b}	1.54±0.24^{c}	28.54±1.57^{c}
Diet B	1308.47±43.83^{ab}	87.68±14.68^{a}	2.07±0.57^{b}	65.84±2.66^{ab}
Diet C	1845.28±109.38^{a}	88.91±20.67^{a}	2.24±0.11^{ab}	70.66±1.68^{a}
Diet D	1753.64±163.87^{a}	95.41±17.28^{a}	2.59±0.09^{a}	65.11±9.29^{ab}

Data analysis

The fluorescence quantitative analysis of target genes was automatically carried out by relative CT (2-ΔΔCT method) with iQ™5 Optical System Software. SPSS 19.0 software was used for data analysis and statistics and ANOVA was used to analyze the data. If there were significant differences between the processing, Duncan's multiple comparisons were compared. The significant level was 0.05.

RESULTS

Comparison of reproductive performance of parent shrimp with different diet combinations

By comparing the gonadal development of parent shrimp before and after Unilateral Eyestalk removal, it was found that the gonadal development of parent shrimp in different groups was different, but the degree of gonadal development was different. The gonad development of the parent shrimp in group C was the best, followed by the group D and group B, and the commercial parent shrimp feed group (Group A) was the worst. Significant analysis showed that there was no significant difference in gonad gain rate between group C, group D and group B ($P > 0.05$), but significant difference between group A and group C ($P < 0.05$).

The spawning rate of parent shrimp in different diet groups was different. Group C had the largest number of consecutive mature oviposition individuals and the largest rate of consecutive oviposition; Group B had the second rate of consecutive oviposition, Group A had the lowest rate of consecutive oviposition. The reproductive performance of parent shrimp in each group is shown in Table II.

VTG mRNA expression changes

The VTG mRNA content in the ovary of the reserve broodstock before the fortification was defined as 1, and the relative expression levels of VTG mRNA in the hepatic pancreas and ovary samples of the other groups were calculated. Under the premise of ensuring bio-safety, the *N. succinea* combination is the best bait combination in the current production application. Therefore, the changes of VTG mRNA expression are sampled at different stages of enhanced cultivation and ripening of the *N. succinea* combination. The results showed that VTG mRNA was induced and expressed in hepatopancreas and ovary after 15 days of broodstock feeding. The expression of VTG mRNA in hepatopancreas and ovary increased first and then decreased. The expression of hepatic pancreas enhanced. On the 15th day, the highest value was reached, and the ovary reached the highest value on the 25th day of intensive culture (Fig. 1). At this time, in addition to the ovarian self-synthesis, VTG mRNA in the ovary should be partially synthesized in the pre-developmental hepatopancreas. The mRNA is gradually transported into the ovaries. After the eye-cutting stalk is the rapid maturity of the broodstock, the expression of VTG mRNA in the hepatopancreas and ovary is not too high. At this stage, the VTG mRNA should be translated and processed to form mature yolk protein.

VTG mRNA expression

The healthy reserve shrimp with 14-month-old, body length 21.64±1.38 cm, body weight 167.05±6.82 g was intensively cultured on the 15th day with parent shrimp bait. Its gonad entered the first round of rapid growth period. At this time, yolk began to accumulate in oocyte, and VTG gene in hepatopancreas and ovaries entered a high

expression state (Fig. 2). On the 25th day of cultivation, the oocytes were further developed, the double-layer follicular cells developed and matured, and a large amount of VTG mRNA was synthesized. The expression level in the ovary reached the maximum, while the expression in the hepatopancreas decreased compared with the 15th day of cultivation (Fig. 3). After cutting the eye stalk, the female prawn enters the rapid maturity stage, and the yolk formation rate is the fastest in the oocyte. At this time, the rapid synthesis of protein consumes VTG mRNA faster than its mRNA synthesis, and the VTG mRNA content in hepatopancreas and ovary is reduced (Fig. 4).

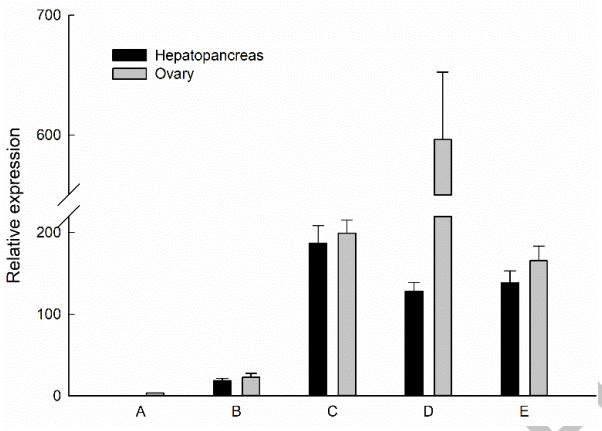

Fig. 1. Relative expression of VTG mRNA in hepatopancreas and ovary of parent shrimps.
Note: In the figure, A represents the time before strengthening cultivation, B represents the time of 5th day after strengthening cultivation, C represents the time of 15th day after strengthening cultivation, D represents the time of 25th day after strengthening cultivation, E represents the time of 5th day after eyestalk removal.

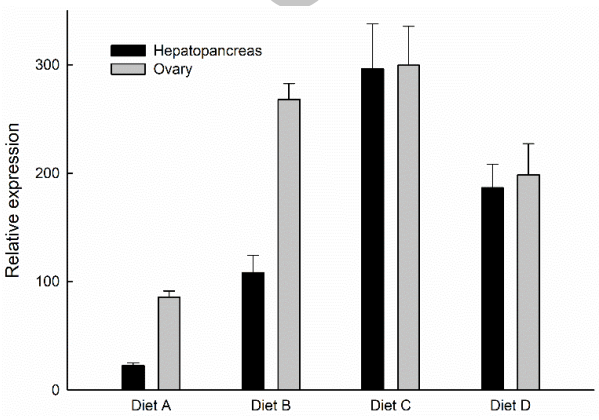

Fig. 2. Relative expression of VTG mRNA in hepatopancreas and ovary of parent shrimps on the 15th day after strengthening cultivation.

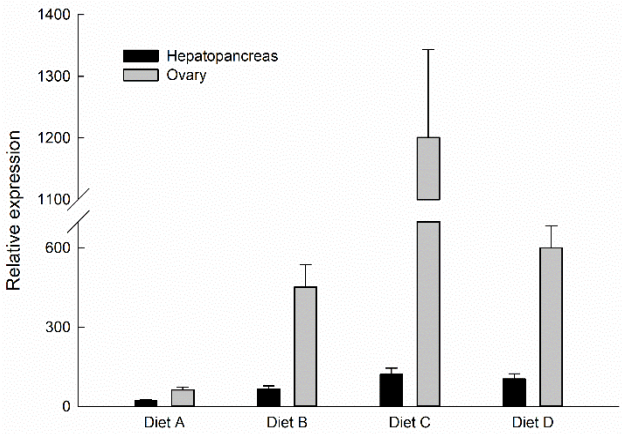

Fig. 3. Relative expression of VTG mRNA in hepatopancreas and ovary of parent shrimps on the 25th day after strengthening cultivation.

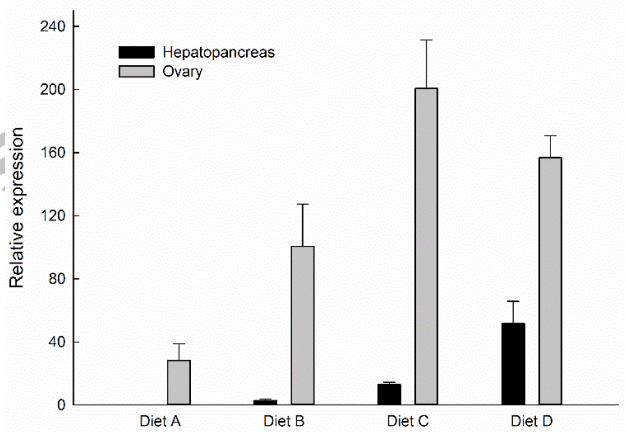

Fig. 4. Relative expression of VTG mRNA in hepatopancreas and ovary of parent shrimps on the 5th day after eyestalk ablation.

DISCUSSION

The possibility of VTG mRNA as indicator for breeding performance of P. monodon

In the experiments of the four bait groups, the expression of VTG mRNA in the hepatopancreas and ovary of *P. monodon* was basically consistent with the observed ripening results. The VTG mRNA expression of shrimp was significantly higher in Group B, Group C and Group D than that of the Group A. The gonad weight gain rate, continuous egg production rate and other reproductive performance were also higher than that of the Group A, which indicating that VTG mRNA can reflect the reproductive performance of shrimp, and hopefully become breeding performance breeding index of broodstock.

VTG mRNA is dynamically expressed in hepatopancreas and ovary of P. monodon

The yolk proteogen synthesis site of shrimp has been reported to be hepatopancreas and ovaries, but the results obtained by different researchers are inconsistent. The ovary of *Penaeus japonicus* is a synthetic site for yolk protein, and the hepatopancreatic extract does not precipitate with the vitellin antibody, so the hepatopancreas is not considered to be involved in the synthesis of yolk (Yano *et al*., 1987). The results of Browdy *et al*. (1990) showed that the ovary is the only yolk proteogen synthesis site of short-spotted shrimp. Fainzilber *et al*. (1992) suggested that ovary is the main site of yolk protein synthesis in *Penaeus scmisulcatus*. The hepatopancreas in the early stage of yolk synthesis also have the function of synthesizing vitellogen, but the synthesis amount is less than 15% of the total protein and it has less effect on the occurrence of yolk. Chang and Jeng (1995) found a vitellogenin in the hemolymph of *Fenneropenaeus chinensis*, indicating that there may also be exogenous synthetic sites in *Fenneropenaeus chinensis*. In the early stage of *Penaeus indicus* ovarian development, the expression of VTG in hepatopancreas and gonads increased, and then decreased in the late stage of ovarian development, but the content of yolk protein in ovary was still increasing rapidly, indicating that hepatic pancreas can synthesize vitellogenin, but mature yolk protein is not only dependent on hepatic pancreas synthesis (Vazquez Boucard, *et al*., 2002).

In this study, VTG mRNA was found in hepatopancreas and ovarian tissues of *P. monodon*. The expression trends in hepatopancreas and ovary increased first and then decreased with ovarian development. The detected VTG in hepatopancreas was lower than that in ovaries, and the peak value of VTG in hepatopancreas was 10 days earlier than that in ovaries. In the early stage of ovarian development, the follicular cells in the main synthesis site of VTG mRNA have not matured, and the synthesis of VTG mRNA in hepatopancreas accounts for a large proportion. It is speculated that VTG mRNA is transported to the ovary after synthesis of hepatopancreas, and the synthesis of yolk material is supplied. After the cells matured, VTG mRNA was mainly synthesized by follicular cells, and the expression level in hepatopancreas gradually decreased.

The P. tschiliensis *can be used as a bait for the cultivation and ripening of* P. monodon

Comparing and analyzing the data of Group B, Group C and Group D, the results showed that *P. tschiliensis* can be used as a substitute of *N. succinea* for the intensive cultivation and ripening of broodstock in the production of *P. monodon*. The feeding amount of *P. tschiliensis* in Group B was smaller than that in group C. The reproductive performance of *P. monodon* in Group B was not as good as that in Group C, indicating that *P. tschiliensis* did have nutrient-promoting nutrients and had a dose effect during the ripening process.

The *P. tschiliensis* and the *N. succinea* are closely related species and may contain similar nutrients that can be ripened. From the test results, the reproductive performance of *P. monodon* was similar to that of the Group C and the Group D. Compared with Group D, Group C has a high rate of gonads, rapid spawning, and insufficient number of consecutive spawning, which may be related to the rapid decline of VTG mRNA in the hepatopancreas and the lack of follow-up supply (Priya *et al*., 2009). The female shrimp in Group A has greater advantages in hepatopancreas index and hepatopancreas weight gain, but there is no corresponding advantage in gonadal weight, gonadal index and gonad weight gain. The expression of VTG gene closely related to performance is also very low. It can be seen that the nutrient content of commercial broodstock feed mainly acts to promote the growth of shrimp muscle or hepatopancreas. This indicates that the commercial broodstock feed lacks a certain ingredient to promote the conversion of nutrients in the female shrimp into the substances required for gonadal development.

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Conflict of interest

The authors have declared no conflict of interests.

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