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# Modulation of innate immunity and gene expressions in white shrimp *Litopenaeus vannamei* following long-term starvation and re-feeding

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ABSTRACT

The survival rate, weight loss, immune parameters, resistance against *Vibrio alginolyticus* and white-spot syndrome virus (WSSV), and expressions of lipopolysaccharide- and ß-glucan-binding protein (LGBP), peroxinectin (PX), prophenoloxidase-activating enzyme (ppA), prophenoloxidase (proPO) I, proPO II, α2-macroglobulin (α2-M), integrin ß, heat shock protein 70 (HSP70), cytosolic manganese superoxide dismutase (cytMnSOD), mitochondrial manganese superoxide dismutase (mtMnSOD), and extracellular copper and zinc superoxide dismutase (ecCuZnSOD) were examined in the white shrimp *Litopenaeus vannamei* (8.18 ± 0.86 g body weight) which had been denied food (starved) for up to 14–28 days. Among shrimp which had been starved for 7, 14, 21, and 28 days, 100%, 90%, 71%, and 59% survived, and they lost 3.2%, 7.3%, 9.2%, and 10.4% of their body weight, respectively. Hyaline cells (HCs), granular cells (GCs, including semi-granular cells), the total haemocyte count (THC), phenoloxidase (PO) activity, respiratory bursts (RBs), and SOD activity significantly decreased in shrimp which had been starved for 1, 1, 1, 5, 14, and 3 days, respectively. The expression of integrin ß significantly decreased after 0.5–5 days of starvation, whereas the expressions of LGBP, PX, proPO I, proPO II, ppA, and α2-M increased after 0.5–1 days. Transcripts of all genes except ecCuZnSOD decreased to the lowest level after 5 days, and tended to background values after 7 and 14 days. Cumulative mortality rates of 7-day-starved shrimp challenged with *V. alginolyticus* and WSSV were significantly higher than those of challenged control-shrimp for 1–7 and 1–4 days, respectively. In another experiment, immune parameters of shrimp which had been starved for 7 and 14 days and then received normal feeding (at 5% of their body weight daily) were examined after 3, 6, and 12 h, and 1, 3, and 5 days. All immune parameters of 7-day-starved shrimp were able to return to their baseline values after 5 days of re-feeding except for GCs, whereas all parameters of 14-day-starved shrimp failed to return to the baseline values even with 5 days of re-feeding. It was concluded that shrimp starved for 14 days exhibited three stages of modulation of gene expression, together with reductions in immune parameters, and decreased resistance against pathogens.



## 1. Introduction

White shrimp *Litopenaeus vannamei* and tiger shrimp *Penaeus monodon* are the dominant penaeid shrimps currently being cultured worldwide. However, shrimp farming has suffered problems linked to deteriorating and stressful environments, subsequently resulting in disease incidences of viral and bacterial etiologies [1,2]. The bacterium *Vibrio alginolyticus* isolated from diseased white shrimp which exhibited whitish musculature and lethargy is considered to be a secondary and opportunistic pathogen, and commonly leads to mortality of shrimp living in stressful temperature and salinity conditions [3–5]. In addition, white-spot syndrome virus (WSSV) is considered to be an important, extremely virulent pathogen, and may cause mortality within a few days after infection [2].

Shrimp farming has increased exponentially since the last two decades, and with 75% of shrimp production coming from the eastern hemisphere due to extensive exploitation of mangrove area in this region in the year 2008 [6]. Shrimp farming is almost performed entirely in land-based ponds, and farmers are likely to increase the land usage by increasing the stocking density. In an intensive pond, feeding has become a major management, and feeding strategy is an important practice that leads to growth, health, survival, and successful shrimp farming [7,8]. Overfeeding may cause deteriorated pond environment, whereas unequal feeding or insufficient feeding may lead to size variation of shrimp. The large and strong shrimp can forcibly occupy the food, and cause devoid of food for small shrimp. Shrimp which have been starved or deprived of food are easily attacked by the opportunistic pathogen, susceptible to disease outbreak, and become a disease breeding ground. In teleost, survival and weight loss

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during the starvation have been reported in European eel *Anguilla anguilla* and Atlantic salmon *Salmo salar* [9,10]. However, little is known on survival, weight loss, and decrease in immunity of shrimp during starvation period. We assume that starved shrimp may weaken its immunity, and lead to mortality infected by pathogen.

In penaeid shrimp, circulating haemocytes play crucial roles in the innate immune defence system [11]. They are involved in a pattern-recognition system, phagocytosis, prophenoloxidase (proPO)-activating system, encapsulation, nodule formation, and release of antimicrobial peptides and lysozymes [12]. It is known that the proPO cascade is triggered by the recognition and binding of pattern-recognition proteins (PRPs) with pathogen-associated molecular patterns (PAMPs) [13–15]. The lipopolysaccharide- and ß-glucan-binding protein (LGBP) is an important PRP [16,17]. Several enzymes including prophenoloxidase, proPO activating enzyme (ppA), peroxinectin (PX), and proteinase inhibitors such as $\alpha$2-macroglobulin ($\alpha$2-M) are important proteins involved in proPO cascade [14,18]. During the course of phagocytosis, superoxide anion is released, and is commonly known as respiratory bursts (RBs) [19]. Superoxide dismutase (SOD) catalyzes superoxide anions to molecular oxygen and hydrogen peroxide and provides antioxidant protection [20]. Peroxinectin (PX), integrin, and SOD are involved in the proPO cascade and post-phagocytosis leading to the generation of cytotoxic products [15,16]. In addition, heat shock proteins (HSPs) are known to be induced under stressful conditions, and HSP70 functions as a fundamental chaperon molecule in cellular physiological processes [21,22].

In mammal, nutritional restriction stress or starvation sometimes augments innate immunity in the function of macrophages and lymphocytes [23]. In decapod crustaceans, the haemocyte count, PO activity, and RBs are affected by moulting stage and nutritional status [24,25]. However, little is known about innate immunity, immune-related gene expressions, and susceptibility to both *Vibrio* and WSSV in shrimp deprived of food or during a starvation period which commonly occurs in pond feeding management and during transportation [7,8]. Accordingly, in this study, eight experiments were conducted. We examined (1) the survival rate, (2) the weight loss, (3) immune parameters, and (4) gene expressions of shrimp during starvation periods of various lengths; determined (5) susceptibility to *V. alginolyticus* and (6) WSSV in starved shrimp; and examined (7) weight recovery and (8) the recovery of immune parameters of starved shrimp that subsequently received normal feeding. Hyaline cells (HCs), granular cells (GCs, including semi-granular cells), the total haemocyte count (THC), PO activity, RBs, and SOD activity were used as indicators of immune parameters [26].

## 2. Materials and methods

### 2.1. White shrimp L. vannamei

White shrimp *L. vannamei* post-larvae ($PL_{5-6}$) obtained from a hatchery farm in Kaohsiung, Taiwan were released into fiberglass tanks filled with filtered natural seawater of 35‰ salinity at room temperature. They were fed live *Artemia* nauplii, and later an artificial diet (36% protein, Tairou Feed, Tainan, Taiwan) until they grew to a weight of about 8–9 g. The IQ2000$^{TM}$ WSSV Detection and Prevention System (GeneReach Biotechnology Corp., Taichung, Taiwan), based on a polymerase chain reaction (PCR) technique, was applied to identify and confirm that shrimp were not infected with WSSV [27]. During the experiment, faeces were removed daily by siphoning.

### 2.2. Experimental design

Eight experiments were conducted. They were (1) survival rate, (2) weight loss, (3) immune parameters assays, and (4) gene expression of shrimp during starvation periods of various lengths; (5) resistance against *V. alginolyticus* in shrimp which had been starved for 7 days and (6) resistance against WSSV in shrimp which had been starved for 7 days; and (7) weight recovery and (8) immune parameters assays of shrimp which had been starved for 7 and 14 days, and then received normal feeding. Around 1000 shrimp with a mean weight 8.18 $\pm$ 0.86 g were used for the experiment. Only shrimp in the intermoult stage were used for the experiments. The moult stage was determined by examining the uropoda, in which partial retraction of the epidermis could be distinguished [28]. During the experiment, the water temperature was maintained at 22–26 °C, and the concentrations of ammonia-N and nitrite-N were 0.09 and 0.02 mg $l^{-1}$, respectively measured by the phenolhypochlorite [29] and sulfanimide methods [30].

### 2.3. Survival rate and weight loss of shrimp during the starvation period

To determine survival rates of shrimp during the starvation period, 100 shrimp were released into 500 l of aerated seawater. Survival of shrimp was checked daily for up to 28 days. To determine the weight loss of shrimp during the starvation period, 24 cages were used, and each cage housed one shrimp. The cages were suspended in 500 l of aerated seawater. Shrimp were sampled and weighed after 0, 1, 3, 5, 7, 14, 21, and 28 days.

### 2.4. Weight recovery of shrimp which had been starved for 7 and 14 days and then subsequently received normal feeding

Twenty-four cages were used, and each cage housed one shrimp. The cages were suspended in 500 l of aerated seawater. After 7 and 14 days of starvation, shrimp were again fed normally (5% of body weight daily) at 10:00 and 18:00. Shrimp were sampled and weighed before initiation of starvation, after 7 and 14 days of starvation, and after 1, 3, 5, and 7 days of subsequent re-feeding.

### 2.5. Immune parameters and gene expressions of shrimp during the starvation period

One hundred and twenty shrimp which had been reared in 500 l of aerated seawater were used for the study of immune parameters, and another 120 shrimp which had been reared in 500 l of aerated seawater were used for the study of gene expressions. After 0, 3, 6, and 12 h, and 1, 3, 5, 7, 14, 21, and 28 days of starvation, eight shrimp were sampled, and immune parameters were measured. After 0 and 12 h, and 1, 3, 5, 7, and 14 days of starvation, 10 shrimp from each time were sampled, and gene expressions were determined.

### 2.6. Immune parameter assays of shrimp which had been starved for 7 and 14 days and then received normal feeding

One hundred and twenty shrimp which had been reared in 500 l of aerated seawater were used for the immune parameter assays of shrimp which had been starved for 7 days and then received normal feeding. Another 120 shrimp which had been reared in 500 l of aerated seawater were used for the immune parameter assays of shrimp which had been starved for 14 days and then received normal feeding. After 0, 3, 6, and 12 h, and 1, 3, and 5 days of re-feeding, eight shrimp from each time were sampled and used to determine immune parameters.

### 2.7. Measurements of immune parameters

Haemolymph sampling, preparation of diluted haemolymph, and haemocyte counts followed previously described procedures [31]. The haemolymph–anticoagulant mixture (diluted haemolymph) was placed in three tubes. Tubes contained 500, 1000, and 1000 µl of diluted haemolymph, and were respectively used to measure: (1)

the haemocyte count and RBs, (2) PO activity, and (3) SOD activity. A drop of diluted haemolymph from the first tube was placed in a haemocytometer to measure HCs, GCs, and the THC using an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems,Wetzlar, Germany). The remainder of the diluted haemolymph mixture was used for subsequent tests.

PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) as previously described [32]. From the second tube, 1000 μl of diluted haemolymph was centrifuged at 800*g* and 4 °C for 20 min. Details of the measurements were previously described [31]. The optical density of the shrimp's PO activity at 490 nm was measured using a spectrophotometer (model U-2000, Hitachi, Tokyo, Japan). PO activity was expressed as dopachrome formation per 50 μl of haemolymph.

RBs of haemocytes were quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anions, as previously described [31,33]. The optical density of a shrimp's RBs at 630 nm was measured using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). RBs were expressed as NBT-reduction per 10 μl of haemolymph.

SOD activity was measured by its ability to inhibit superoxide radical-dependent reactions using a Ransod kit (Randox, Crumlin, UK). Details of the measurement were previously described [31]. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. Specific activity was expressed as SOD units $ml^{-1}$ [34].

### 2.8. Total RNA isolation and quantitative real-time (q)PCR analysis of gene expressions

Five hundreds microlitres of haemolymph was individually withdrawn similarly to that described above, placed in a tube containing 500 μl of an anticoagulant solution, and centrifuged at 800*g* and 4 °C for 20 min. The haemocyte pellet was washed with an anticoagulant solution and centrifuged again. One milliliter Trizol reagent (Invitrogen, Carlsbad, CA, USA) was added to the haemocyte pellet to isolate total RNA. First-strand complementary (c)DNA was generated in a 25-μl reaction volume containing 3 μg DNase I-treated total RNA, 400 pM oligo $dT_{18}$, 0.4 mM dNTP, 20 U of an RNase inhibitor (Invitrogen), 100 U ReverTra Ace RTase (Toyobo, Tokyo, Japan), and 1 × reverse-transcription (RT) buffer. The reaction was conducted at 42 °C for 1 h. After first-strand cDNA synthesis, a PCR of the housekeeping gene, elongation factor (EF)1α, was performed to check the RT reaction.

Transcripts of target genes (LGBP, PX, ppA, proPO I, proPO II, α2-M, integrin ß, HSP70, cytMnSOD, mtMnSOD, and ecCuZnSOD), and the internal control (EF1α) were measured by a qPCR. Primer sets for each gene were designed based on published *L. vannamei* genes using Beacon Designer Software vers. 6.0 (Table 1). The recombinant plasmids containing LGBP, PX, ppA, proPO I, proPO II, α2-M, integrin ß, HSP70, cytMnSOD, mtMnSOD, and ecCuZnSOD qPCR fragments were all quantified to 1 μg $\mu l^{-1}$. A series of concentrations of recombinant plasmids of $10^{-5}$–$10^{-11}$ μg $\mu l^{-1}$ was diluted with DEPC-treated water to construct the LGBP, PX, ppA, proPO I, proPO II, α2-M, integrin ß, HSP70, cytMnSOD, mtMnSOD, and ecCuZnSOD qPCR standard curves. Relationships between the threshold concentration (Ct) and copy number calculated based on the molecular weight of the target genes were established. Target gene expressions were quantified based on their relationships with the Ct and copy number.

All real-time PCRs used 10 μl 2 × power SYBR GREEN PCR master mix (Applied Biosystems, Framingham, MA, USA) with 4 μl of sample, and 0.2 μM each of the forward and reverse primers. Real-time PCRs were carried out on an ABI 7500 real-time PCR system (Applied Biosystems) using a program of denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55–62 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. After amplification, a melting-curve analysis was conducted to ensure that a single product was amplified. Each RNA sample and standard curve were examined in duplicate.

### 2.9. Preparation of V. alginolyticus

A pathogenic strain of *V. alginolyticus* isolated from diseased *L. vannamei*, which displayed symptoms of anorexia, lethargy, and whitish musculature, was used for the study [3]. The bacterium was cultured in tryptic soy broth (TSB supplemented with 2% NaCl, Difco, Sparks, MD, USA) for 24 h at 28 °C, and then centrifuged at 7155*g* for 20 min at 4 °C [31]. The supernatant was removed, and the bacterial pellet was resuspended in a phosphate-buffered saline (PBS) solution at 1.9 × $10^8$ colony-forming units (cfu) $ml^{-1}$ as the bacterial suspension for the *Vibrio* challenge test.

### 2.10. Preparation of WSSV inoculum

The WSSV inoculum and test solution were prepared based on a previously described method [27]. Briefly, 100 μl of haemolymph was withdrawn from WSSV-infected white shrimp and diluted with 400 μl of PBS. The viral titer of the inoculum was determined by a real-time PCR using the IQ2000™ WSSV Detection and Prevention System (GeneReach Biotechnology, Taipei, Taiwan) according to instructions provided by the manufacturer. This was then diluted with PBS to 2.5 × $10^7$ copies $ml^{-1}$, which served as a stock solution which was stored at −80 °C. The inoculum stock was unfrozen at 4 °C before use and centrifuged at 1000*g* for 10 min, and the supernatant was filtered through a 0.45-μm membrane filter. Ten microlitres of the filtrate was diluted with PBS to make a test solution of 0.75 × $10^5$ copies $ml^{-1}$ for the WSSV-challenge test.

### 2.11. Susceptibility of L. vannamei to V. alginolyticus

Two unchallenged groups (control shrimp and 7-day-starved shrimp) and two challenged groups (control shrimp and 7-day-starved shrimp) were set up. Challenge tests were conducted in triplicate with 10 shrimp per replicate following previously described methods [31]. Into the ventral sinus of the cephalothorax of each shrimp, 20 μl of a bacterial suspension (1.9 × $10^8$ cfu $ml^{-1}$) was injected resulting in 3.8 × $10^6$ cfu $shrimp^{-1}$. For the unchallenged groups, control shrimp and 7-day-starved shrimp were injected with an equal volume of a sterile saline solution. After the injection, shrimp were kept in separate 40-l aquaria (10 shrimp each) containing 20 l of aerated water at 35‰ salinity with three replicates. Therefore, there were four treatments in total with 30 shrimp in each treatment. Survival of shrimp was examined every 12 h during the first day, then every day after that until the end of the experiment at 7 days.

### 2.12. Susceptibility of L. vannamei to WSSV

Two unchallenged groups (control shrimp and 7-day-starved shrimp) and two challenged groups (control shrimp and 7-day-starved shrimp) were set up. Challenge tests were conducted in triplicate with 10 shrimp per replicate following previously described methods [35]. A challenge test was conducted by injecting 20 μl of a WSSV suspension of 0.75 × $10^5$ copies $ml^{-1}$ resulting in 1.5 × $10^3$ copies $shrimp^{-1}$ into the ventral sinus of the cephalothorax. For the unchallenged groups, control shrimp and 7-day-starved shrimp were injected with an equal volume of sterile saline solution. Experimental and control shrimp (10 shrimp $aquarium^{-1}$) were kept in 40-l aquaria containing 20 l of seawater (35‰) with three replicates. There were four treatments in total with 30 shrimp in each treatment. Survival of shrimp was examined every 12 h during the first day, and then every day after that until the end of the experiment at 7 days.

**Table 1**
Primers used for the quantitative real-time PCR study of elongation factor (EF) 1α and 11 immune-related genes of the white shrimp *Litopenaeus vannamei*.

| Gene | Primer name | Sequence 5′ – 3′ | Amplicon | Reference/GenBank |
|---|---|---|---|---|
| LGBP | Liva LGBP qPCR F | CGG CAA CCA GTA CGG AGG AAC | 115 bp | [17] |
| | Liva LGBP qPCR R | GTG GAA ATC ATC GGC GAA GGA G | | |
| Peroxinectin | Liva PX qPCR F | ATC CAG CAG CCA GGT ATG | 147 bp | [50] |
| | Liva PX qPCR R | CAG ACT CAT CAG ATC CAT TCC | | |
| ppA | Liva ppA qPCR F | CTA GAG ACG TCG GTG TCA TCA CC | 151 bp | AY368151 |
| | Liva ppA qPCR R | AAC TTG CCG TCC GAA GTG CG | | |
| proPO I | Liva proPO I qPCR F | ACG TCA CTT CCG GCA AGC GA | 156 bp | AY723296 |
| | Liva proPO I qPCR R | CCT CCT TGT GAG CGT TGT CAG G | | |
| proPO II | Liva proPO II qPCR F | ACC ACT GGC ACT GGC ACC TCG TCT A | 161 bp | EU373096 |
| | Liva proPO II qPCR R | TCG CCA GTT CTC GAG CTT CTG CAC | | |
| α2-macroglobulin | Liva A2M qPCR F | GCA CGT AAT CAA GAT CCG | 204 bp | DQ988330 |
| | Liva A2M qPCR R | CCC ATC TCA TTA GCA CAA AC | | |
| Integrin ß | Liva It ß qPCR F | TTG GGC ATC GTG TTC GGA CTC | 184 bp | GQ889365 |
| | Liva It ß qPCR R | TGA AGG TGT TGG TCG CAG GTC | | |
| HSP70 | Liva Hsp70 qPCR F | CCT CCT ACG TCG CCT TCA CAG ACA | 233 bp | AY645906 |
| | Liva Hsp70 qPCR R | GGG GTA GAA GGT CTT CTT GTC TCC C | | |
| cytMnSOD | Liva cytMnSOD qPCR F | TGA CGA GAG CTT TGG ATC ATT CC | 155 bp | DQ029053 |
| | Liva cytMnSOD qPCR R | TGA TTT GCA AGG GAT CCT GGT T | | |
| mtMnSOD | Liva mtMnSOD qPCR F | CAG ACT TGC CCT ACG ATT AC | 216 bp | DQ005531 |
| | Liva mtMnSOD qPCR R | AGA TGG TGT GAT TGA TGT GAC | | |
| ecCuZnSOD | Liva CuZnSOD qPCR F | CGC GGG AGA CAC AGC TGA TTT C | 164 bp | HM371157 |
| | Liva CuZnSOD qPCR R | GAA ATC CAG GGT GCC GGA GA | | |
| EF1α | Liva EF1α F | ATG GTT GTC AAC TTT GCC CC | 500 bp | GU136229 |
| | Liva EF1α R | TTG ACC TCC TTG ATC ACA CC | | |

### 2.13. Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA). If significant differences were indicated at the 0.05 level, then a multiple-comparisons (Tukey's) test was used to examine significant differences among treatments using the SAS computer software (SAS Institute, Cary, NC, USA). The percent data (susceptibility test and weight recovery percentage) were normalized using arcsine-transformation before the analysis. Statistical significance of differences required that $p$ be $<0.05$.

## 3. Results

### 3.1. Survival and weight loss of shrimp that had been starved for 28 days

All shrimp survived during the first week. Survival rates of shrimp which had been starved for 14, 21, and 28 days weeks were 90%, 71%, and 59%, respectively (Fig. 1A). Weights of shrimp starved for 7, 14, 21, and 28 days decreased by 3.2%, 7.3%, 9.2%, and 10.4%, respectively (Fig. 1B).

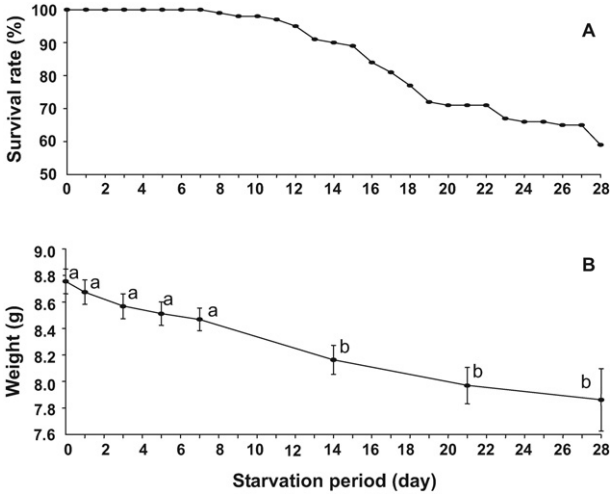


**Fig. 1.** Survival rates of white shrimp *Litopenaeus vannamei* starved for 1–28 days (A). Weights of white shrimp *L. vannamei* starved for 1–28 days (B). Values are presented as the mean ± standard error (SE). Data with different letters significantly differ ($p<0.05$) among different starvation periods.

### 3.2. Immune parameters of shrimp during the starvation period

No significant changes in HCs, GCs, and the THC were observed in shrimp starved for 3–12, 3–12, and 3–12 h, respectively. However, HCs, GCs, and the THC respectively decreased by 48%, 40%, and 46% in shrimp starved for 7 day (Fig. 2). No significant differences in PO activity, RBs, and SOD activity were observed in shrimp starved for 3–72, 3–168, and 3–24 h, respectively. However, PO activity, RBs, and

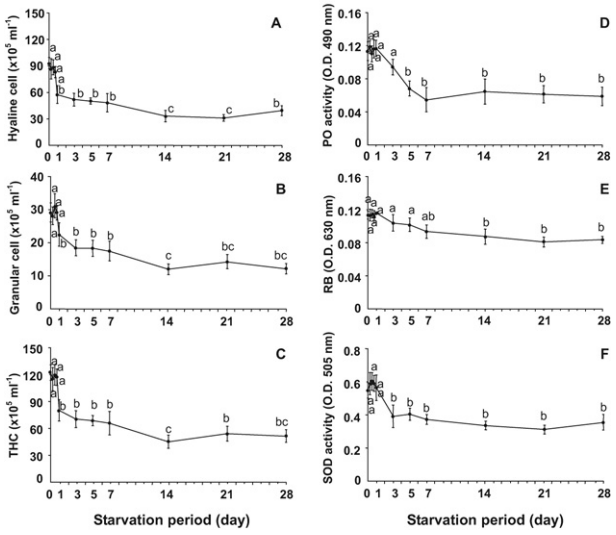


**Fig. 2.** Hyaline cells (HCs) (A), granular cells (GCs, including semi-granular cells) (B), the total haemocyte count (THC) (C), phenoloxidase (PO) activity (D), respiratory bursts (RBs) (E), and superoxide dismutase (SOD) activity (F) of white shrimp *Litopenaeus vannamei* starved for 3, 6, 12h, 1, 3, 5, 7, 14, 21, and 28 days. Each bar represents the mean value from eight determinations with the statistical error (SE). Data with different letters significantly differ ($p<0.05$) among different starvation time.

SOD activity respectively decreased by 51%, 18%, and 32% in shrimp starved for 7 days (Fig. 2).

### 3.3. Expressions of LGBP, PX, ppA, proPO I, proPO II, α2-M, integrin ß, HSP70, cytMnSOD, mtMnSOD, and ecCuZnSOD by shrimp

The integrin ß transcript significantly decreased after 0.5–5 days, whereas transcripts of LGBP, PX, ppA, proPO I, proPO II, and α2-M increased after 0.5–1 days. Transcripts of all these genes except ecCuZnSOD had decreased to the lowest levels after 5 days, and then tended to background values after 7 and 14 days. However, ppA expression was significantly higher, whereas expressions of integrin ß, HSP70, cytMnSOD and mtMnSOD of shrimp which had been starved for 7 and 14 days were significantly lower than levels in control shrimp (Figs. 3 and 4).

### 3.4. Susceptibility to V. alginolyticus in shrimp that had been starved for 1 week

All unchallenged control-shrimp survived for 7 days. The cumulative mortality rate of challenged 7-day-starved shrimp was significantly higher than that of challenged control-shrimp over 1–7 days (Fig. 5A).

### 3.5. Susceptibility to WSSV in shrimp that had been starved for 1 week

All unchallenged control-shrimp survived for 7 days. After 2 days, two and six out of 30 shrimp respectively died among the challenged control-shrimp and challenged 7-day-starved shrimp. The cumulative mortality rate of challenged 7-day-starved shrimp was significantly higher than that of challenged control-shrimp over 1–4 days (Fig. 5B).

### 3.6. Weight recovery of shrimp which had been starved for 7 and 14 days and then received normal feeding

Weights of shrimp which had been starved for 7 and 14 days and then received normal feeding are shown in Fig. 6. Weight recovery percentage of 7-day-starved shrimp that then received normal feeding respectively was 0.41%, −0.06%, 1.07%, and 1.57% at 1, 3, 5, and 7 days after re-feeding began. However, weight recovery percentage of 14-day-starved shrimp that then received normal feeding was −1.09%, −2.20%, −3.04%, and −2.07% at 1, 3, 5, and 7 days after re-feeding began.

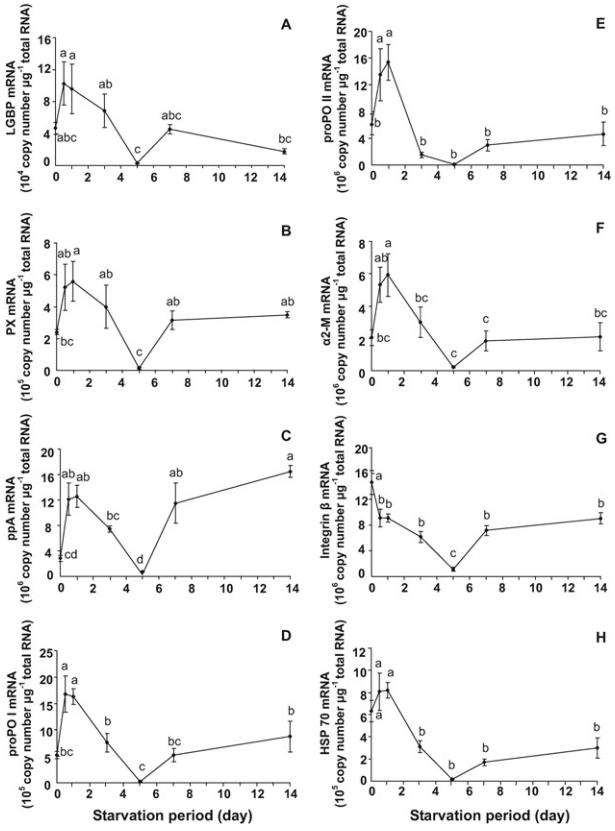


**Fig. 3.** Real-time RT-PCR analysis of lipopolysaccharide- and ß-glucan-binding protein (LGBP) (A), peroxinectin (PX) (B), prophenoloxidase-activating enzyme (ppA) (C), prophenoloxidase I (proPO I) (D), proPO II (E), α2-macroglobulin (α2-M) (F), integrin ß (G), and HSP70 expressions (H) in haemocytes of white shrimp *Litopenaeus vannamei* that starved for 0, 0.5, 1, 3, 5, 7, and 14 days. Each bar represents the mean value from 10 determinations with the statistical error (SE). See Fig. 2 for statistical information.

### 3.7. Immune parameters of shrimp which had been starved for 7 and 14 days and then received normal feeding

The immune parameters of 7-day-starved shrimp that then received normal feeding gradually increased with time. HCs, the THC, PO activity, RBs, and SOD activity of 7-day-starved shrimp that then received normal feeding were able to return to their original values at 5 days after re-feeding began (Figs. 7 and 8). However, GCs of 7-day-starved shrimp that then received normal feeding did not return to its original value after 5 days of re-feeding.

The immune parameters of 14-day-starved shrimp that then received normal feeding did not return to their original values after 5 days of feeding (Figs. 7 and 8). HCs of 14-day-starved shrimp that then received normal feeding slightly increased after 6–24 h of feeding, but still remained at 56% of the baseline value after 5 days of feeding. Similarly, GCs, the THC, PO activity, RBs, and SOD activity of 14-day-starved shrimp that then received normal feeding seemed to increase after 12 h of feeding, but still remained at 42%, 53%, 61%, 88%, and 69% of the respective baseline values after 5 days of feeding.

## 4. Discussion

Starvation in animals results in decreased immunity. In mice, the number of lymphocytes in liver, spleen and thymus greatly decreased

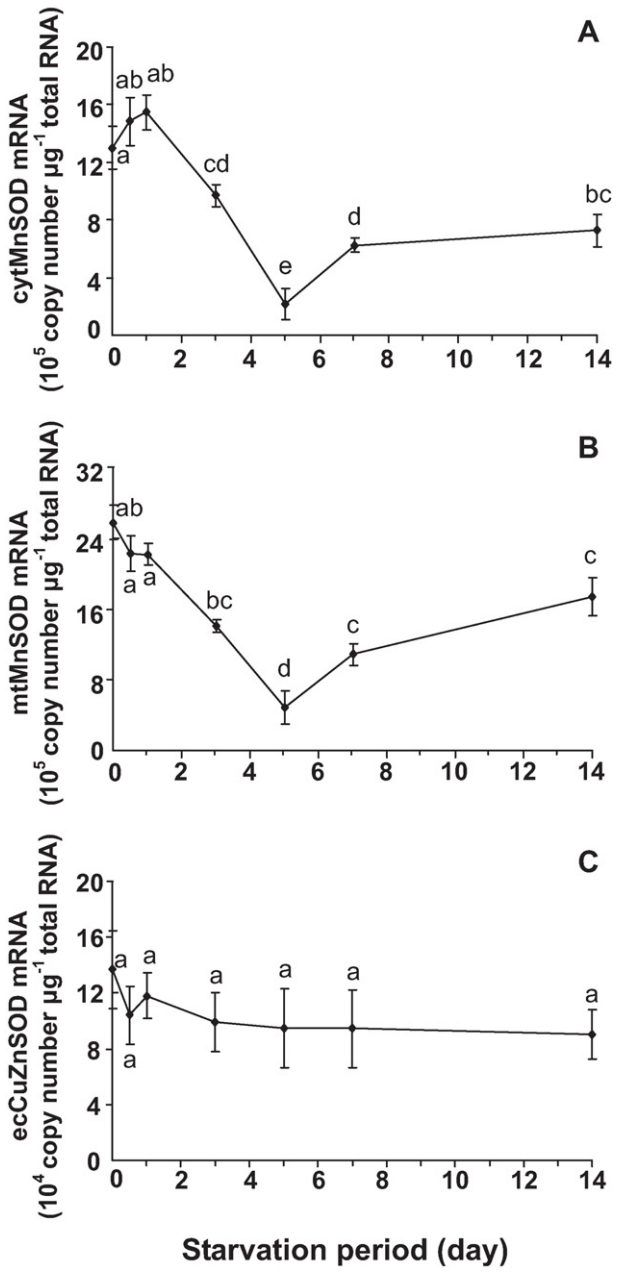


**Fig. 4.** Real-time RT-PCR analysis of cytMnSOD (A), mtMnSOD (B), and ecCuZnSOD expressions (C) in haemocytes of white shrimp *Litopenaeus vannamei* that starved for 0, 0.5, 1, 3, 5, 7, and 14 days. See Fig. 3 for statistical information.

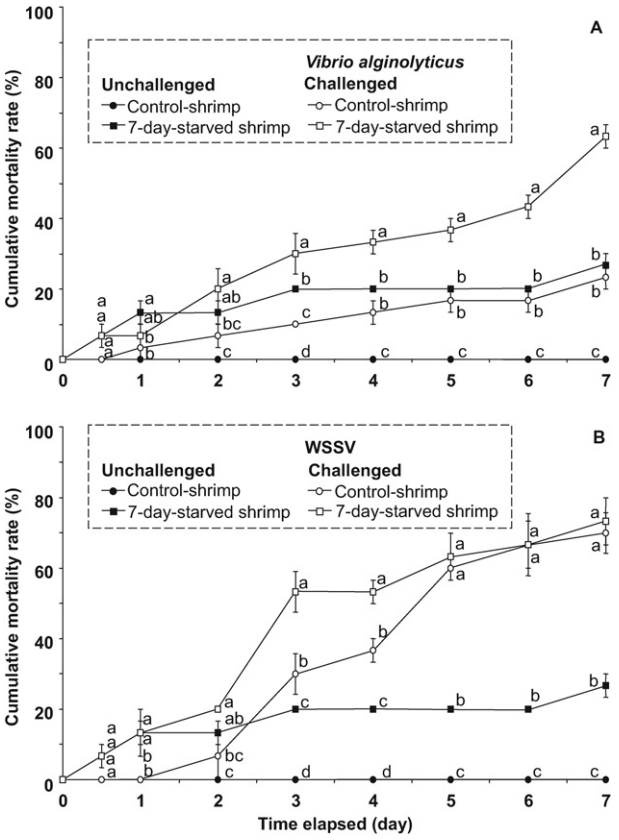


**Fig. 5.** Cumulative mortality rates of control white shrimp *Litopenaeus vannamei* and 7-day-starved shrimp, challenged with *Vibrio alginolyticus* at $3.8 \times 10^6$ colony-forming units (cfu) shrimp$^{-1}$ and then released into 35% seawater after 0.5–7 days (A), and cumulative mortality rates of control shrimp and 7-day starved shrimp, challenged with WSSV at $1.5 \times 10^3$ copies shrimp$^{-1}$ and then released into 35% seawater after 0.5–7 days (B). Data in the same time with different letters significantly differ ($p<0.05$) among different treatments. Values are presented as the mean ± standard error (SE).

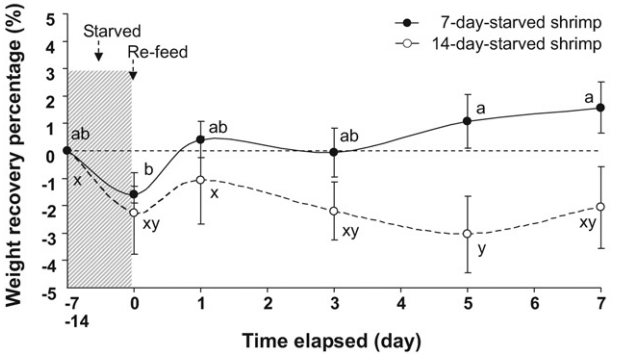


**Fig. 6.** Weight recovery percentage of white shrimp *Litopenaeus vannamei* that had been starved for 7 and 14 days, and then received normal feeding for 1, 3, 5, and 7 days. Data with different letters significantly differ ($p<0.05$) among different starvation time. See Fig. 2 for statistical information.

when starved for 3 days [23]. In humans, decrease in SOD activity with an increase in superoxide anion was observed in fasted-person [36]. In teleost, decreases of haemolytic activity and haemagglutinating titre were observed in the starved European eel *Anguilla anguilla* [37]. In insects, decrease in PO activity was observed in the starved worm beetle *Tenebrio molito* [38]. The haemocyte count, PO activity, and RBs of white shrimp which had been fed a diet (40% protein) significantly decreased after 21, 14, and 21 days of starvation, respectively [25]. In the present study, white shrimp which had been deprived of food for 7 days showed significant decrease in all the immune parameters (Figs. 2, 7 and 8). Furthermore, the 7-days-starved shrimp showed increased mortality when infected by *V. alginolyticus* and WSSV (Fig. 5). A frequent feeding schedule that prevents shrimp from suffering a food deficiency leading to reduced immunity is suggested.

HCs, GCs, PO activity, RBs, and SOD activity respectively decreased to ≤21%, 21%, 18%, 46%, and 28% of the original values, which could cause death of the shrimp due to weakened immunity, as these are considered as critical components of a shrimp's innate immunity [39]. In the present study, despite a 90% survival rate, HCs, GCs, the THC, PO activity, RBs, and SOD activity respectively remained at 36%, 60%, 37%, 58%, 77% and 61% of the original values after 14 days of starvation. Difference in survival rate of shrimp which had been starved for 14 days between 90% (Fig. 1), and 78% (Fig. 5) is considered due to the water volume and density. It is suggested that shrimp should be

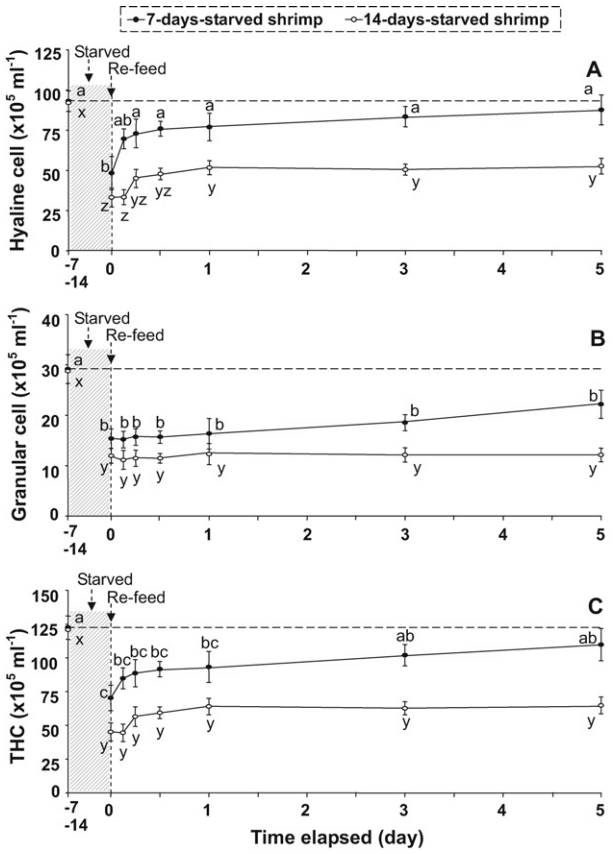


**Fig. 7.** Hyaline cells (HCs) (A), granular cells (GCs, including semi-granular cells) (B), and the total haemocyte count (THC) (C) of white shrimp *Litopenaeus vannamei* that had been starved for 7 and 14 days, received normal feeding for 3, 6, and 12h, and 1, 3, and 5 days. See Fig. 2 for statistical information.

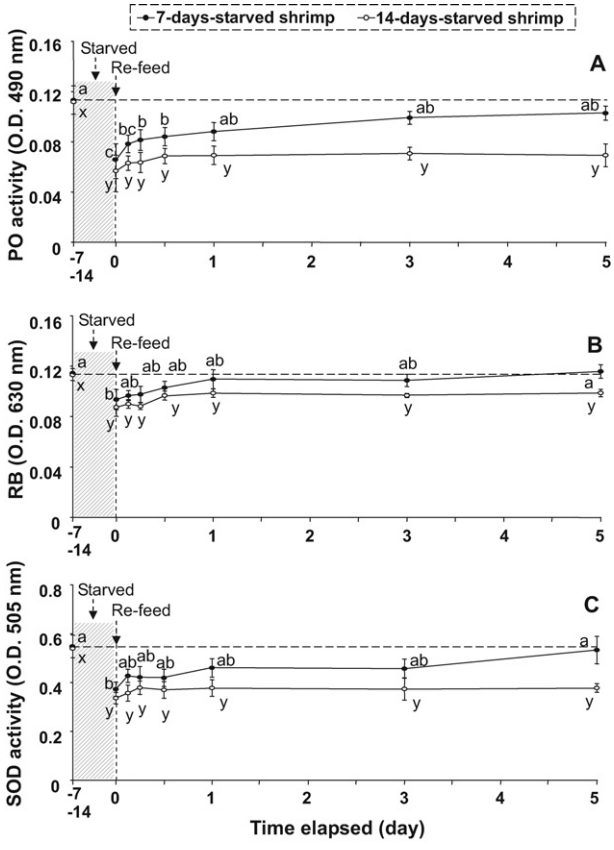


**Fig. 8.** Phenoloxidase (PO) activity (A), respiratory bursts (RBs) (B), and superoxide dismutase (SOD) activity (C) of white shrimp *Litopenaeus vannamei* that had been starved for 7 and 14 days, received normal feeding for 3, 6, and 12h, and 1, 3, and 5 days. See Fig. 2 for statistical information.

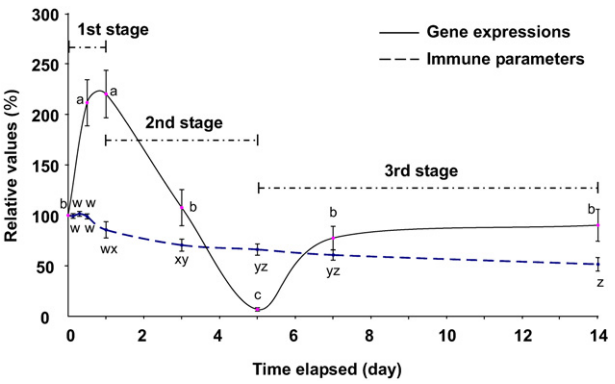


**Fig. 9.** Patterns of relative immune parameters (HC, GC, THC, PO activity, RB and SOD activity) (broken line) of white shrimp *Litopenaus vannamei* that had been starved for 3, 6, 12h, 1, 3, 5, 7, and 14 days, and patterns of relative expressions of genes (LGBP, peroxinectin, proPO I, proPO II, α2-macroglobulin, HSP70, and cytMnSOD) (solid line) of white shrimp *L. vannamei* that starved for 0.5, 1, 3, 5, 7, and 14 days. See Fig. 2 for statistical information.

maintained to avoid another stress like pathogen infection, and environment change that may cause exacerbation of immunity. Furthermore, the fact that the immune parameters of 14-day-starved shrimp were not able to return to their baseline values even after 5 days of refeeding indicates that shrimp following long-term starvation might lose their capability to retrieve immunity indicating immune fatigue.

The transcripts of genes which are involved in metabolism were induced in starved-human and mice [40,41]. Trypsin transcript of white shrimp increased after 24 h of starvation, followed by a 1.5-fold decrease after 72 h, and then remained unchanged after 120 h [42]. The transcript of high density lipoprotein-beta glucan binding protein (HDL-BGBP) of white shrimp significantly decreased in white shrimp which had been starved for 24 h, while the ribosomal L8 levels remained constant suggesting that the synthesis of the lipoprotein is less required in the absence of food [43]. HDL-BGBP is a dual function protein, and serves as (1) a transporter of lipid, and (2) pattern recognition protein (PRP) [44–46]. Starvation of animal greatly affects both functional response and gene expression.

Following long-term culture in unfavorable salinity condition (2.5‰ and 5‰), white shrimp were able to up-regulate their immune related gene expressions including LGBP, PX, integrin ß, and α2-M to facilitate the normal immunological functions [35]. The present study indicated that shrimp which had been starved for 0.5–1 day had significantly increased expressions of LGBP, PX, ppA, proPO I, proPO II, α2-M, and HSP70, but showed a significant decrease in integrin ß expression. Integrin is a cell surface receptor. PX has peroxidase and cell-adhesion activities, and is associated with integrin in mediating haemocyte–microbe binding in the proPO activating system and cellular immunity [15]. It is suggested that shrimp which had been deprived of food for 0.5–1 day were able to up-regulate expression of LGBP, and modulate gene expressions of proPO-activating system proteins.

We would like to discuss the innate immune response of shrimp following long-term starvation (up to 14 days of starvation). We normalized values of the immune parameters to their baseline values, and presented them as percentage, and we also normalized values of gene expressions to their baseline values, and presented them as percentage except for integrin ß, ppA, mtMnSOD, and ecCuZnSOD. We found that shrimp following 14 days of starvation showed three stages of gene expression of immune-related proteins (Fig. 9).

In the first stage (starved for 0.5–1 day), shrimp exhibited a reduction in the immune parameters including haemocyte count, but increased PO activity, RBs, and SOD activity per haemocyte, and also up-regulated compensation of LGBP, PX, ppA, proPO I, proPO II, and α2-M expressions. Shrimp which had been deprived of food for 0.5–1

day encountered the first threshold. In the second stage (starved for 1–5 days), shrimp suffered starvation showing moderate stress and reductions in both immune parameters and gene expressions except ecCuZnSOD. At the end of the second stage, most immune related gene transcripts decreased to the lowest levels and encountered a second threshold. In the third stage (starved longer than 5-days), shrimp showed continuous reductions of immune parameters with a second up-regulated compensation of gene expressions. In this stage, shrimp modulated gene expressions to prevent further injury to immunological functions. Allostasis or allostasis load, an adaptable component of stress response is commonly used in maintenance of homeostasis [47]. In the present study, the starved shrimp had capability to modulate the homeostasis of immunity to prevent over deepened perturbation through three stages of gene regulations.

It is interesting to note that the expression of integrin ß is different to the expressions of other immune genes in the first stage. During the first and second stages, integrin ß was negatively related to starvation period (0.5–5 days). Integrin ß is known to be associated with integrin $\alpha$ to form a functional receptor on the cell surface, and it plays up-stream regulation of immunity [48]. A knockdown of integrin ß caused the attenuated immune parameters including haemocyte count, RB, SOD activity, and lysozyme activity (data not shown). It is suggested that decrease of integrin ß expression guides to cause decrease in immune parameters, whereas up-regulated expressions of other immune related genes contribute less effect on the immune parameters and appear to be a pseudo-morph during the first stage of starvation.

It is well known that gene expression does not necessarily refer to functional proteins, and does not necessarily correlate to the amount of expressed protein [49]. The present study indicated that the up-regulation of gene expressions did not guarantee an increase of immune parameters. Shrimp which had been starved for 7 and 14 days showed recovery of expressions of all genes related to proPO cascade, and with higher ppA expression (Figs. 3 and 4). Shrimp which had been deprived of food for 5 days encountered the second threshold of trying to prevent excessively low expressions of genes leading to immune fatigue in immunological responses. Shrimp which had been starved longer than 5 days might cause some unknown mechanism to trigger the expression of immune related genes, but reduction in immune parameters.

In conclusion, 100% of white shrimp *L. vannamei* which had been starved for 7 days survived, and lost 3.2% of their weight, but showed significantly decreased immunity and increased mortality when infected by *V. alginolyticus* and WSSV. Shrimp which had been starved for 0.5–1 day showed increased expressions of LGBP, PX, ppA, proPO I, proPO II, HSP70, and $\alpha$2-M, but decreased expression of integrin ß. Shrimp which had been deprived of food for 5 days were at a critical point, and shrimp subjected to longer-term (longer than 5 days) starvation had the capability to up-regulate gene expressions. Furthermore, immune parameters of shrimp starved for 7 days were able to return to their background values after the shrimp received normal re-feeding, whereas shrimp starved for 14 days exhibited immune fatigue that subsequently resulted in immune parameters not being able to recover even after 5 days of re-feeding.

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