Decreased level of crustacean hyperglycemic hormone (CHH) in black tiger shrimp Penaeus monodon suffering from Monodon Slow-Growth Syndrome (MSGS)

Benjamart Pratoomthai, Waraporn Sakaew, Apinunt Udomkit, Kanokpan Wongprasert, Ernest S. Chang, Boonsirm Withyachumnarnkul

A B S T R A C T

Monodon Slow-Growth Syndrome (MSGS), a pathological condition in the black tiger shrimp Penaeus monodon, is associated with Laem–Singh virus (LSNV) infection. Infected shrimp grow slowly when the virus invades the part of the retina called the zona fasciculata. Since molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH) are synthesized in the optic lobe and are related to molting activity and growth of the shrimp, the purpose of this study is therefore to determine the levels of these hormones and related parameters in MSGS shrimp. P. monodon juveniles were sampled from normal and MSGS ponds and were divided into small-negative, large-negative, small-positive and large-positive groups, depending on the size of the shrimp and whether they were LSNV-negative or LSNV-positive. Individual shrimp were measured for duration of each molt stage and molt intervals. Levels of MIH1 and CHH1 transcripts were determined from the optic lobe, using real-time reverse transcriptase-polymerase chain reaction. The results revealed that the small-positive and the small-negative shrimp did not differ in durations of molt stages or molt intervals, as well as on the levels of MIH1 transcript in the optic lobe, MIH1 peptide in the optic lobe and hemolymph, or ecdysteroids in the hemolymph. Differences in molting activities and related transcript and hormones were observed between the small- and large-sized shrimp, but not in the status of LSNV infection. While levels of CHH1 transcript in the optic lobe of both small-negative and small-positive shrimp did not differ, levels of CHH1 peptide, as well glucose, in the hemolymph of the small-positive shrimp were significantly lower than those of the small-negative group. Levels of glycogen in the hepatopancreas of the small-positive shrimp were also significantly higher than that of the small-negative ones. The results suggest that growth retardation in MSGS shrimp is related to the suppression of the release of CHH1 peptide by LSNV invasion in the zona fasciculata, consequently causing decreased hepatopancreatic glycogenolysis and persistent hypoglycemia, resulting in growth stunting.

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1. Introduction

Monodon Slow-Growth Syndrome (MSGS) in the black tiger shrimp Penaeus monodon has been observed in shrimp farms in Thailand since 2002. The most prominent feature of a MSGS pond is growth retardation of the shrimp. At similar stocking densities, the average growth rate in MSGS ponds is approximately half that normally expected by shrimp farmers, and size variation among individual shrimp is abnormally high, with a coefficient of variation greater than 35% (Sritunyalucksana et al., 2006; Withyachumnarnkul et al., 2004). The syndrome has been associated with an infection by Laem–Singh virus (LSNV) (Anantasomboon et al., 2006; Sritunyalucksana et al., 2006) and probably co-infected by another virus-like particle called integrase-containing element (Panphut et al., 2011). In an MSGS pond, most shrimp were infected by LSNV, however, some of the infected shrimp grow normally, while others have retarded growth (Withyachumnarnkul et al., 2004). The growth-retarded shrimp are characterized by intensely dark and yellow stripes of body color and appendages, and striped and brittle antennae (Withyachumnarnkul et al., 2004). Compared to the normal-growth infected shrimp, the growth-retarded infected shrimp show a significant pathology in the area of the eyestalk called the zona fasciculata, or retinopathy (Pratoomthai et al., 2008). The finding suggests that LSNV does not cause slow growth of the shrimp, unless the infection causes damage to the zona fasciculata, and possibly interferes with the synthesis and release of...
certain neurohormones of the optic lobe that lies behind the zona fasciculata.

In crustaceans, the optic lobe contains several types of neurosecretory cells that synthesize and store neuropeptides important for normal physiology, such as for molting, body color, controlling levels of glucose in the hemolymph, etc. (for review, see Keller, 1992). The most highly-studied neuropeptide hormones are molt-inhibiting hormone (MIH), crustacean hyperglycemic hormone (CHH), gonad-inhibiting hormone (GIH) and mandibular organ-inhibiting hormone (MOIH); these hormones share some parts of their amino acid sequences and thus are grouped as hormones of the CHH family (de Kleijn and van Herp, 1995; Keller, 1992). MIH controls molting by inhibiting the synthesis and release of ecdysteroids from Y-organ. CHH plays a major role in controlling glucose levels in the hemolymph that are involved in growth and important events during molting and reproductive cycles (Avarre et al., 2001; Cooke and Sullivan, 1982; Khayat et al., 1998; Yasuda et al., 1994). CHH elevates glucose levels in the hemolymph by stimulating glycogenolysis and inhibiting glycogenesis in the hepatopancreas (Chang and O’Connor, 1985; Sedlmeier, 1982).

In this study, we hypothesized that LSNV-induced retinopathy in P. monodon causes alterations in the levels of MIH and/or CHH transcripts. Any of these alterations may subsequently affect the molt cycle, ecdysteroid level, glucose level, and finally growth retardation.

2. Materials and methods

2.1. Animals and housing

Three commercial ponds culturing black tiger shrimp P. monodon in Thailand were selected as representatives of normal and MSGS ponds. Two ponds raised shrimp with a normal growth rate, and the shrimp were LSNV-negative by reverse transcriptase polymerase chain reaction (RT-PCR) determination. Shrimp in one of the two ponds were previously cultured for one month and their body weights (BW) were 5–10 g; these shrimp were designated small-negative shrimp. The other pond contained shrimp that were three months in culture and their BWs were 20–25 g; these shrimp were designated large-negative shrimp. The third pond raised three month cultured shrimp that were LSNV-positive, and the shrimp BW averaged 10 g with a coefficient of variation (CV) of 40%, and many of them had external features typical of MSGS shrimp (Sritunyalucksana et al., 2006; Withyachumnarnkul et al., 2004). Shrimp in this pond were divided into two groups: small-positive (5–10 g BW) and large-positive (20–25 g BW). These animals were individually placed in compartments of fiberglass tanks held vertically in an enclosed cabinet (Fig. 1). Two sets of such cabinets were built to accommodate the shrimp; one accommodated LSNV-negative shrimp (small-negative and large-negative groups) and the other accommodated LSNV-positive shrimp (small-positive and large-positive groups).

Each cabinet was composed of four 0.3×2.5×0.4 m (width×length×depth) fiberglass tanks. Each tank was divided into 10 compartments, each was 0.25 m long. The compartments were separated by a 0.5 cm fiberglass plates with multiple 0.5 cm holes to allow free flow of water between compartments. The four tanks were connected with PVC pipes and water was re-circulated through all the tanks and filtered through polyvinyl alcohol beads (Nagadomi et al., 1999) contained in a 0.5×0.5×0.5 m fiberglass tank. Levels of total ammonia nitrogen and nitrite were monitored and controlled at ≤0.5 ppm throughout the experiment. Brackish seawater (15 ppt) was used for

![Fig. 1. Diagram of a cabinet housing four fiberglass aquaria with recirculatory water system that was filtered by polyvinyl alcohol beads. Each aquarium was divided into 10 compartments partitioned by sieved plates to allow water circulation. Each compartment housed one black tiger shrimp Penaeus monodon, which were divided into four groups; they were individually studied for molting activities (see text for explanation). P, water pump; arrows show direction of water flow.](image-url)
rearing and for water exchange as necessary. Water temperature was kept at 28 ± 1 °C by aquarium heaters, aeration was provided to keep dissolved oxygen at > 6 ppm. Water alkalinity was maintained at 130–150 ppm and pH at 8.4–8.6. Light was provided with a 12 h:12 h light–dark cycle, with 750–800 lx intensity. Feed pellets (Charoen Pokphand Foods Public Company) were provided at 5% BW, two meals a day, at 07.00 and 19.00 h.

2.2. Molt stages and intervals

Molt stages were determined twice daily according to the method described by Promwikon et al. (2004), which was by observation of the uropod under light microscopy at low (10×) magnification. In this study, the following molt stages were determined: postmolt (A–B stages), intermolt (C), early premolt (D0,2) and late premolt (stage D1–4). Shrimp were monitored for two molt cycles and the durations of individual molt stages and molt intervals were calculated from means of two cycles (about 1 month).

2.3. Experimental plan

Two sets of experiments were designed. In the first experiment, the shrimp were reared for one month to determine the durations of molt stages and molt intervals. In the second experiment, shrimp were sampled at each molt stage and tissues were isolated and determined for MIH1 and CHH1 transcripts (from optic lobe), MIH1 and CHH1 peptides (from optic lobe and hemolymph), ecdysteroids (from hemolymph), glucose (from hemolymph) and glycogen (from hepatopancreas). For the determinations of MIH1 transcript, MIH1 peptide and ecdysteroids, shrimp were sampled during postmolt, intermolt, early premolt and late premolt. For the determinations of CHH1 transcript, CHH1 peptide, glucose and glycogen, shrimp were sampled only during intermolt and they were not fed for two days before sampling to avoid any effects of differential feeding.

MIH1 transcript and MIH1 peptide were chosen to represent MIH activity in P. monodon in this study because MIH1 has close identity to the MIH of Metapenaeus japonicus, Fenneropenaeus chinensis and Metapenaeus ensis, and the hormone was found to be biologically active (Yodmuang et al., 2004). Likewise, CHH1 transcript and CHH1 peptide have been reported in P. monodon and found to be biologically active (Treerattrakool et al., 2003; Udomkit et al., 2004).

2.4. MIH1 and CHH1 transcripts

The eyestalks were isolated from shrimp samples and the optic lobe was dissected out from the compound eyes and optic nerve under a stereomicroscope. It was then immediately submerged in ice-cold water. Total RNA was extracted from the optic lobe by using TRIZOL (Invitrogen, Carlsbad, CA), according to the manufacturer protocol and cDNA was synthesized from 100 ng RNA by using superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA) with an oligo-dT primer. cDNA was synthesized from 100 ng RNA by using superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA) with an oligo-dT primer.

The comparative threshold cycle (Ct) method was used to determine transcript levels of MIH1 and CHH1 transcripts and elongation factor-1α (EF-1α) transcript was used as reference (Qiu et al., 2007). Primers for the amplification of MIH1 were F 5′catagcaccgcctgccgag3′ and R 5′cctgttgccagcctttagac3′, and for CHH1, F 5′cacaagctctctctggag3′ and R 5′aacaagggggctgtacg3′ (Treerattrakool et al., 2003; Udomkit et al., 2004). Primers for amplification of EF-1α [F (5′gaacctggctgacagcagacag 3′); R (5′gagatctggctgacagcagacac 3′)] were designed based on GenBank accession no. DQ021452. The PCR mixture consisted of 1 μL of cDNA sample, 300 nM of each primer, and 12.5 μL of Syber® Green Master mix (QIAGEN, Hilden, Germany) in a final volume of 25 μL. All amplification protocols comprised 1 initial step at 94 °C for 1 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 31 s. The specificity of primers and the absence of nonspecific PCR products were examined by dissociation assays after PCR, as recommended by the ABI Prism 7500 detection system protocol (Applied Biosystems, Foster City, CA).

2.5. MIH1 and CHH1 peptides

MIH1 and CHH1 peptides were determined from the optic lobe and hemolymph. The optic lobe was homogenized in lysis buffer containing 135 mM NaCl, 20 mM Tris–HCl, 2 mM EDTA and 1 mM phenylmethanesulfonyl fluoride, pH 7.4 (Watson et al., 2001). The homogenate was centrifuged at 8000 × g, at 4 °C for 10 min; the supernatant was isolated in a microtube and stored at −20 °C until subsequent analysis. A small piece (about 1 mg) of abdominal muscle was processed identically and used as negative control.

For MIH1 and CHH1 peptide determinations in the hemolymph, 100 μL of hemolymph was withdrawn from the ventral sinus of the shrimp into a 1 mL syringe. The mixture was centrifuged (1 min at 12,000 × g); supernatant was isolated in a microtube and stored at −20 °C until use.

Determinations of MIH1 and CHH1 peptides were carried out by enzyme-linked immunosorbent assay (ELISA). Standard recombinant MIH1 and CHH1 (Treerattrakool et al., 2006) were gifts from Dr. Panyim. Polyclonal antibodies against the two peptides were produced in rabbits and mice at the Biomedical Technology Research Unit, Faculty of Associated Medical Sciences, Chiangmai University, Thailand.

The procedure was carried out as follows. The microtiter plates (Nunc, Roskilde, Denmark) were coated for 16 h at 4 °C with 100 μL of the rabbit anti-rMIH1/CHH1 IgG (1:2000 in 15 mM Na2CO3, 35 mM NaHCO3, pH 9.6). After being coated with the primary antibody, the wells were washed five times with PBS, pH 7.4, and successively blocked with 400 μL 2% BSA in PBS for 8 h at 4 °C. After being blocked, the plates were incubated with 100 μL of the hemolymph sample for 16 h at 4 °C and washed seven times with 400 μL 0.1% Tween 20 in PBS (PBS-T). After addition of the secondary antibody (polyclonal mouse anti-rMIH1/CHH1, 1:1000 in PBS containing 2% BSA), the plates were incubated for 2 h at 37 °C and washed with PBS-T. After addition of 100 μL of polyclonal antibody to mouse IgG–biotin (1:1000 in PBS containing 2% BSA), the plates were incubated for 1 h at 37 °C. Finally, 100 μL streptavidin–peroxidase conjugate (1:1000 in PBS containing 2% BSA) was added and the sample was incubated for 1 h at 37 °C and washed with 400 μL PBS-T. The enzymatic reaction was then initiated by the addition of 100 μL tetramethyl benzidine (TMB) for 15 min in room temperature in darkness and the reaction was stopped by adding 100 μL 6 N H2SO4. The optical density was measured at 450 nm with a microplate reader.

2.6. Ecdysteroid level in the hemolymph

The withdrawn hemolymph (100 μL) was added into 300 μL of 95% methanol before storage at −20 °C. Determination of ecdysteroids was carried out by radioimmunoassay (RIA).

The RIA process of ecdysteroid determination was carried out as described previously (Chang and O’Connor, 1978). Briefly, samples were thawed, vortexed, and centrifuged at 14,000 × g, 4 °C for 20 min. The supernatant (100 μL) was added into RIA tubes (USA Scientific, Ocala, FL), heated and vacuum dried. The radiolabel, [3H]-ecdysone (1:1000 in PBS containing 2% BSA), the plates were incubated for 2 h at 37 °C and washed with PBS-T. After addition of 100 μL of polyclonal antibody to mouse IgG–biotin (1:1000 in PBS containing 2% BSA), the plates were incubated for 1 h at 37 °C. Finally, 100 μL streptavidin–peroxidase conjugate (1:1000 in PBS containing 2% BSA) was added and the sample was incubated for 1 h at 37 °C and washed with 400 μL PBS-T. The enzymatic reaction was then initiated by the addition of 100 μL tetramethyl benzidine (TMB) for 15 min in room temperature in darkness and the reaction was stopped by adding 100 μL 6 N H2SO4. The optical density was measured at 450 nm with a microplate reader.
Hepatopancreas was isolated from the shrimp, quickly frozen in liquid nitrogen and stored at −80 °C until the time of analysis. At analysis, it was thawed on ice, homogenized in 1.5 mL of ice-cold distilled water and centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatants obtained were used for glycogen determination (Dubois et al., 1956). Briefly, a 100 μL sample of supernatant was placed in a microtube with 2 mL of 20% trichloroacetic acid and centrifuged at 3000 rpm for 20 min. The volumes obtained were transferred into tubes with 2 mL ether and centrifuged again. The upper layers were carefully discarded and the rest of the volumes were placed in tubes with 1.5 mL distilled water, 500 μL of 80% phenol and 5 mL 12 N H2SO4. The samples were allowed to incubate for 30 min at room temperature. Ten minutes before the end of the incubation period, 200 μL of the reaction mixture was transferred to a microplate. The absorbance was determined at 492 nm in a spectrophotometer. Known glucose standards were processed in the same way as the samples and used to construct a calibration curve.

2.7. Glycogen level in the hepatopancreas

Hepatopancreas was isolated from the shrimp, quickly frozen in liquid nitrogen and stored at −80 °C until the time of analysis. At analysis, it was thawed on ice, homogenized in 1.5 mL of ice-cold distilled water and centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatants obtained were used for glycogen determination (Dubois et al., 1956). Briefly, a 100 μL sample of supernatant was placed in a microtube with 2 mL of 20% trichloroacetic acid and centrifuged at 3000 rpm for 20 min. The volumes obtained were transferred into tubes with 2 mL ether and centrifuged again. The upper layers were carefully discarded and the rest of the volumes were placed in tubes with 1.5 mL distilled water, 500 μL of 80% phenol and 5 mL 12 N H2SO4. The samples were allowed to incubate for 30 min at room temperature. Ten minutes before the end of the incubation period, 200 μL of the reaction mixture was transferred to a microplate. The absorbance was determined at 492 nm in a spectrophotometer. Known glucose standards were processed in the same way as the samples and used to construct a calibration curve.

2.8. Glucose level in the hemolymph

Hemolymph glucose was determined by using a glucose assay kit (Sigma, St. Louis, MO). The hemolymph was diluted in distilled water (1:50) and loaded (50 μL/well) onto 96-multiwell plates in duplicate. A mixture of glucose assay reagent (100 μL) was added to each well and the plates were incubated at 37 °C for 30 min. The reaction was stopped by adding 100 μL of 12 N H2SO4 and the plates were read on a microplate reader at 540 nm. The data were quantified with standards (20 to 120 μg/mL).

2.9. Statistical analysis

Data were expressed as means ± standard deviation and analyzed with a one-way analysis of variance (ANOVA) and Tukey’s test. All statistical analyses were conducted using the SPSS (V.16 for Windows). For MIH and ecdysteroid level, the data were analyzed by using three-way ANOVA with SAS system (V. 9.2 for Windows). For MIH1 peptide in the optic lobe, highest levels were also detected during early premolt (Fig. 2a). Highest levels of MIH1 were observed during early premolt, and were lowest during intermolt. During early premolt, the amount of MIH1 in the small-positive shrimp was significantly (P<0.05) lower than that of the large-negative and large-positive shrimp, but comparable to that of the small-negative shrimp. The MIH1 levels of all groups of shrimp during postmolt, intermolt and late premolt did not differ significantly.

When comparing MIH1 transcript levels during different molt stages of the same shrimp group, variable statistical results were detected. In large-positive, large-negative and small-negative shrimp, the early premolt level was significantly (P<0.05) higher than that of postmolt and intermolt. During early premolt, the amount of MIH1 in the small-positive shrimp was significantly (P<0.05) lower than that of the large-negative and large-positive shrimp, but comparable to that of the small-negative shrimp. The MIH1 levels of all groups of shrimp during postmolt, intermolt and late premolt did not differ significantly.

When comparing MIH1 transcript levels during different molt stages of the same shrimp group, variable statistical results were detected. In large-positive, large-negative and small-negative shrimp, the early premolt level was significantly (P<0.05) higher than that in all other stages. But in small-positive shrimp, the early premolt level was significantly (P<0.05) higher than that of postmolt and intermolt, but not that of late premolt (Fig. 2a).

For MIH1 peptide in the optic lobe, highest levels were also detected during early premolt (Fig. 2b). When comparing the levels among shrimp groups during the same molt stages, no significant differences were detected. When comparing within the same shrimp group but during different molt stages, the levels in the large-positive and large-negative shrimp during early premolt were significantly (P<0.05) higher than those during all other stages. Whereas in small-positive and small-negative shrimp, early premolt levels were significantly (P<0.05) higher than those during intermolt and late premolt.

In the hemolymph, highest levels of MIH1 peptide were found during intermolt in all shrimp groups [Fig. 2c], followed by the levels during early premolt. When comparing the levels among shrimp groups, but within the same molt stage, significant differences were detected during the intermolt and premolt. During intermolt, small-negative shrimp had significantly (P<0.05) lower levels than those of the large-positive and large-negative shrimp, but not significantly different from that of the small-negative shrimp. The same pattern was detected during early premolt, but at lower levels of the peptide in all groups of shrimp.

When comparing MIH1 peptide level in the hemolymph within the same shrimp group but during different molt stages, in the

| Table 1  |
| Body weights, durations of molting stages and molt intervals of Penaeus monodon that were large sized and Laem-Singh virus-positive (large-positive), small sized and Laem-Singh virus-negative (small-negative), large sized and Laem-Singh virus-negative (large-negative) and small sized and Laem-Singh virus-negative (small-negative). The shrimp were kept individually in compartments for one month (see text for explanation). N=20 in each group. Different superscripts in the same row indicate statistical significance. |
| Large-positive | Small-positive | Large-negative | Small-negative |
| Body weights (g) | | | |
| Initial | 21.3±3.0a | 7.4±1.8b | 20.7±4.5b | 8.3±2.0b |
| Final | 21.9±4.8a | 8.5±3.6a | 21.9±5.7a | 13.4±3.7a |
| Specific growth rate (%) | 3.1±1.5a | 14.8±5.7a | 4.5±1.8b | 60.7±15.1b |
| Durations of molting stages (days) | | | | |
| Postmolt (A–B) | 1.6±0.4a | 1.1±0.3b | 1.4±0.5b | 1.0±0.2b |
| Intermolt (C) | 2.8±0.6a | 2.2±0.4b | 2.7±0.4b | 1.9±0.3b |
| Early premolt (D–C) | 5.8±0.8a | 4.5±0.7b | 5.6±0.9b | 4.1±0.6b |
| Late premolt (D–A) | 6.1±0.8a | 4.8±1.0b | 6.1±0.8b | 4.7±0.7b |
| Molting interval (days) | 16.4±1.6a | 12.8±1.8b | 15.9±1.8b | 11.9±1.7b |

3.2. MIH1, transcript, MIH1 peptide and ecdysteroids

To validate real-time RT-PCR in the determinations of MIH1/CHH1 transcripts, the sample titration curve (log dilution factor vs. threshold cycle number, Ct) was plotted and its slope was used to calculate amplification efficiency (Ginzinger, 2002). The amplification efficiency was always greater than 90%. In order to check amplification specificity, samples were subject to melting curve analysis after PCR amplification. Results of melting curve analyses showed a single peak indicating the specificity of PCR amplification (data not shown).

All shrimp groups had similar patterns of MIH1 transcript expression during the molt cycle (Fig. 2a). Highest levels of MIH1 were observed during early premolt, and were lowest during intermolt. During early premolt, the amount of MIH1 in the small-positive shrimp was significantly (P<0.05) lower than that of the large-negative and large-positive shrimp, but comparable to that of the small-negative shrimp. The MIH1 levels of all groups of shrimp during postmolt, intermolt and late premolt did not differ significantly.

When comparing MIH1 transcript levels during different molt stages of the same shrimp group, variable statistical results were detected. In large-positive, large-negative and small-negative shrimp, the early premolt level was significantly (P<0.05) higher than that in all other stages. But in small-positive shrimp, the early premolt level was significantly (P<0.05) higher than that of postmolt and intermolt, but not that of late premolt (Fig. 2a).

For MIH1 peptide in the optic lobe, highest levels were also detected during early premolt (Fig. 2b). When comparing the levels among shrimp groups during the same molt stages, no significant differences were detected. When comparing within the same shrimp group but during different molt stages, the levels in the large-positive and large-negative shrimp during early premolt were significantly (P<0.05) higher than those during all other stages. Whereas in small-positive and small-negative shrimp, early premolt levels were significantly (P<0.05) higher than those during intermolt and late premolt.

In the hemolymph, highest levels of MIH1 peptide were found during intermolt in all shrimp groups [Fig. 2c], followed by the levels during early premolt. When comparing the levels among shrimp groups, but within the same molt stage, significant differences were detected during the intermolt and premolt. During intermolt, small-negative shrimp had significantly (P<0.05) lower levels than those of the large-positive and large-negative shrimp, but not significantly different from that of the small-negative shrimp. The same pattern was detected during early premolt, but at lower levels of the peptide in all groups of shrimp.

When comparing MIH1 peptide level in the hemolymph within the same shrimp group but during different molt stages, in the
large-positive, small-positive and large-negative groups, the level during intermolt was significantly (P<0.05) higher than those during postmolt and late premolt. Whereas in the small-negative shrimp, no significant difference was detected during any of the molt stages.

Profiles of the hemolymph ecdysteroid levels in the molt cycles of all shrimp groups were comparable, with basal levels at postmolt, intermolt and early premolt, and a sharp rise at late premolt (Fig. 2d), which was significantly (P<0.05) higher than the values during other stages.

3.3. CHH1 transcript, CHH1 peptide, glycogen and glucose

The relative expression levels of CHH1 transcript and CHH1 peptide levels in the optic lobe of all shrimp groups did not differ (Table 2). However, the hemolymph level of CHH1 peptide in the small-positive shrimp was significantly (P<0.05) lower than those of other groups, including that of the small-negative shrimp; the level in the small-positive group was about one-third of the other groups.

Glycogen level of the hepatopancreas of small-positive shrimp was 2–3 times higher than and significantly (P<0.05) higher than those during other stages (Table 2). The levels in the large-positive, large-negative and small-negative groups did not differ.

The glucose level in the hemolymph of small-positive shrimp was significantly (P<0.05) lower than that of other groups, among which no significant differences were observed.

4. Discussion

In our attempt to find out the effect of slow growth in small-positive shrimp, we set up a hypothesis that MIH synthesis/release in the optic lobe of the shrimp is reduced, causing a decrease in ecdysteroids and thus higher frequency of molting or shorter molt interval. The increase in molt frequency would require energy and energy received from feed intake may be spent on this unnecessary molting activities. Alternatively, they might have reduced molt frequency and thus affecting growth rate. Our results indicated that neither of these events occurred. Our results proved that these hypotheses were wrong; all molting activities of the small-positive shrimp were comparable to the small-negative shrimp although they were significantly different from the large-negative and large-positive shrimp. These findings suggested that it is the size of the shrimp that make the difference in molting activities, not the age of the shrimp or disease condition. On the contrary, it was CHH that was significantly decreased in the hemolymph and caused high glycogen level in the hepatopancreas. The retention of glycogen resulted in low hemolymphatic glucose level, and thus rendering low energy for sufficient growth of the shrimp.

This study also revealed for the first time that MIH1 transcript in P. monodon was increased significantly during early premolt. The increase was not in agreement with MIH transcript levels observed in the molt cycle of other crustacean species. In the crab Callinectes sapidus and the shrimp Litopenaeus vannamei, MIH transcripts were high during postmolt and intermolt (Chen et al., 2007; Lee et al., 1998); this pattern of MIH transcripts corresponds to a conventional theory that MIH transcript (through its translated MIH peptide) suppresses ecdysteroids synthesis/release and only allows ecdysteroids to rise at premolt, resulting in ecdysis. On the contrary, in the crab Carcinus maenas and the Japanese shrimp M. japonicus, MIH transcript

| Table 2 |
| Levels of CHH1 transcript, CHH1 peptide, glycogen and glucose at intermolt of Penaeus monodon that were large sized and Laem–Singh virus-positive (large-positive), small sized and Laem–Singh virus-negative (small-negative); N = 10 in each group. Different superscripts in the same row indicate statistical significance (P<0.05). |

<table>
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<th>Large-positive</th>
<th>Small-positive</th>
<th>Large-negative</th>
<th>Small-negative</th>
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<td>CHH1 transcript</td>
<td>1.5 ± 0.8a</td>
<td>1.4 ± 0.4a</td>
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<td>1.4 ± 0.9a</td>
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<tr>
<td>CHH1 peptide</td>
<td>9.0 ± 2.1a</td>
<td>11.6 ± 4.1a</td>
<td>9.1 ± 4.9a</td>
<td>8.9 ± 3.1a</td>
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<tr>
<td>In the optic lobe</td>
<td>33.7 ± 7.2a</td>
<td>11.5 ± 6.1a</td>
<td>29.4 ± 5.9a</td>
<td>28.2 ± 13.7a</td>
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<tr>
<td>In the hemolymph</td>
<td>9.9 ± 2.4a</td>
<td>20.0 ± 8.1a</td>
<td>6.4 ± 4.1a</td>
<td>7.7 ± 3.3a</td>
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<td>Glycogen (mg/g dry weight of hepatopancreas)</td>
<td>46.0 ± 10.1ab</td>
<td>29.3 ± 12.5b</td>
<td>46.8 ± 9.1a</td>
<td>45.7 ± 5.4a</td>
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<tr>
<td>Glucose (μg/mL hemolymph)</td>
<td>33.7 ± 7.2a</td>
<td>11.5 ± 6.1a</td>
<td>29.4 ± 5.9a</td>
<td>28.2 ± 13.7a</td>
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*Fig. 2. Relative expression of the levels of MIH1 transcript (compared to the levels of elongation factor-1α) in the optic lobe (a), MIH1 peptide in the optic lobe (b), MIH1 peptide in the hemolymph (c) and ecdysteroids in the hemolymph (d) of Penaeus monodon that were large sized and Laem–Singh virus-positive (large-positive), small sized and Laem–Singh virus-negative (small-negative), large sized and Laem–Singh virus-negative (large-negative) and small sized and Laem–Singh virus-negative (small-negative). Different superscript letters within a molt stage indicate significant differences (P<0.05).*
levels and its encoded MIH peptide remained the same throughout molt cycle (Chung and Webster, 2003; Ohira et al., 1997), and it was suggested that changes in ecdysteroid synthesis in the Y-organ was controlled by susceptibility of the gland itself to MIH peptide, not by the level of MIH as conventionally believed.

Levels of MIH1 peptide in the optic lobes of small-positive shrimp were similar to other shrimp groups that showed highest levels during early premolt and dropped significantly during late premolt, postmolt, and intermolt. This pattern was similar to that of MIH peptide in the sinus gland of the crayfish Procambarus clarkii (Nakatsuji et al., 2000). In this study, levels of MIH1 peptide in hemolymph of P. monodon were highest during intermolt and decreased during early premolt before they dropped to basal levels during late premolt and postmolt. The reason why the MIH1 peptide levels in the hemolymph did not follow those in the optic lobe was not known. During intermolt, low levels of MIH1 peptide in the optic lobe and high levels in the hemolymph could be due to the release of MIH1 from the storage site at the sinus gland into hemolymph; and the high levels of MIH1 in the optic lobe during early premolt might be due to storage of MIH1 in the sinus gland, where a fraction of the peptide was released into the hemolymph.

The hemolymph levels of MIH1 peptide in this study were different from that reported in the hemolymph of P. clarkii, which showed the lowest level during early premolt (Nakatsuji and Sonobe, 2004). The drop in early premolt coincided with a rise in ecdysteroid levels in the hemolymph in that species, but the rise in MIH peptide during middle and late premolt is concomitant with a rise in ecdysteroid levels. This did not suggest a conventional MIH-suppressive effect on ecdysteroid synthesis. In the crab C. maenas, levels of MIH1 peptide remained the same throughout molt cycle (Chung and Webster, 2005), suggesting unresponsiveness of Y-organ to MIH peptide. This drop in the study, drop in MIH1 peptide in the hemolymph coincided with a sharp rise in ecdysteroid levels in the same stage. The findings suggest that, in P. monodon, MIH peptide acted in a conventional way to suppress the ecdysteroid level.

In the present study, levels of MIH1 transcript (in the optic lobe) and MIH1 peptide (in the hemolymph) of small-positive shrimp were comparable to that of small-negative shrimp, but significantly lower than those of large-negative and large-positive shrimp from both ponds during intermolt and early premolt. The results indicated that the size, not the age, of shrimp had influence on the levels of both parameters. While large-sized shrimp had higher levels of MIH1 in the hemolymph than small-sized shrimp, levels of ecdysteroids in all the shrimp of different sizes were comparable. It is possible that the Y-organ in P. monodon became less sensitive in response to MIH1 peptide as the size of the shrimp increased. In this respect, it is interesting to explore further how age and size affect MIH control of ecdysteroid synthesis/release from the Y-organ in crustaceans, which has been a controversial topic recently. Peak levels of ecdysteroids during late premolt in this study agreed well with those reported in the blue crab C. sapidus and in the crayfish P. clarkii (Lee et al., 1998; Nakatsuji and Sonobe, 2004).

In the present study, both CHH1 transcript and CHH1 peptide in the optic lobe in all shrimp groups under study were comparable, whereas CHH1 peptide in the hemolymph in the small-positive shrimp was significantly lower than that of the small-negative shrimp, as well as the large-negative and large-positive shrimp. The result was substantiated by the finding of significantly high glycogen level in the hepatopancreas and low hemolymphatic glucose levels. It is well-known that CHH in crustaceans functions as an agent that increases hemolymph glucose level, mainly by breaking down glycogen in the hepatopancreas (Chang and O’Connor, 1985; Keller and Orth, 1990; Sedlineier, 1982, 1988). A decreased level of CHH1 peptide in P. monodon thus caused a decrease in glycogen breakdown and decreased hemolymph in the small-positive shrimp. It can be stated that small-positive shrimp suffer from a chronic hypoglycemic condition, resulting in growth retardation.

It is interesting to find a decreased level of CHH1 peptide level in the hemolymph of small-positive shrimp despite a normal level of the peptide in the optic lobe. This could be due to an inhibition of CHH1 peptide being released from the neurosecretory cells or from the sinus gland of the optic lobe, or by an increasing metabolic degradation of the circulating CHH1 peptide. Regarding the first possibility, several lines of evidence suggest that the neurotransmitter serotonin is involved in the stimulation of CHH peptide release from the optic lobe in several crustacean species, including in the crabs C. maenas and Chasmagnathus granulatus and in the crayfish P. clarkii and Orconectes limosus (Escamilla-Chimal et al., 2002; Luschen et al., 1993; Santos et al., 2001). A defect in serotonin induction of CHH neuronsecretory cells may cause a decrease in CHH release from the optic lobe. Immunoreactive studies have demonstrated the expression of serotonin in retinular cell axons of juvenile crayfish, P. clarkii (Escamilla-Chimal et al., 2001). The same study also showed expression of CHH in tapetal cells of the retina and the axons and terminals of the X-organ sinus gland tract and in the sinus gland. From these studies it is therefore possible that the infection of LSNV in zona fasciculata (Pratoomthai et al., 2008) that comprise the retinular cell axons running from the retina to the lamina ganglionaris of the optic lobe may reduce serotonin induction of CHH peptide secretion from the X-organ/sinus gland complex and finally lead to growth retardation in small-positive P. monodon. This interesting hypothesis is currently under investigation.

5. Conclusions

The study is aimed at finding the cause of growth retardation in black tiger shrimp P. monodon suffering from Monodon Slow Growth Syndrome (MSGS). Previous finding suggested that MSGS shrimp is associated with Laem–Singh virus (LSNV) infection. The virus infects several organs and tissues of the shrimp but a large fraction of infected shrimp did not show MSGS unless the infection is extended into an area in the shrimp retina called zona fasciculata. This area links retinular cells to other areas in the shrimp optic lobe that contains several neuroendocrine cells. We hypothesized that molt-inhibiting hormone (MIH) and/or crustacean hyperglycemic hormone (CHH) may be altered by the lesion. We have found that levels of MIH transcript, MIH peptide, ecdysteroids, as well as durations of molt stages and interval of the small-MSGS shrimp, were in normal range of small-sized shrimp. The level of CHH peptide, however, was significantly lowered in the hemolymph, in association with an increase in glycogen in the hepatopancreas and a decreased glucose level in the hemolymph. We conclude that in LSNV cause growth retardation in P. monodon by damaging zona fasciculata, and subsequently inhibiting the release of CHH from the optic lobe, decreasing glycogen breakdown in the hepatopancreas and causing chronic hypoglycemia.

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