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# Molecular investigation on the presence of monodon slow- growth syndrome (MSGS) in the green tiger shrimp *Penaeus semisulcatus* in Egypt; biofloc as a control measure

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## ABSTRACT

Juveniles of *Penaeus semisulcatus* were sampled from shrimp farms and were sorted into four categories; small-negative, large-negative, small-positive and large-positive groups based on the size of the shrimp. Levels of MIH-1 and CHH-1 transcripts, peptide, ecdysteroid, glucose and glycogen were determined. The highest levels of MIH-1 were noticed at early pre-molt stage, while the lowest was noticed at inter-molt stage. However, the relative expression level of CHH-1 transcript in the optic lobe from the inter-molt stage of all different shrimp categories (large-negative, small-negative, large-positive and small-positive, was significantly different ($P$<0.05). Rabbit anti-MIH-1 antibody at 1:10,000 dilutions was bound to rMIH-1 without cross reactivity to with rCHH-1 or rGIH, whereas the anti-CHH antibody was bound to rCHH-1, without cross reactivity to rMIH or rGIH. The haemolymph ecdysteroid level was 90-100 ng/ml at late pre-molt stage and significantly different ($P$<0.001) from the post-molt, inter-molt and early pre-molt stages. The average glycogen concentration of the hepatopancreas of large-positive shrimp was not significantly different ($P$>0.05) from small- and large-negative shrimp. Similarly, no significant difference ($P$>0.05) was noticed in the average glycogen concentration of hepatopancreas between small-negative and large-negative shrimps. The average glucose concentration in haemolymph of large-positive shrimp was not significantly different ($P$>0.05) from small-negative and large-negative shrimps. Two low protein diets ($^{\sim 20}BFd_{20.13}$ and $^{\sim 22}BFd_{22.20}$) and two biofloc ($BFd_{Sucrose}$ and $BFd_{Glucose}$) were prepared in addition to control (35% crude protein). The lowest glucose concentration was estimated in the control (13.45±2.15 µg/ml), and the highest glucose concentration was found in $BFd_{Glucose}$ (75.01±2.31 µg/ml), followed $BFd_{Sucrose}$ (45.32±1.32 µg/ml), $^{\sim 20}BFd_{20.13}$ (43.51±3.21 µg/ml) and $^{\sim 22}BFd_{22.20}$ (40.21±3.12 µg/ml). Pleopod tissues from shrimp *P. semisulcatus* grown in biofloc, low protein diets, and control were tested by RT-PCR. LSNV was not detected in *P.semisulcatus* grown in biofloc, low protein diets, compared to control. This study revealed that biofloc treatments and low protein diets could enhance growth rates and control MSGS in *P. semisulcatus*.

## INTRODUCTION

Penaeid shrimp culture has attracted Egypt's attention in recent years for food security and as a foreign exchange earner. Thus, Egypt has given shrimp farming a

priority in its aquaculture programs. Shrimp farming in Egypt starts in the early 1980s, where a first shrimp farm were established near Alexandria (Megahed *et al*., 2013a, 2013b). In Egypt, most hatcheries collect wild gravid females to produce shrimp postlarvae (PL) or collection of wild PL of *Penaeus semisulcatus* and *Marsupenaeus japonicus* for growout and this puts farmers at risk as a result of unpredictability in the abundance of gravid broodstock and PL, in addition to transmission of diseases (Megahed *et al*., 2013a, 2013b). The farming of *Penaeus semisulcatus* still depends on collection of wild broodstock and the first trial to domesticate this important species in Egypt was established between 2009-2010 at Shrimp and Fish International Company (SAFICO), Gulf of Aqaba, Nabq Protectorate, Sharm EL Sheikh, Egypt (Megahed *et al*., 2013a, 2013b). Thus, sustainability of shrimp farming requires continuous controlled reproduction maintained in captivity. The *P. semisulcatus* was the oldest and preferable farmed shrimp in Egypt since 1980s. However, farming of *P. semisulcatus* suffers from low growth and low survival. After the recognition of this problem in Egypt, a detailed survey of known shrimp pathogens and environmental factors was carried out to identify the factors behind this problem.

A detailed screening of known shrimp pathogens such as, white spot syndrome virus (WSSV), and early mortality syndrome (EMS) revealed that their presence was not correlated to the abnormal slow growth and low survival of *P.semisulcatus*. The study of water quality parameters did not reveal any association with abnormal slow growth and low survival and the values recorded were normally measured and considered acceptable for tropical shrimp farming (Chen and Lei, 1990; Forteath, 1990; Muir *et al*., 1991; Tsai and Chen, 2002; Lin and Chen, 2003). A similar case occurred in Thailand since 2002 and diagnosed to be monodon slow growth syndrome (MSGS) caused by a new virus called Laem-Singh virus (LSNV) (Prakasha *et al*., 2007; Sritunyalucksana *et al*., 2006). The LSNV is RNA virus and is icosahedral viral like particles 25 nm in diameter detected in *P. monodon* from MSGS affected farms in Thailand and detected in the present study in *P. semisulcatus* in Egypt.

Biological, physiological and molecular investigation of *P. semisulcatus* shrimp farms, revealed four shrimp categories according to previously described by (Sritunyalucksana *et al*., 2006), shrimp grow normally and LSNV-free (large-negative, LSNV- LN), shrimp grow slowly and LSNV-free (small-negative, LSNV-SN), shrimp grow normally and LSNV-positive (large-positive, LSNV- LP) and shrimp grow slowly and LSNV-positive (small-positive, LSNV- SP). The common measures used for prevention and treatment include application of chemicals and drugs to control aquatic pathogens have failed to be long term strategy for disease control. One of the alternative strategies to control disease outbreaks in aquaculture is the application of probiotics. Recently, biofloc technology considered a biosecurity farming technology (Megahed and Mohamed, 2014; Megahed *et al*., 2018). Biofloc technology depends on manipulation of carbon nitrogen ratio (C:N ratio) whether by addition of external carbon source into the water of culture systems or increasing carbon level in the feed (Avnimelech, 1999; McIntosh, 2000; Ballester *et al*., 2010; Crab *et al*., 2012; Anand *et al*., 2013; Megahed, 2010; Megahed and Mohamed, 2014; Megahed *et al*., 2018).

At high C: N ratio, heterotrophic bacteria immobilize ammonia for production of microbial protein which can be used by the cultured animals as supplemental food and reduces inorganic nitrogen level (Avnimelech, 1999; Megahed, 2010; Megahed and Mohamed, 2014; Megahed *et al*., 2018). Several research work indicated that

biofloc are rich source of growth promoters and bioactive compounds (Ju *et al*., 2008a, 2008b) which enhance digestive enzymes (Xu and Pan, 2012) and shrimp health (Bright *et al*., 2005; Megahed and Mohamed, 2014; Megahed *et al*., 2018). The objective of the present study is to understand the mechanism of MSGS in *P. semisulcatus* and the effect of biofloc as a control measure.

## MATERIALS AND METHODS

### Investigated site and shrimp

This study was carried out at Dibah Triangle Zone (DTZ), Egypt. The estimated total area under production is around 10,000 ha used for marine shrimp and finfish farming and faced several constraints led to the failure of the shrimp production. Three farms raised *P. semisulcatus* were selected for the present study and investigated for MSGS by biological, physiological and molecular methods according to (Sritunyalucksana *et al*., 2006). Shrimp in the first farm was thirty days from date of culture (DOC-30) with average body weight (ABW) ranged from 4-9 g (LSNV-SN). The second farm raised *P. semisulcatus* was thirty days of culture DOC-30 with ABW ranged from 21-27 g (LSNV-LN). The third farm assigned as MSGS infected farm ninety days of culture DOC-90 with two groups , one with ABW ranged from 4-9 g (LSNV-SP) and second group of ABW ranged from 21-27g (LSNV-LP) with external clinical signs of MSGS shrimp as described in the literature by (Sritunyalucksana *et al*., 2006). Twenty- five random individual shrimp were taken from each farm to confirm the presence of LSNV infection according to (Sritunyalucksana *et al*., 2006).

### RT-PCR screening of *P. semisulcatus* samples

Total RNAs were extracted from shrimp pleopods using TriReagent (Qiagen), following the manufacturer's instructions. The RT-PCR reagent consisted of 2x reaction mix (10µM sense primer, 10µM anti-sense primer and SuperScript™III RT/Platinum® *Taq* Mix (Invitrogen™, USA). 11.5µl of the RT-PCR reagent was added into each 0.5 ml Eppendorf tube. 1 µl of each of extracted RNA, DEPC-water (negative control) and positive control was added into each reaction mixture. Optimal amplification conditions with 35 cycles amplification: cDNA synthesis at 50°C for 30 min; denaturation at 94°C for 2 min followed by PCR amplification of denaturing at 94°C for 20 sec, annealing at 55°C for 35 sec, an extension at 68°C for 1 min and final extension at 67°C for 6 min.

### The mRNA expression of MIH-1, CHH-1 and EF-1α from *P. semisulcatus* eyestalk

In the present study, primers for MIH-1*:* F 5́catagacggcacttgtcgag 3́ and MIH-1: R 5́ cctgttggcagcctttagac 3́ and EF-1α: F 5́gaactgctgaccaagatcgacagg 3́ and EF-1α: R 5´gagcatactgttggaaggtctcca 3´ were used to study the levels of MIH-1 and CHH-1 transcripts and the expected amplicon sizes were 172 and 140 bp, respectively (Sritunyalucksana *et al*., 2006). Specific primer CHH-1: F 5́ ccagaagcctctcctgtgac 3́ and CHH-1: R 5' acaactgggtgggttactgc 3' for CHH gene was used and the expected amplicon sizes were 199 bp (Sritunyalucksana *et al*., 2006). The sequence of CHH-1 was detected by purification of PCR products using QIAquick PCR purification kit (QIAGEN, Germantown, USA), then ligated to pGEM®-T easy vector system (Promega, Madison, USA). 10 µL of a mixture consisted of (1 µL of 200 ng DNA fragment, 3 µL of 50 ng pGEM ®-T easy vectors, 5 µL of 2 x ligation buffers, and 1 µL of T4 DNA ligase) was incubated overnight at 4 °C, then transformed into the competent cells *E. coli* XL1 blue (GenomeWeb LLC, New York, USA). 50 µL of prepared competent cells was added to ligation mixture and cooled on ice for 30 min.

The reaction tube was incubated in water bath at 42 °C for 2 min, and then cooled on ice for 2 min. A mixture of 500 µL of LB medium (10 g Bacto-Tryptone (VWR Corporate, Atlanta, GA, USA), 5 g Bacto-yeast extract (VWR Corporate, Atlanta, GA, USA), 10 g NaCl, $ddH_2O$ to 1 L) was added to the ligation tube, mixed and incubated on shaker for 1 h at constant temperature 37 °C. 200 µL of the transformed reaction of was plated on the LB/ampicillin/X-gal plate (Sigma-Aldrich, St. Louis, USA), prepared by mixing 10 g Bacto-Tryptone, 5 g Bacto-yeast extract, 10 g NaCl, 15 g Bacto-agar and $ddH_2O$ to a volume of 1 L with addition of 100 µg/ml ampicillin, and 80 µg/ml X-gal. The plate was incubated at 37 °C for 16 h. Bacteria colonies of *E. coli* was analyzed using 25 µL PCR volume containing 18 µL $ddH_2O$, 2.5 µL 10 x buffer, 0.75 µL $MgCl_2$, 0. 5 µL dNTP, 1 µL sense primer, 1 µL antisense primer, 0.25 µL *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, USA) and *E. coli* cells. 25 cycles amplification of denaturing at 94 °C for 5 min, annealing at 55 °C for 30 sec, an extension at 72 °C for 30 sec and final extension at 72 °C for 5 min. PCR products were analyzed on a 2% agarose gel stained with ethidium bromide and visualized by UV-trans-illumination (Major Science, USA). Single colony was selected and cloned in LB medium contain ampicillin on shaker overnight at 37 °C. Cells containing inserted plasmid DNA were extracted using QIAGEN Plasmid Mini Kit (QIAGEN, Germantown, USA). PCR was carried out to confirm the insertion using gene specific primers before DNA sequencing. The sequence of a single colony was compared with the CHH-1 sequences using CLUSTAL-W software package http:// www.ebi. ac.uk / Tools/clustalw2/index.html.

**RT-PCR of the optic lob of *P. semisulcatus* Eyestalks**

Eyestalks of *P. semisulcatus* from each molting stage were used to determine MIH-1 and CHH-1 transcript level. The RNA from the optic lobe was isolated using RNeasy Plus Mini Kit (QIAGEN, Germantown, USA), following the manufacturer's instructions. RT-PCR was carried using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies Corporation, NY, USA) according to the manufacturer instructions The reaction mixture (5-10 µg of total RNA, 1 µl of oligo dT and 1 µl of dNTP) incubated at 65°C for 5 min, then cooled on ice for 1 min. The reaction mixture was added to a mixture of (4 µl of 5 x first-strand buffer, 1 µl of 0.1 M DDT, 1 µl of RNase Inhibitor and 1 µl of superscript III (200 unit/µl). The reaction was mixed and incubated at 55°C for 60 min and at 72°C for 15 min in order to inactivate the reverse transcriptase. The PCR mixture consisted of (1 µl of cDNA sample, 300 nM of each primer, and 12.5 µl of Fast SYBR™ Green Master Mix (Applied Biosystems™, CA, USA) in a final volume of 25 µl. RT- PCR was carried out on ABI Prism 7500 Sequence Detection System (Applied Biosystems™, CA, USA). Optimal amplification was obtained with initial denaturation at 94 °C for 15 min, then followed by 40 cycles of denaturing at 94 °C for 15 sec, annealing at 55°C for 30 sec and an extension step at 72 °C for 31 sec. The amplifications of MIH-1, CHH-1 and EF-1α cDNAs were performed simultaneously in separate tubes. The validity of the RT-PCR in the determinations of MIH-1/CHH-1 transcripts was carried out according to Kubista *et al*. (2006) and Sritunyalucksana *et al*. (2006). Briefly, sample titration curve was plotted and its slope was used to calculate amplification efficiency using equation $E = (10^{-1/slope})-1$. The comparative threshold cycle method was used to estimate transcript levels of the target genes (MIH-1 and CHH-1) and the reference gene (EF-1α). The transcript level of MIH-1/CHH-1 was determined by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), by subtracting Ct value of MIH-1/CHH-1 from the Ct of EF-1α (ΔCt) to normalize for any difference in RNA used for each RT-PCR reaction. The maximum value of ΔCt for

MIH-1/CHH-1 was set as calibrator. The ΔΔCt was obtained by subtracting ΔCt of MIH-1 or CHH-1 from ΔCt of calibrator.

**Production of polyclonal antibodies for MIH-1 and CHH-1**

A single colony of rMIH-1 *and* rCHH-1 was incubated in 30 ml of LB medium (Invitrogen™, USA) contain 100µg/µl ampicillin, 34µg/µl chloramphenicol and incubated at 37°C with shaking, 200 rpm for 14-16 h. The overnight culture was diluted at 1:50 in LB medium and incubated at 37°C with shaking, 200 rpm until OD600 reached 0.4-0.6. The expressed recombinant protein of MIH-1/CHH-1 is induced by 0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, Milwaukee, WI, USA). The culture was incubated at 30°C for 4 h with shaking. The cell culture was harvested by centrifugation at 6,000 rpm for 15 min at 4°C. The supernatant was discarded and the pellets were stored at -20°C. After expression of rMIH-1/rCHH-1, 80 ml of *E. coli* culture was centrifuged at 8,000xg for 10 min at 4°C. The pellet was resuspended in 25 ml of 1X Sodium Chloride-Tris-EDTA (STE) buffer pH 8.0 (10mM tris, 1mM EDTA, and 100mM NaCl) (Thermo Fisher Scientific, New Hampshire, USA) with 100 µg/µl lysozyme and cooled on ice for 15 min. 2.5 ml of 10% Triton X-100 (Sigma-Aldrich, Milwaukee, WI, USA) was added to the cell suspension in STE buffer and sonicated for 3-5 times until the supernatant become clears. The sonicated supernatant was centrifuged at 6,000 rpm for 30 min at 4°C to separate inclusion fraction from soluble fraction. The inclusion fraction was washed with $ddH_2O$ and sonicated for 3-5 times then divided into 4 small aliquot in 2 ml microcentrifuge tube and stored at -20°C.

**Protein electrophoresis**

A protein sample was prepared by mixing optic lob with 5x sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.1% (w/v) Bromophenol Blue), boiling for 10 min and centrifuged at 13,000 rpm for 5 min. Glycine SDS-PAGE was prepared according to Laemmli (1970). Tricine SDS-PAGE was used to separate protein of size of less than 20 kDa according to Judd (1994). The concentration of protein was determined using Bradford's method using Bio-Rad Protein Assay kit (BIO-RAD, USA) according to the manufacturer instruction.

**Preparation of rMIH-1/rCHH-1 for antibody production**

The inclusion fraction of rMIH-1/rCHH-1 was run on Glycine SDS-PAGE as describe above. The gel was fixed in 10 (v/v) acetic acid and 40% (v/v) methanol in $ddH_2O$ for 2 h. The gel was stained in 4 volumes of (910 g ammonium sulfate, 1 g Coomassie blue G-250, 20 ml $H_3PO_4$ in 1 L of $ddH_2O$) to 1 volume of methanol for 1h and destained in $ddH_2O$ until the background was clear. The gel slice containing rMIH-1/rCHH-1 was excised from the gel, minced into small pieces and loaded into the Model 422 Electro-Eluter (BIO-RAD, USA). The rMIH-1/rCHH-1 was eluted in 25 mM Tris, 192 mM Glycine and 0.04% (w/v) SDS at current of 10 mA/glass tube for 7 h. The eluted rMIH/rCHH-1 was used as an antigen to raise anti-MIH-1/CHH-1 antibody in mouse. For production of anti-MIH-1/CHH-1 antibody in mouse, 50 µg of purified proteins were used as antigen to immunize mice by intradermal injection once every 15 days. Antigen was mixed with an equal volume of Freund's complete adjuvant (InvivoGen, San Diego, USA) for the first injection. Subsequent injections were conducted by using antigen mixed with an equal volume of Freund's incomplete adjuvant. One week after the last injection, the serum was collected from each mouse by the eye bleeding in 2 ml microcentrifuge tube and centrifuged at 5,000 *g* for 10 min and the supernatant was then transferred into a new tube, divided into aliquots of

100 µL and stored at -20°C. The specificity of the antibodies was determined by western blot analysis.

**Western Blot Analysis**

Western blot analysis was used to determine the specificity of the antibodies. Proteins (rMIH-1 and rCHH-1) eluted from Glycine SDS-PAGE and recombinant gonad-inhibiting hormone (rGIH) was tested for binding with the antibodies. The eyestalks containing peduncular neural ganglia (XOSG complex) were dissected under a Stereo Zoom dissection microscope (Microscope World, Carlsbad, USA). The optic lobes were quickly excised, cleaned, and placed in a medium containing (10 mM $KH_2PO_4$, 0.02 M HEPES, 0.25 M sucrose, and 0.5 mM EDTA, and 5% fatty acid free BSA, adjusted to pH 7.5 at 25°C). The tissue sample was homogenized in 10 volumes of the medium. The homogenate was transferred to a centrifuge tube and centrifuged at 800*xg* for 10 min. The supernatant was collected and transferred to a new micro centrifuge tube, centrifuged at 9000*xg* for 10 min. The pellet obtained was washed in a medium containing (10 mM $KH_2PO_4$, 0.02 M HEPES, 0.25 M sucrose, and 0.5 mM EDTA, and adjusted to pH 7.5 at 25°C), then resuspended in a final volume of 1 ml of a medium containing (10 mM $KH_2PO_4$, 0.02 M HEPES, 0.25 M sucrose, and 0.5 mM EDTA, and adjusted to pH 7.5 at 25°C). The protein sample was prepared and run on SDS-PAGE as describe by Laemmli (1970). The Mini Trans-Blot® electrophoresis transfer cell (BIO-RAD, USA) was used to transfer protein samples from acrylamide gel to a membrane. PVDF membrane (Invitrogen™, USA) was pre-wet in methanol for a 15 seconds then equilibrated for 5-10 min in transfer buffer containing (25 mM Tris-HCl, pH 9.2, 192 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol). The transblotting sandwich was assembled and electroblotted at a 30 volts for overnight at 4°C. After blotting, the membrane was removed and rinsed with $ddH_2O$ prior to staining with 1.0% (w/v) Coomassie Brilliant Blue R-250.

**Determination of haemolymph ecdysteriods**

Haemolymph of *P.semisulcatus* was withdrawn from the arthropodial membrane with disposal insulin needle into 1.5 ml microcentrifuge tube contain 300 µl of 95% methanol alcohol, vortexed and stored at -20°C. For radioimmunoassay (RIA), stored sample tubes were thawed at room temperature, vortexed and centrifuged at 14,000*xg*; 4°C for 20 min. 100 µl of supernatant were added into 1.2 ml RIA tube (PerkinElmer, OH, USA), then heated and vacuumed dye. 100 µl of each supernatant was added into triplicate tubes and then vacuum dried. 100 µl of the radiolabel (3H)-ecdysone was added to both sample and standard tubes containing a series of concentrations (25, 50, 125, 250, 500, 1000, 2000, and 4000 pg) of 20-hydroxyecdysone (Sigma-Aldrich,Milwaukee, WI, USA), except control. Ecdysteroid antisera, M-20 ecdysone antiserum was added to sample and standard tubes, while normal rabbit serum was added to control tubes and all tubes were incubated at 4°C overnight. The antigen-antibody complex was precipitated with cold saturated ammonium sulfate. The precipitate was dissolved in 25 µl of $ddH_2O$ and analyzed for radioactivity with 600 µl of CytoScint ES (Thermo Fisher Scientific, Waltham, MA, USA) in a TRI-CARB 4910TR 110 V Liquid Scintillation Counter (PerkinElmer, Akron, OH, USA). The circulating levels of ecdysteroids were expressed as edysteroids equivalent (ng/ml haemolymph).

**Measurement of glycogen content and plasma glucose concentration**

Glycogen in the digestive gland was extracted in the presence of sulphuric acid and phenol (Dubois *et al*., 1965). To do so, the digestive gland was first homogenized in 5% trichloroacetic acid for 2 min at 3340 *g*. The supernatant was

quantified and 200 µl were pipetted into a tube and mixed with 5 volumes of 95% ethanol. Tubes were placed in an oven at 37-40°C for 3h. After precipitation, the tubes were centrifuged at 7000 *g* for 15 min. The glycogen pellets were then dissolved by adding 0.5 ml of boiling water. One ml of concentrated sulphuric acid and phenol (5%) was added and mixed. Tube contents were transferred to microplates in duplicate and read at 490 nm with a microplate reader (Bio-Rad 550). To obtain hemolymph glucose concentrations, plasma was extracted from hemolymph, which had been previously diluted in an isotonic solution with a complex to avoid coagulation of the hemolymph (SIC-EDTA) anticoagulant (NaCl 450 mM, KCl 10 mM, HEPES 10 mM, EDTA 10 mM; pH 7.3) at 2-8°C according to Vargas-Albores *et al*. (1993) considering a hemolymph-anticoagulant ratio equal to 1:2. The haemolymph with anticoagulant was centrifuged at 2800 rpm for 2 min at 4°C and the supernatant was collected in 2 ml tubes. Glucose determinations were made in plasma aliquots of 20µl with 200µl of the glucose assay kit (MilliporeSigma, MO, USA), placed in microplates and read in an ELISA lector (Elicrom, Ecuador) at 540 nm. SIC-EDTA was used as a blank. The metabolite concentration was calculated using a calibration curve, in which the standard was the substrate that acts as the reagent in the kit.

**Effect of biofloc on growth and disease prevention of MSGS in small-positive *P. semisulcatus***

***Experimental conditions***

Healthy juvenile green tiger shrimp *P. semisulcatus* of an average weight of 2.30 ± 0.06 g, tested negative for WSSV and EMS (Lightner, 1996) (Fig. 1a,b) were obtained from a shrimp farm (DTZ, PortSaid, Egypt).

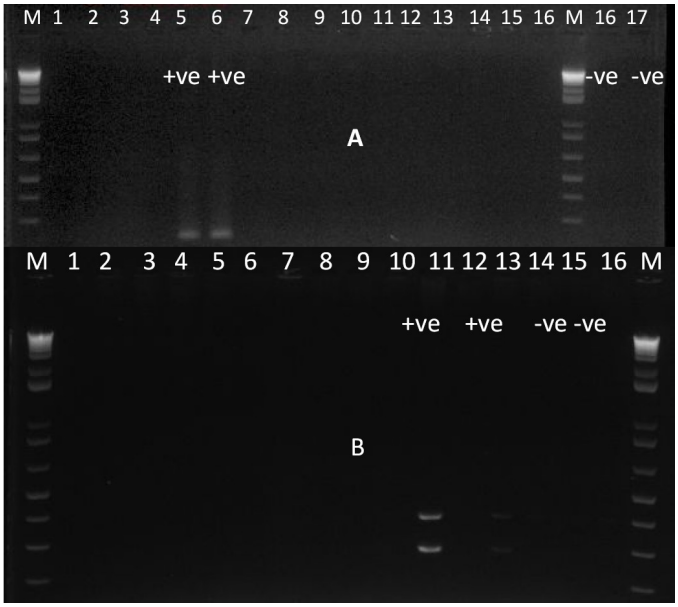


Fig. 1. Diagnostic results of A) EMS, B) WSSV of shrimp samples used in LSNV study.

The shrimp was stocked at an initial density of 15 individuals per tank. The feeding experiment consisted of two low protein diets ($^{\sim 20}BFd_{20.13}$ and $^{\sim 22}BFd_{22.20}$), two biofloc treatments using sucrose ($BF_{Sucrose}$) and glucose ($BF_{Glucose}$) as a carbon sources in plastic tanks of 100 L in capacity; in addition to control 35% crude protein (CP). The compositions of experimental diets are presented in Table 1 and feed were stored at 4 °C until use. A peristaltic pump distributed the biofloc influent continuously and hydraulic retention time was 1 day. The feed was given at 5% of the

shrimp biomass. The feed was given two times a day at 9:00 AM and at 5:00 PM. The amount of biofloc given as feed was determined based on the crude protein content and the dry weight of biofloc to obtain the same level of protein input in each treatment (isonitrogenous diet), which was measured every 5 days. The water quality parameters such as, salinity, temperature and pH were measured using an ATAGO (ATAGO CO., LTD. Tokyo, Japan), hand refractometer, thermometer and pH meter, respectively. Dissolved oxygen; total ammonia-N (TAN), nitrite-N ($NO_2N$) and nitrate-N ($NO_3N$) were analyzed immediately after sample collection following the procedures described in APHA (1998). Sludge volume index (SVI) in biofloc fed tanks were determined by sampling 1000 mL water into a series of Imhoff cones. The growth performance was assessed in terms of weight gain (%), specific growth rate (SGR, %/day), feed conversion ratio (FCR), and survival (%) using the following formulae: Weight gain (%) = (Final weight – Initial weight/Initial weight) X 100; SGR (%/day) = (ln (final weight in grams) - ln (initial weight in grams) x100) / t (in days); FCR = Feed applied/Body weight gain; and Survival rate (S%) = (Total number of shrimps survived (harvested)/total number of shrimp stocked) X 100.

**Proximate and biochemical analyses of biofloc and low protein diets**

The proximate composition of the biofloc and experimental diets were determined following AOAC (1995). The moisture content was determined by drying at 105 °C to a constant weight, and the difference in weight of the sample indicated the moisture Content. Crude protein was determined by Kjeldahl method (Kelplus, Pelican equipments, India). Crude lipid was determined by the solvent extraction method by Soxtec system (Soxtec system, SCS–6, Pelican equipments, India) using diethyl ether (boiling point, 40–60 °C) as a solvent. Ash content was determined by incinerating the samples in a muffle furnace at 600 °C for 6 h. Crude fiber was determined based on the weight loss on ignition of the oven dried residue remaining after sequential digestion of a sample with $H_2SO_4$ and NaOH solution using Fibretec (Foss Tecator 2022, Sweden). Gross energy was determined as per (Jobling, 1983). Total nitrogen free extract (NFE) was determined as per the formula described in Hastings and Dupree (1969).

NFE (%) = 100 - (Crude protein + Ether extract + Ash + Fiber); Gross energy (GE), Kcal was calculated by multiplying CP x 5.65 + fat x 9.45 + (Carbohydrate +Fiber) x 4.1

Total lipids in the biofloc were extracted according to Folch *et al*. (1957). Fatty acid methyl esters were prepared according to AOAC (1995). Briefly, the lipid extract was boiled in a condenser with 2 mL methanolic NaOH for first 5 min followed by 2 mL BF3-methanol for the next 2 min. To recover the fatty acid methyl ester (FAME) in organic phase, 5 mL heptane was added and heated for 8 min, and washed with saturated NaCl solution into a separating funnel. The upper FAME layer was stored in glass vials at 0 °C for further analysis in GC–MS. The methylated fatty acids were separated using GC–MS (QP2010, Shimadzu, USA) equipped with DB Wax (30 m × 0.25 mm internal diameter × 0.25 µm film thickness) capillary column (Cromlab SA, Spain). Helium was used as carrier gas. Injector and detector temperatures were set at 250 °C. Injection was performed in split mode (1:15) with an injection volume of 1 µl FAME. The initial column temperature was maintained at 50 °C for 2 min. The temperature was set to increase at the rate of 10 °C per minute until the final temperature reached 230 °C and kept for 35 min. Fatty acid methyl esters were separated at a constant pressure of 82.5 KPa. The peaks were identified by comparing a constant pressure of 82.5 KPa. The peaks were identified by comparing the mass spectra with the mass spectral data base. Amino acid content in the samples

was quantified by high performance liquid chromatography (HPLC) (Teshima *et al*., 1986).Glucose concentration was determined using a glucose assay kit (MilliporeSigma, MO, USA) as described previously. Standard curve were produced using glucose concentrations of 20, 40, 60 and 80 µg/ml from a glucose assay kit (MilliporeSigma, MO, USA).

***Detection of Laem-Singh virus (LSNV) in small-positive P. semisulcatus grown in control, biofloc treatments and low protein diets in the presence of biofloc***

In the present study, samples of *P. semisulcatus* collected from low protein diets, biofloc and control diet were tested by RT-PCR and RT-nested PCR for the presence of LSNV infection according to (Sittidilokratna *et al*., 2009;). Eyestalk tissues were collected in RNAlater (Invitrogen™, USA) and stored at –20°C. Total RNA was extracted using TRIzol Reagent (Invitrogen™, USA) according to manufacturer instructions and stored at –80°C. Screening for the presence of LSNV was conducted by RT-PCR as described above (Sritunyalucksana *et al*. 2006). For cDNA synthesis, a TaqMan® Gold RT-PCR Kit (Applied Biosystems, CA, USA) was used in a 10 µl volume (100 ng total RNA, TaqMan RT Buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM random hexamer primers, 4 U of RNase inhibitor and 12.5 U of MultiScribe reverse transcriptase). The reverse transcription amplification cycle was as follows: 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. PCR was conducted in a 25 µl volume containing 1 µl of first-strand cDNA, 2.5 U of Platinum® Taq DNA Polymerase (Invitrogen™, USA), Platinum PCR Buffer, 200 µM of each dNTP and 0.4 µM of each primer BLF (5́-CGT TGC CTT CTC CCG AGT GGT-3́) and LR1 (5'-AAT CTC ACC ATG AAG CTC CTC AC-3'). The cycle amplification included an initial activation step of 94°C for 2 min, followed by 35 cycles amplification of denaturing at 94°C for 30 s, annealing at 60°C for 30s and extension at 72°C for 30 s, and a final extension at 72°C for 7 min. According to Sittidilokratna *et al*. (2009), the nested PCR used 1 µl of the primary PCR and the same reaction components, except for the substitution of nested primers LF2 (5́-AGA TCA TGC TGC ATA TGC TTG C-3́) and LR2 (5́-GTG TAG ATT GGT TGC ATG GCG-3́) and a reduced annealing temperature (58°C). Amplified DNA products of 357 and 205 bp were visualized in a 2% agarose TAE gel containing 0.5 µg ml–1 ethidium bromide alongside a 1 kb DNA ladder (Promega,WI,USA) and visualized using a UV transilluminator (Major Science, USA).

**Statistical analysis**

Data were analyzed using (SAS version 9.2 for Windows, Cary, North Carolina, US, 2002–2004). Means between treatments and control were compared using one-way analysis of variance (ANOVA). Tukey's HSD was employed to check for differences between means according to the method described by Zar (1996). The 5% significance level was used for all tests.

## RESULTS

**MIH-1 and CHH-1 transcripts expression level**

The shrimp optic lobe was screened for the expression of MIH-1, CHH-1 and EF-1α transcripts for product sizes of 172, 199 and 140 bp, respectively (Fig. 2a,b,c).

The specificity of CHH-1 primers was determined by cloning the PCR product which gave exactly a product size of 199bp similar to CHH-1 PCR. Relative RT-PCR was used to compare the expression of MIH-1 and CHH-1 in the shrimp optic lobe using EF-1α as internal control gene (Fig.2c). The patterns of MIH-1 transcript expression during the molt cycle are shown in (Fig. 3a).

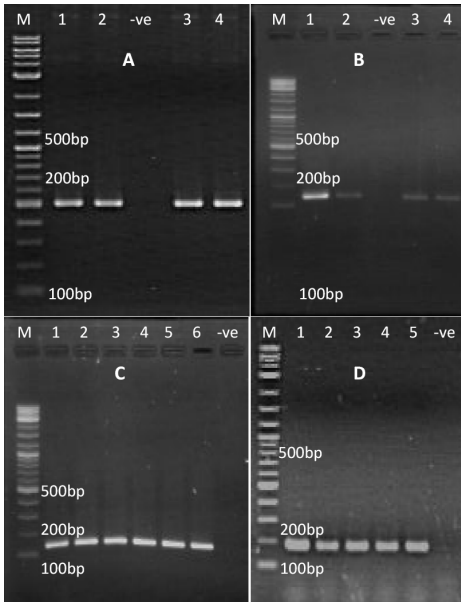


Fig. 2: Gel electrophoresis of RT-PCR product. (A) RT-PCR specific for MIH-1 (B), CHH-1, (C) EF-1α from *Penaeus semisulcatus* optic lobe and (D) Plasmids DNA gel electrophoreses amplified by specific primers for CHH-1. (M) DNA ladder (–) negative control (sterile $ddH_2O$).

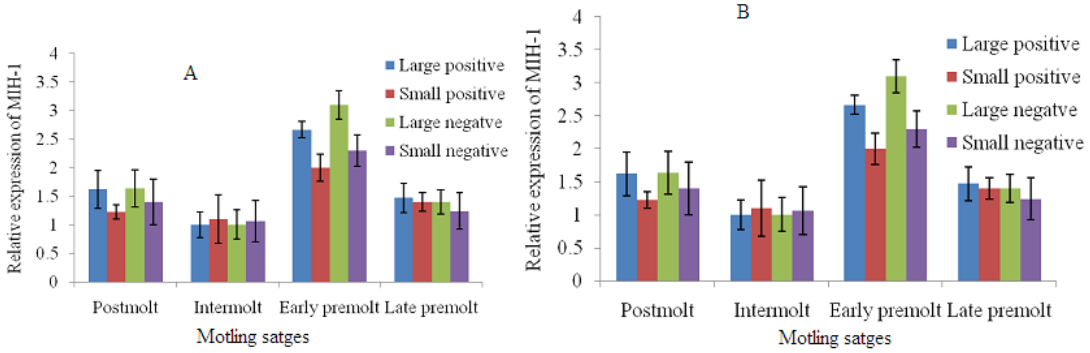


Fig. 3 A: Relative expression of the levels of the MIH-1 transcript compared to the levels of EF-1α in the optic lobes of four groups of *P. semisulcatus* during different molting stages. B) Relative expression of the levels of CHH-1 transcript compared to the levels of EF-1α in the optic lobes of four groups of *P. semisulcatus*, during different molting stages.

The highest levels of MIH-1 were noticed at early pre-molt stage, while the lowest was noticed at inter-molt stage. The patterns of MIH-1 transcript expression at the early pre-molt stage of the small-positive shrimp was significantly ($P$<0.05) lower than that of the large-negative and large-positive shrimp, but almost similar to that obtained for the small-negative shrimp. It has been noticed that, when comparing MIH-1 transcript levels in the different stages of the molt cycle of the different shrimp categories, the levels during early pre-molt stage were significantly higher ($P$<0.05) than other molt stages. However, the relative expression level of CHH-1 transcript in the optic lobe from the inter-molt stage of all different shrimp categories (large-negative, small-negative, large-positive and small-positive, was significantly different ($P$<0.05) (Fig. 3b).

**Production of polyclonal antibodies against MIH-1 and CHH-1**

Recombinant proteins rMIH-1 and rCHH-1 were prepared by eluting from Glycine SDS-PAGE (Fig. 4a,b), using Electro Eluter (Model 422, BIO-*RAD,* USA) and showed single band of rMIH-1 and rCHH-1 of 19 kDa.

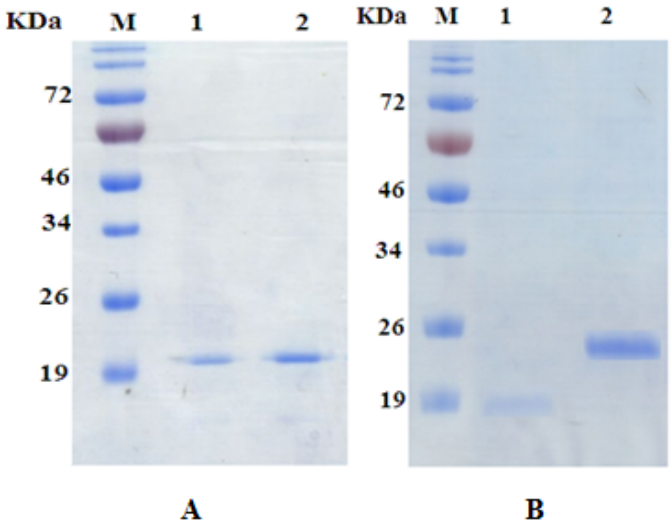


Fig. 4: Glycine SDS-PAGE of (A) Lane 1: rMIH-1 and Lane 2: rCHH-1 from *E. coli* transformants. (B) The recombinant proteins Lane 1: rMIH and Lane 2: rCHH. M: protein ladder.

The concentration of eluted rMIH-1 and rCHH-1 is 0.5 µg/µl, and the eluted proteins were used as an antigen to produce polyclonal anti-MIH-1 and anti-CHH-1 antibodies in mice. Both sensitivity and specificity of anti-MIH-1 and anti-CHH-1 antibodies was determined using western blot analysis. Rabbit anti-MIH-1 antibody at 1:10,000 dilutions was bound to rMIH-1 without cross reactivity to with rCHH-1 or rGIH, whereas the anti-CHH antibody was bound to rCHH-1, without cross reactivity to rMIH or rGIH (Fig. 5a, b, c).

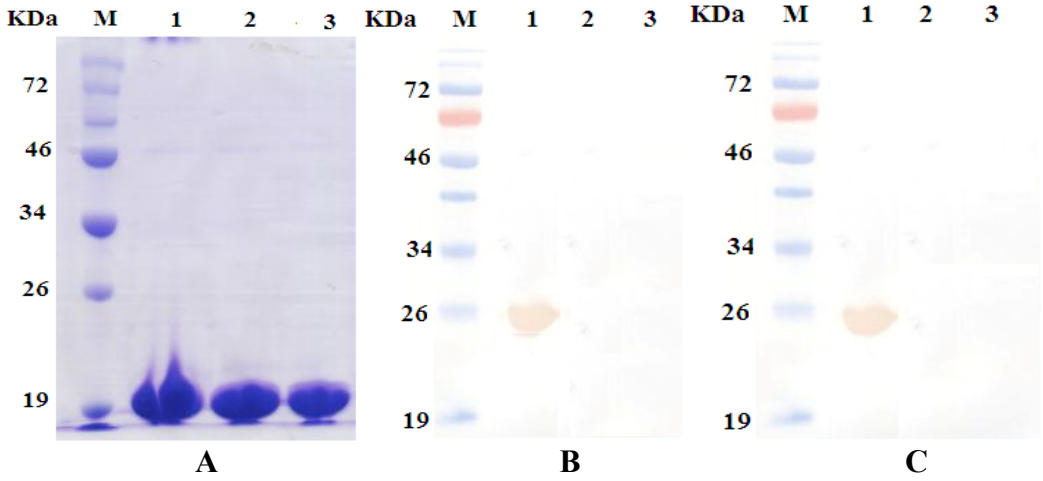


Fig. 5: Western blot analyses of A) anti-MIH-1 and anti-CHH-1 antibodies to rMIH-1, rCHH-1 and rGIH at dilution factor of 1:10,000. B) 15% Glycine SDS-PAGE of binding test with anti-MIH antibody. C) Binding test with CHH antibody. M: protein ladder. Lane 1: purified rMIH-1 (1 µg), Lane 2: purified rGIH (0.5 µg); Lane 3: purified rCHH-1 (0.5 µg); Tricine SDS-PAGE, western blot analysis of protein of CHH-1, MIH-1, GIH and the eyestalk extract from *Penaeus semisulcatus.*

The eyestalks were extracted by acetronitrile and analyzed by western blot analysis to estimate the specificity of the antibodies, and showed a single band detected by rabbit anti-MIH-1 antibody at a size of 97 kDa and cross reacted with rGIH, rabbit anti-GIH antibody, but the signal derived from rabbit anti-MIH antibody was stronger than that from anti-GIH antibody (Fig. 6a, b, c).

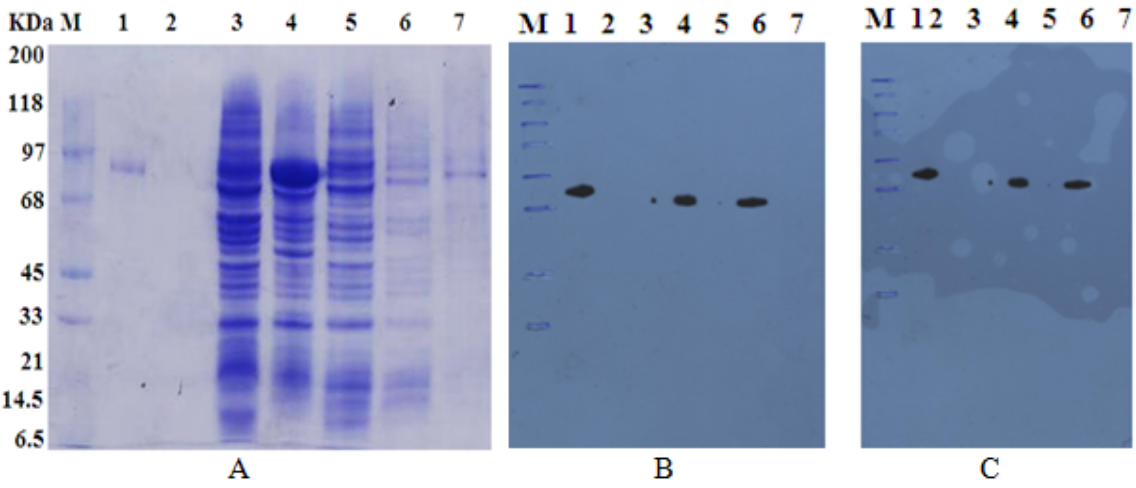


Fig. 6. A) Recombinant protein and the partially purified eyestalk were analyzed by 16.5% Tricine SDS-PAGE. B) Western blot analysis of a 1:10,000 dilution of rabbit anti-GIH antibody. C) A 1:10,000 dilution of rabbit anti-MIH-1 antibody was used in western blot analysis to determine its specificity. M: protein ladder. Lane 1: 50 ng of purified rMIH-1. Lane 2: 50 ng of purified rGIH and Lane 3: flow-through. Lane 4 represents protein fraction no.1. Lanes 5-7: protein fractions 2-4, respectively.

**Ecdysteroid Profiles**

There was no significant difference ($P>0.001$) in the haemolymph ecdysteroid levels (1-12 ng/mL) in molt cycle of small-positive shrimp were similar to large-positive, small-negative and large-negative at stages of post-molt, inter-molt and early pre-molt. However, the level was 90-100 ng/mL at late pre-molt stage and significantly different ($P<0.001$) from the post-molt, inter-molt and early pre-molt stages (Fig. 7).

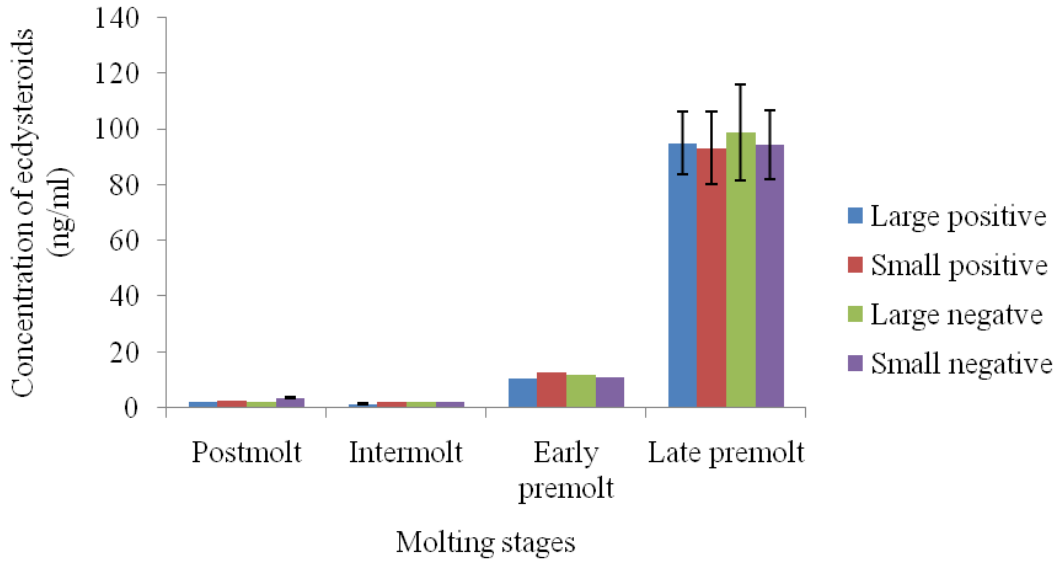


Fig. 7. Concentration of ecdysteroids (ng/ml) in the haemolymph of large-positive, small-positive, large-negative and small-negative *Penaeus semisulcatus*. Values are expressed as means±S.D.

**Glycogen and glucose concentration**

The average glycogen concentration of the hepatopancreas of small-positive shrimp (20.06±8.1 mg/g DW) was significantly ($P$<0.05) higher than large-positive (9.9±2.4 mg/g DW), small-negative (7.70±3.3 mg/g dry weight) and large-negative (6.4±4.1 mg/g DW) shrimps. However, the average glycogen concentration of the hepatopancreas of large-positive shrimp was not significantly different ($P$>0.05) from small- and large-negative shrimp. Similarly, no significant difference ($P$>0.05) was noticed in the average glycogen concentration of hepatopancreas between small-negative and large-negative shrimps (Fig. 8). Standard curve were produced using glucose concentrations of 20, 40, 60 and 80 µg/ml from a glucose assay kit (MilliporeSigma,MO,USA).The average concentration of glucose in the haemolymph of small-positive shrimp (29.3±12.5µg/ml) was significantly lower ($P$<0.05) than large-positive (46.0±10.1µg/ml), small-negative (45.7±5.4 µg/ml) and large-negative (46.8±9.1µg/ml) shrimps. However, the average glucose concentration in haemolymph of large-positive shrimp was not significantly different ($P$>0.05) from small-negative and large-negative shrimps.Similarly, no significant difference ($P$>0.05) in average glucose concentration of haemolymph was noticed between small-negative and large-negative shrimps (Fig.8).

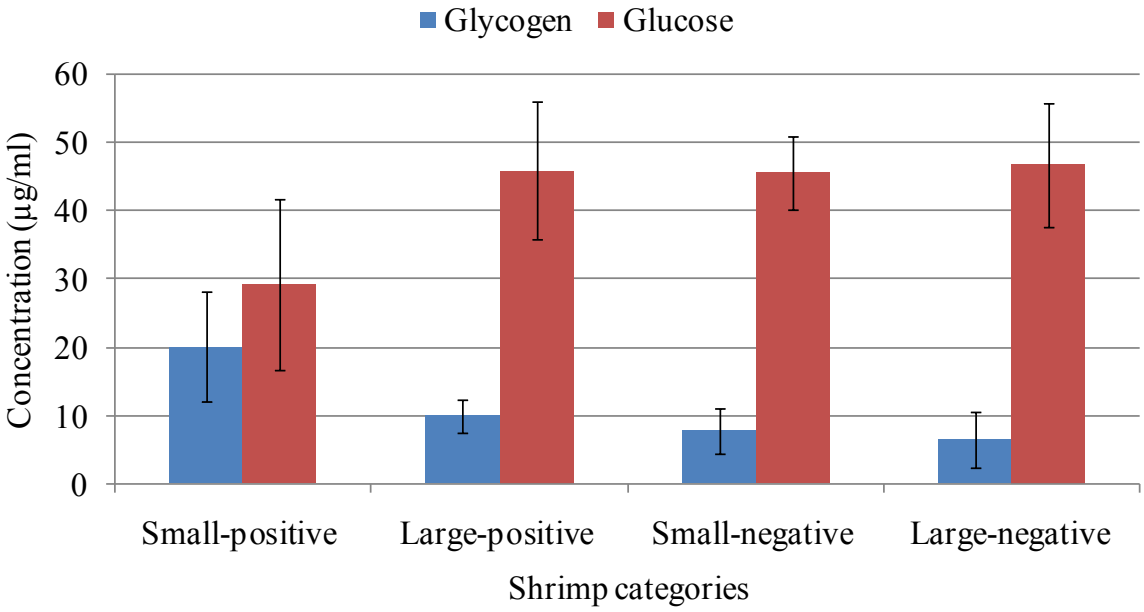


Fig.8. Concentration of glycogen (mg/g dry weight) in the hepatopancreas and concentration of glucose (µg/ml) in the haemolymph of large-positive, small-positive, large-negative and small-negative *Penaeus semisulcatus.*

**Effect of biofloc on growth and disease prevention of MSGS in small-positive *P. semisulcatus***

***Proximate and biochemical composition of the of biofloc and shrimp body***

The composition of experimental diets is presented in Table 1.

Table 1. Composition of experimental diets (g/kg$^{-1}$).

| Ingredients | Control $_{35}$ (9.27)[c] | $^{\sim 20}$BFd$_{20.13}$ (16.28)[1] | $^{\sim 22}$BFd$_{22.20}$ (14.89)[2] |
|---|---|---|---|
| **Fish meal** | 210 | 60 | 80 |
| **shrimp meal** | 260 | 100 | 130 |
| **soybean meal** | 249 | 139 | 139 |
| **Corn** | 80 | 210 | 190 |
| **Wheat** | 40 | 300 | 280 |
| **Wheat bran** | 70 | 100 | 90 |
| **Fish oil** | 30 | 30 | 30 |
| **Cholesterol** | 1 | 1 | 1 |
| **Lecithin** | 10 | 10 | 10 |
| **Vitamin & mineral mix** [a] | 30 | 30 | 30 |
| **Binder (CMC)**[b] | 20 | 20 | 20 |
| **Total** | 1000 | 1000 | 1000 |

[a] Vitamin & mineral mix (quantity/kg): Vitamin A, 20,00,000IU; Vitamin D3, 400,000 IU; Vitamin B2, 800 mg; Vitamin C, 1000 mg ;Vitamin E, 300 unit; Vitamin K, 400 mg; Vitamin B6, 400 mg; Vitamin B12, 2.4 mg; Calcium Pantothenate, 1 g; Nicotinamide, 4 g; Choline Chloride, 60 g; Mn, 10.8 g; Iodine, 0.4 g; Fe, 3 g; Zn, 6 g; Cu, 0.8 g; Co, 0.18 g.

b CMC: Carboxymethyle Cellulose.

[C] Control 35 (9.27): Control diet with subscript refers to protein percentage, superscript refers to approximation of protein percentage in the diet and C/N ratio between the brackets; $^{\sim 20}$BFd$_{20.13}$ (16.28)[1] and $^{\sim 22}$BFd$_{22.20}$ (14.89)[2]: Diets 1 and 2 with subscript refers to protein percentage; superscript refers to approximation of protein percentage in the diet and C/N ratio between the brackets.

The results of proximate and biochemical composition of the of biofloc and shrimp body are shown in Table (2). There was significant difference ($P$<0.05) in the crude protein content of the biofloc between treatments during the experiment. It ranged from 20.13±2.80 to 37.37±1.13% DW in average (Table 2). The biofloc grown on sucrose (BFd$_{Glucose}$), which showed increase in protein content during the culture (37.37±1.13). There was significant difference ($P$<0.05) in ash content of all treatments that ranged from 6.36±1.00 - 9.50±2.30%. The ash content of the biofloc throughout this experiment are presented in Table 2. There was a significant differences in the lipid content ($P$<0.05). The highest lipid content was recorded in the BFd$_{Glucose}$ treatment (8.26±1.10). The PUFAs content is presented in Table 2. The total n-3 PUFAs of the biofloc of all treatments were not significantly different ($P$>0.05) from each other and were all within the range of 0.93±0.02 - 0.97±0.01 mg/g DW. With respect to the total n-6 PUFAs, the biofloc grown with glucose as a carbon source had a higher level than other treatments and control (Table 2). The LA (18:2(n-6)) was the most abundant PUFA in the biofloc compared to the control and than that with low protein diets. The shrimp in the biofloc treatments (BFd$_{Glucose}$ and BFd$_{sucrose}$) showed a higher LA (18:2(n-6)), LNA (18:3(n-3)) and AA (20:4(n-6)) than the low protein treatments and control.

Table 2: Proximate composition (%) of the control and the experimental diets based on dry weight basis (g/kg$^{-1}$) and PUFAs content (mg/g DW) of biofloc grown in low protein diets and different carbon source (sucrose and glucose) (mean±SE).

| Nutrient | Proximate composition (%) | | | | |
|---|---|---|---|---|---|
| | Control 35% CP | $^{\sim 20}BFd_{20.13}$ | $^{\sim 22}BFd_{22.20}$ | $BFd_{Sucrose}$ | $BFd_{Glucose}$ |
| **Crude protein** | 350.0±0.00$^{c}$ | 201.3±2.80$^{e}$ | 222.0±2.10$^{d}$ | 362.17±1.05$^{b}$ | 373.7±1.13$^{a}$ |
| **Crude lipid** | 91.6±2.20$^{a}$ | 72.8±3.00$^{c}$ | 76.2±2.10$^{c}$ | 79.4±2.00$^{c}$ | 82.6±1.10$^{b}$ |
| **Ash** | 141.0±0.13$^{a}$ | 63.6±1.00$^{e}$ | 74.3±3.00$^{d}$ | 81.3±2.10$^{c}$ | 95.0±2.30$^{b}$ |
| **Crude fiber** | 38.0±0.10$^{c}$ | 44.1±2.00$^{b}$ | 49.1±1.00$^{b}$ | 50.19±1.70$^{b}$ | 70.49±1.53$^{a}$ |
| **NFE[1]** | 379.4±1.6$^{d}$ | 618.2±2.00$^{a}$ | 578.4±2.10$^{b}$ | 426.94±2.5$^{c}$ | 378.21±1.9$^{d}$ |
| **Gross energy (Kcal/100 g)[2]** | 4512.72 | 4474.50 | 4484.39 | 4551.31 | 4446.41 |
| **Total n-3 PUFA (mg/g DW)** | 0.77±0.01$^{b}$ | 0.93±0.02$^{a}$ | 0.95±0.06$^{a}$ | 0.97±0.01$^{a}$ | 0.94±0.00$^{a}$ |
| **Total n-6 PUFA (mg/g DW)** | 32.11±2.71$^{b}$ | 35.29±3.11$^{a}$ | 29.17±2.99$^{c}$ | 37.15±4.28$^{a}$ | 32.00±1.53$^{b}$ |
| **LA (18:2(n-6))** | 6.99±1.50$^{c}$ | 7.59±1.25$^{b}$ | 5.71±1.8$^{c}$ | 30.83±1.3$^{a}$ | 33.59±1.2$^{a}$ |
| **LNA (18:3(n-3))** | 0.93±0.01$^{a}$ | 0.91±0.03$^{a}$ | 0.75±0.02$^{c}$ | 0.99±0.00$^{a}$ | 0.89±0.01$^{b}$ |
| **AA (20:4(n-6))** | 0.21±0.01$^{c}$ | 0.26±0.00$^{b}$ | 0.29±0.00$^{b}$ | 0.31±0.00$^{a}$ | 0.30±0.00$^{a}$ |
| **EPA (20:5(n-3))** | 0.30±0.03$^{b}$ | 0.32±0.00$^{b}$ | 0.50±0.01$^{a}$ | 0.33±0.00$^{b}$ | 0.47±0.00$^{a}$ |
| **DHA (22:6(n-3))** | 0.21±0.00$^{c}$ | 0.28±0.00$^{b}$ | 0.20±0.00$^{b}$ | 0.35±0.00$^{a}$ | 0.37±0.00$^{a}$ |

[1]NFE (%) = 100 - (Crude protein + Ether extract + Ash + Fiber).
[2]Gross energy (GE), Kcal was calculated by multiplying CP x 5.65 + fat x 9.45 + (Carbohydrate + Fober) x 4.1 (Jobling, 1983).
CP X5.65; FAT X 9.45; C X 4.1; and Fiber X4.1

### *Water quality*

The water quality parameters were measured are presented (mean±SE) in Table 3. They were significant difference ($P$<0.05) between control, low protein diets, and biofloc treatments. Average water temperature (29 °C), DO concentrations (6 mg l$^{-1}$) and pH (8.01). The biofloc treatments reduced water TAN, $NO_3$–N and $NO_2$–N concentrations. The TAN concentrations were significantly higher in the control tanks compared to low protein fed treatments and biofloc treatments. The biofloc with $BFd_{Sucrose}$ as the carbon source tended to have a higher SVI than those grown with $BFd_{Glucose}$. The highest SVI were 720±72 and 721±17 mg l$^{-1}$ in the ($^{\sim 22}BFd_{22.20}$) and $BFd_{Sucrose}$, respectively.

Table 3: Average physicochemical parameters of water in shrimp rearing tanks during the feeding experiment (mean ± SE).

| Parameters | Control 35% CP | $^{\sim 20}BFd_{20.13}$ | $^{\sim 22}BFd_{22.20}$ | $BFd_{Sucrose}$ | $BFd_{Glucose}$ |
|---|---|---|---|---|---|
| **Temperature (°C)** | 29.17±0.21 | 29.15 ±0.26 | 29.15±0.17 | 29.13 ±0.10 | 29.16 ±0.10 |
| **DO (mg/L)** | 5.27±0.11$^{b}$ | 6.61±0.10$^{a}$ | 6.17 ±0.13$^{a}$ | 6.22±0.28$^{a}$ | 6.87±0.50$^{a}$ |
| **pH** | 8.01±0.13$^{a}$ | 8.11±0.17$^{a}$ | 8.00 ±0.15$^{a}$ | 8.05±0.18$^{a}$ | 8.00±0.13$^{a}$ |
| **Salinity (ppt)** | 35.00±0.00$^{a}$ | 35.26 ±0.05$^{a}$ | 35.33±0.09$^{a}$ | 35.58 ±0.20$^{a}$ | 35.58 ±0.01$^{a}$ |
| **Nitrite-N ($NO_3$ µg L$^{-1}$)** | 0.03 ±0.00$^{a}$ | 0.08 ±0.00$^{c}$ | 0.07±0.00$^{b}$ | 0.08 ±0.00$^{c}$ | 0.09 ±0.00$^{c}$ |
| **Nitrate-N ($NO_2$ µg L$^{-1}$)** | 0.40 ±0.03$^{c}$ | 0. 13 ±0.01$^{a}$ | 0.19 ±0.00$^{b}$ | 0.11±0.00$^{a}$ | 0.11 ±0.00$^{a}$ |
| **Total ammonia-N (µg L$^{-1}$)** | 0.20 ±0.01$^{c}$ | 0.12 ±0.00$^{b}$ | 0.14 ±0.00$^{b}$ | 0.10 ±0.00$^{a}$ | 0.10 ±0.00$^{a}$ |
| **SVI(mg/L)** | 654 ± 773$^{b}$ | 664 ± 81$^{b}$ | 720 ± 72$^{a}$ | 721 ± 17$^{a}$ | 519 ± 17$^{c}$ |

Mean values in same row with a different superscript differ significantly ($P$<0.05)

### *Shrimp performance and feed efficiency of P. semisulcatus*

The growth performance and feed efficiency of *P. semisulcatus* are presented in Table 5. Average initial body weight was recorded as 2.30±0.06 (g) in all treatments. There was no significant difference ($P$>0.05) among the SGR which

recorded as 1.12±0.03 in (Control), 1.43±0.01 in ($^{\sim 20}BFd_{20.13}$), 1.43±0.03 in ($^{\sim 22}BFd_{22.20}$), 1.17±0.00 in ($BFd_{Glucose}$) and 1.15±0.01 in ($BFd_{sucrose}$). There was significant difference ($P<0.05$) in the FCR values (2.42 ± 0.32; 1.25±0.04; 1.32±0.06; 1.04±0.12 and 1.35±0.12 for Control,$^{\sim 20}BFd_{20.13}$,$^{\sim 22}BFd_{22.20}$, $BFd_{Sucrose}$ and $BFd_{Glucose}$, respectively). There was significance difference ($P<0.05$) in the survival rate (SR%) among the different feeding treatments (57.28±3.60, 64.24±5.00, 72.13±1.14, 89.11±3.15 and 90.50±4.18 for Control, $^{\sim 20}BFd_{20.13}$,$^{\sim 22}BFd_{22.20}$, $BFd_{Sucrose}$ and $BFd_{Glucose}$, respectively). The highest survival was obtained by the ($BFd_{Glucose}$) treatment, which was 90.50±4.18%. The control treatment showed the lowest survival 57.28±3.60% and was significantly different ($P<0.05$) from all other biofloc treatments (Table 4).

Table 4:Growth performance (mean±SE) of small-positive *Penaeus semisulcatus* receiving control, low protein diets and biofloc developed with sucrose and glucose as carbon sources for 60 days.

| Growth parameters | Control 35% CP | $^{\sim 20}BFd_{20.13}$ | $^{\sim 22}BFd_{22.20}$ | $BFd_{Sucrose}$ | $BFd_{Glucose}$ |
|---|---|---|---|---|---|
| **Initial wt. (g)** | 2.30 ± 0.06 | 2.31 ± 0.06 | 2.33 ± 0.11 | 2.31 ± 0.01 | 2.30 ± 0.04 |
| **Weight gain/week (AWG,** | 0.24 ± 0.01$^{c}$ | 0.44 ± 0.02$^{b}$ | 0.45 ± 0.02$^{b}$ | 0.33 ± 0.03$^{a}$ | 0.33 ± 0.03$^{a}$ |
| **SGR** | 1.12 ± 0.03$^{b}$ | 1.43 ± 0.01$^{a}$ | 1.43 ± 0.03a | 1.17 ± 0.00$^{b}$ | 1.15 ± 0.01$^{b}$ |
| **FCR** | 2.42 ± 0.32$^{c}$ | 1.25 ± 0.04$^{b}$ | 1.32 ± 0.06$^{b}$ | 1.04 ± 0.12$^{a}$ | 1.35 ± 0.12$^{b}$ |
| **Survival (%)** | 57.28 ± 3.60$^{e}$ | 64.24 ± 5.00$^{d}$ | 72.13 ± 1.14$^{c}$ | 89.11 ±3.15$^{b}$ | 90.50 ±4.18$^{a}$ |
| **Final wt. (g)** | 6.15 ± 0.16$^{d}$ | 19.20 ± 0.12$^{c}$ | 18.60 ± 0.21$^{c}$ | 23.11 ± 0.13$^{b}$ | 24.17 ± 0.22$^{a}$ |

Mean values in same row with a different superscript differ significantly ($P<0.05$)

There were significant difference ($P<0.05$) in the final body weight (g) between Control and $^{\sim 20}BFd_{20.13}$, $^{\sim 22}BFd_{22.20}$, $BFd_{Sucrose}$ and $BFd_{Glucose.}$ The low protein diets $^{20}BFd_{20.13}$ (19.20±0.12), $^{\sim 22}BFd_{22.20}$ (18.60±0.21) and biofloc treatments $BFd_{Sucrose}$ (23.11±0.13), $BFd_{Glucose}$ (24.17±0.22) were characterized by high final body weight than control (6.15 ± 0.16).

### *Glucose level in haemolymph of shrimp*

The average concentration of glucose in the haemolymph of small-positive shrimp was significantly different ($P<0.05$) between control, low protein diets and biofloc treatments (Table 5). The lowest glucose concentration was for the control (13.45±2.15 µg/ml), and the highest glucose concentration was for $BFd_{Glucose}$ (75.01±2.33 µg/ml), followed $BFd_{Sucrose}$ (45.32±1.32 µg/ml), $^{\sim 20}BFd_{20.13}$ (43.51±3.21 µg/ml) and $^{\sim 22}BFd_{22.20}$ (40.21±3.12 µg/ml).

Table 5: Mean glucose level (µg/ml) of small-positive *Penaeus semisulcatus* grown in control, low protein diets and biofloc developed with sucrose and glucose as carbon sources at different intervals for 60 days (mean±SE).

| DOC* | Control 35% CP | $^{\sim 20}BFd_{20.13}$ | $^{\sim 22}BFd_{22.20}$ | $BFd_{Sucrose}$ | $BFd_{Glucose}$ |
|---|---|---|---|---|---|
| **10** | 12.15±1.23$^{d}$ | 39.21±3.45$^{c}$ | 36.21±2.20$^{c}$ | 42.08±4.00$^{b}$ | 75.25±1.01$^{a}$ |
| **20** | 13.12±1.32$^{c}$ | 39.51 ±3.20$^{b}$ | 40.21±1.11$^{b}$ | 43.01±2.18$^{b}$ | 75.31±1.82$^{a}$ |
| **30** | 12.01±2.01$^{d}$ | 40.00 ±1.03$^{c}$ | 40.00±1.02$^{c}$ | 45.11±2.31$^{b}$ | 80.32±1.37$^{a}$ |
| **40** | 14.21±1.32$^{d}$ | 45.42 ±3.00$^{b}$ | 40.02±1.20$^{c}$ | 45.10±1.22$^{b}$ | 78.11±1.23$^{a}$ |
| **50** | 15.08±1.02$^{d}$ | 42.53 ±3.17$^{c}$ | 40.13±1.72$^{c}$ | 46.00±1.21$^{b}$ | 78.32±1.03$^{a}$ |
| **60** | 13.45±2.15$^{d}$ | 43.51 ±3.21$^{b}$ | 40.21±3.12$^{c}$ | 45.32±1.32$^{b}$ | 75.01±2.33$^{a}$ |

*DOC: days of culture.

Means with same superscripts in the same row indicate no statistical significance (P<0.05).

### *Detection of Laem-Singh virus (LSNV) in small-positive Penaeus semisulcatus grown in control, low protein diets and biofloc treatments*

Screening by RT-PCR and RT-nested PCR detected no evidence of LSNV infection in Pleopod tissue of *P. semisulcatus* grown in biofloc system (Table 6). The RT-nested PCR was also used to retest *P. semisulcatus* samples from biofloc treatments that had previously been screened by the original RT-PCR. The increased analytical sensitivity of the test allowed detection of LSNV in control samples, while no LSNV detected in biofloc treatments.

Table 6: Detection of Laem-Singh virus (LSNV) by RT-PCR in 50 *P. semisulcatus* samples collected from control, low protein diet and biofloc developed with sucrose and glucose as carbon sources at different times throughout the experiment. (+): LSNV-positive. (-): LSNV-negative.

| Treatment | DOC* | Health status** | LSNV RT-PCR | | Treatment | DOC* | Health status** | LSNV RT-PCR | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Primary | Nested | | | | Primary | Nested |
| **Control** 35% CP | 10 | Diseased | + | + | **$BFd_{Sucrose}$** | 10 | Healthy | | |
| | 20 | Healthy | - | - | | 20 | Healthy | - | - |
| | 30 | Healthy | - | - | | 30 | Healthy | - | - |
| | 40 | Healthy | - | - | | 40 | Healthy | - | - |
| | 50 | Healthy | - | - | | 50 | Healthy | - | - |
| | 60 | Healthy | - | - | | 60 | Healthy | - | - |
| **$^{\sim 20}BFd_{20.13}$** | 10 | Healthy | - | - | **$BFd_{Glucose}$** | 10 | Healthy | - | - |
| | 20 | Healthy | - | - | | 20 | Healthy | - | - |
| | 30 | Healthy | - | - | | 30 | Healthy | - | - |
| | 40 | Healthy | - | - | | 40 | Healthy | - | - |
| | 50 | Healthy | - | - | | 50 | Healthy | - | - |
| | 60 | Healthy | - | - | | 60 | Healthy | - | - |
| **$^{\sim 22}BFd_{22.20}$** | 10 | Healthy | - | - | | | | | |
| | 20 | Healthy | - | - | | | | | |
| | 30 | Healthy | - | - | | | | | |
| | 40 | Healthy | - | - | | | | | |
| | 50 | Healthy | - | - | | | | | |
| | 60 | Healthy | - | - | | | | | |

*DOC: days of culture.
**Health status: diseased if it is positive to (EMS and WSSV) and healthy if it is negative to (EMS and WSSV) (Lightner, 1996).

## DISCUSSION

The aim of the present study was to find out the reason behind growth retardation in the green tiger shrimp *P. semisulcatus*. Previous findings suggested that MSGS in shrimp is associated with LSNV infection (Prakasha *et al*., 2007; Pratoomthai *et al*., 2008). The virus infects several organs and tissues of the shrimp. However, majority of infected shrimp do not show MSGS unless the infection extends into the zona fasciculate in the shrimp retina and in the shrimp optic lobe that contains several neuro-endocrine cells (Pratoomthai *et al*., 2008). The result of the present study revealed that MIH and/or CHH may be altered by LSNV infection, causing a change in molt activities and/or carbohydrate metabolism. There was no significant difference between molting activities of the small-positive and small-negative shrimp, but both of them are significantly different from both large-negative and large-positive shrimp, and the differences in molting activities was associated with shrimp size not the age of the shrimp. The retention of high glycogen resulted in

lower levels of glucose in the haemolymph, thus low energy available to support shrimp growth. In the white leg shrimp *L. vannamei,* MIH-1 transcript level increased during post-molt and inter-molt stages (Lee *et al*., 1998; Chen *et al*., 2007).

This pattern of MIH-1 transcripts supports the conventional theory that MIH-1 transcripts through its translated MIH-1 peptide suppress ecdysteriods synthesis/release and allow ecdysteriods to rise only at pre-molt stage, resulting in ecdysis. The result of the present study indicated that changes in ecdysteriods synthesis in the Y-organ were controlled by susceptibility of the gland itself to MIH-1 transcript rather than the level of MIH-1. There was no significant difference in the levels of MIH-1 transcript in the optic lobes of all shrimp categories. Small-positive shrimp showed highest levels of MIH-1 transcript during early pre-molt stage and decreased during late pre-molt, post-molt and inter-molt stages, which agrees with similar patterns of MIH-1 transcript levels in the sinus gland of the crayfish *Procambarus clarkii* (Nakatsuji *et al*., 2000). The levels of MIH-1 transcript in the haemolymph of *P. semisulcatus* increased during inter-molt stage and decreased during early pre-molt stage and decreased to the basal level during the late pre-molt and post-molt stages, which shows that the MIH-1 transcript levels in the shrimp haemolymph did not follow the same pattern in the shrimp optic lobe. The results showed that during inter-molt, low levels of MIH-1 transcript in the optic lobe and high levels in the haemolymph might be due to the release of MIH-1 transcript from the storage site at the sinus gland into haemolymph, which agrees with the results obtained by Sritunyalucksana *et al*. (2006). This would explain that, the high levels of MIH-1 transcript in the optic lobe during early pre-molt stage due to the storage of MIH-1 transcript in the sinus gland and release small fraction into the haemolymph. There was no significant difference in the levels of ecdysteriods in all the shrimp categories, as it is concluded that the Y-organ in *P.semisulcatus* became less sensitive in response to MIH-1 peptide as the size of the shrimp *P.semisulcatus* increased and agreed well with those reported in the blue crab *C. sapidus* and in the crayfish *P. clarkia* (Nakatsuji and Sonobe, 2004) and *P. monodon* (Sritunyalucksana *et al*., 2006).

The result showed that the anti-MIH-1 antibody was specific to both rMIH-1 and rGIH, but was not specific to rCHH-1 as has been explained by Treerattrakool *et al*. (2008). The eyestalk of the shrimp *P.semisulcatus* during early pre-molt was used to study, the specificity of anti-MIH-1 antibody due to the high level of MIH-1 transcript at this stage by western blot analysis, which showed signal at 9 kDa and 75 kDa, indicating the presence of CHH-1. The small-positive shrimp had high glycogen levels in the hepatopancreas and low glucose level in the haemolymph. It is well-known that CHH-1 in crustaceans responsible for the increases of haemolymph glucose level by the breakdown of glycogen in the hepatopancreas (Chang and O'Connor, 1985, 1978; Keller and Orth, 1990). Thus, the decreased level of CHH-1 decreases glycogen breakdown and result in a decrease in the glucose level in the haemolymph of the shrimp and this as the case of the small-positive shrimp that has chronic hypoglycemic case which cause retardation of the growth of shrimp *P.semisulcatus* growth. The results of the present study revealed that, infection of the zona fasciculata by LSNV reduces serotonin induction of CHH-1 secretion from the XOSG complex and causes growth retardation in small-positive *P.semisulcatus* (Pratoomthai *et al*., 2008).

Bacteria in the water column play important role in ammonium uptake and played significant role in reducing the TAN and nitrite-N of nitrogen metabolites by their uptake for microbial protein synthesis, which indicated that the biofloc had

converted the inorganic nitrogen into organic nitrogen (De Schryver *et al*. 2008; Megahed, 2010; Megahed and Mohamed, 2014). In the biofloc treatments,the nitrification bacteria were possibly outcompeted by the heterotrophic bacteria. The presence of TAN in these treatments indicated that there was not sufficient organic carbon available to convert all inorganic nitrogen into bacterial biomass. The growth parameter (AWG, SGR and FCR) in the present study are comparable with commercially viable values (Allan and Maguire, 1992). The AWG values of the present study were comparable with the results obtained by Wyban and Sweeney (1989) who worked on *L.vannamei*. There was slightly improvement in the FCR in the low protein diet and biofloc compared to the control. According to the present study, the FCRs in bioflocs treatments were significantly better than that of the control. The survival rate in the present study agrees with similar results observed by Nunes and Parsons (1998) for semi-intensive culture system. Feeding shrimp with low protein diets and biofloc found to improve the growth performance of shrimp (Ju *et al*., 2008a, 2008b; Kuhn *et al*., 2010; Megahed, 2010; Megahed and Mohamed, 2014).

The results of the present study indicate that biofloc can be used as a control measure to LSNV in *Penaeus semisulcatus*. This was evident from the no detection of LSNV in biofloc treatments. In contrast, LSNV infection has been detected in *Penaeus semisulcatus* grown in control diet. The LSNV positive shrimp displayed signs of loose shell syndrome with no particular association between LSNV infection and other diseases in the shrimp. The field observations of the disease were; low feed consumption, abnormal activity of the shrimp including swimming, in addition to high mortality rate.

## CONCLUSION

Based on the result, it can be concluded that *P. semisulcatus* infected with LSNV and showing retarded growth did not have abnormal molting activities or levels of molt-regulating hormones. Instead, the shrimp were found to have abnormalities in glycogen metabolism, with low levels of CHH that cause of hypoglycemia. It is possible that this hypoglycemic condition caused growth retardation in LSNV-infected *P. semisulcatus*. Feeding with biofloc of sucrose and glucose as carbon sources improved the growth rate of the shrimp. The highest survival was found in the shrimp fed biofloc.

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## REFERENCES

Allan, G.L. and Maguire, G.B. (1992). Effects of stocking density on production of *Penaeus Monodon* model farming systems. Aquaculture, 107 (1): 49–66. https://doi.org/10.1016/0044-8486 (92)90049-Q.

Anand, P. S.; Kohli, M. P. S.; Roy, S. D.; Sundaray, J. K.; Kumar, S.; Sinha, A. and Pailan, G. H. (2013). Effect of dietary supplementation of periphyton on growth performance and digestive enzyme activities in *Penaeus monodon*. Aquaculture, 392: 59-68. http://dx.doi.org/10.1016/j.aquaculture.2013.01.029.

AOAC (1995). Official methods of analysis. 15th edition, Association of Official Analytical Chemists, Arlington, VA, pp. 1-45

APHA (1998). Standard Methods for the Examination of Water and Wastewater. 20th Edition, American Public Health Association, American Water Works Association and Water Environmental Federation, Washington DC.

Avnimelech, Y. (1999). Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture, 176: 227-235. http://dx.doi.org/10.1016/S0044-8486(99)00085-X.

Ballester, E.L.C.; Abreu, P. C.; Cavalli, R. O.; Emerenciano, M.; Abreu, L. and Wasielesky, W. (2010). Effect of practical diets with different protein levels on the performance of *Farfantepenaeus paulensis* juveniles nursed in a zero exchange suspended microbial flocs intensive system. Aquaculture Nutrition, 16: 163-172. http://dx.doi.org/10.1111/j.1365-2095.2009.00648.x.

Bright Singh, I.S.; Manjusha, M.; Somnath Pai, S. and Philip, R. (2005). *Fenneropenaeus indicus* is protected from white spot disease by oral administration of inactivated white spot syndrome virus. Diseases of Aquatic Organisms, 66: 265-270.

Chang, E.S. and O'Connor, J.D. (1978). In vitro secretion and hydroxylation of alpha-ecdysone as a function of the crustacean molt cycle. Gen. Comp. Endocrinol. 36: 151-210.

Chang, E.S. and O'Connor, J.D. (1985). Metabolism and transport of carbohydrates and lipids; in The Biology of Crustacea, Mantel, L. H. (ed.), pp. 263-281, Academic.

Chen, H.Y.; Watson, R.D.; J.C. Chen, Liu, H.F. and Lee, C.Y. (2007). Molecular characterization and gene expression pattern of two putative molt-inhibiting hormones from *Litopenaeus vannamei*. General and Comparative Endocrinology, 151: 72-81.

Chen, J.C. and Lei, S.C. (1990). Toxicity of ammonia and nitrite to *Penaeus monodon* juveniles. Journal of World Aquacultural Society, 21: 300-305.

Crab, R.; Defoirdt, T.; Bossier, P. and Verstraete, W. (2012). Biofloc technology in aquaculture: beneficial effects and future challenges. Aquaculture, 356: 351-356. http://dx.doi.org/10.1016/j.aquaculture., 2012.04.046.

De Schryver, P.; Crab, R.; Defoirdt, T.; Boon, N. and Verstraete, W. (2008). The basics of bio- flocs technology: the added value for aquaculture. Aquaculture, 277: 125–137.

Folch, J.; Lees, M. and Stanley, G.H.S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226 (1): 497-509.

Forteath, N. (1990). A handbook on recirculating systems for aquatic organisms. Fishing Industry Training Board of Tasmania Inc., Hobart, Australia

Hastings, W. and Dupree, H. K. (1969). Formula feeds for channel catfish. Prog.
Fish-Culturist, 31: 187-196.

Jobling, M. (1983). A short review and critique of methodology used in fish growth and nutrition studies. Journal of Fish Biology, 23: 685-703.

Ju, Z.Y.; Forster, I.; Conquest, L. and Dominy, W. (2008a). Enhanced growth effects on shrimp (*Litopenaeus vannamei*) from inclusion of whole shrimp floc or floc

fractions to a formulated diet. Aquaculture Nutrition, 14: 533-543. http://dx.doi.org/10.1111/j.1365-2095.2007.00559.x.

Ju, Z.Y.; Forster, I.; Conquest, L.; Dominy, W.; Kuo, W.C. and Horgen, F.D. (2008b). Determination of microbial community structures of shrimp floc cultures by biomarkers and analysis of floc amino acid profiles. Aquaculture Research, 39: 118–133.

Judd, R.C. (1994). Eleltrophoresis of peptides. In: Basis Protein and Peptide Protocols. Humana Press, New Jersey, 39-48.

Keller, R. and Orth, H.P. (1990). Hyperglycemic neuropeptides in crustaceans. Prog. Clin. Biol. Res. 342: 265-271.

Kubista, M.; Andrade, J.M.; Bengtsson, M.; Forootan, A.; Jonak, J.; Lind, K.; Sindelka, R.; Sjoback, R.; Sjogreen, B. and Strombom, L. (2006). The real-time polymerase chain reaction. Molecular Aspects of Medicine, 27: 95-125.

Kuhn, D. D.; Lawrence, A. L.; Boardman, G. D.; Patnaik, S.; Marsh, L. and Flick Jr, G. J. (2010). Evaluation of two types of bioflocs derived from biological treatment of fish effluent as feed ingredients for Pacific white shrimp, *Litopenaeus vannamei*. Aquaculture, 303: 28-33.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lee, K.J.; Watson, R.D. and Roer, R.D. (1998). Molt-inhibiting hormone mRNA levels and ecdysteroid titer during a molt cycle of the blue crab, Callinectes sapidus.Biochemical and Biophysical Research Communications,249: 624-627.

Lightner, D.V. (1996). A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Society, Baton Rouge, LA.

Lin, Y.C. and Chen, J.C. (2003). Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. Aquaculture, 224: 193-201.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C (T)) Method. Methods, 25: 402-408.

McIntosh, R. P. (2000). Changing paradigms in shrimp farming: low protein feeds and feeding strategies. Global Aquacult. Advocate, 3: 44-50.

Megahed, M.E. (2010). The effect of microbial biofloc on water quality, survival and growth of the green tiger shrimp *Penaeus semisulcatus* fed with different crude protein levels. Journal of the Arabian Aquaculture Society, 5(2): 119–142.

Megahed, M.E.; Ghoneim, S.; Desouky, G. and Dakar, A.E. (2013a). Pilot Experiment on Shrimp *P. semisulcatus* Production at Al – Deba Farm Site. Journal of the Arabian Aquaculture Society, 8: 331-340.

Megahed, M.E.; Ghoneim, S.; Desouky, G. and Dakar, A.E. (2013b). Major Constraints Facing Development of Marine Shrimp Farming in Egypt. Journal of the Arabian Aquaculture Society, 8: 321-330.

Megahed, M.E. and Mohamed, K. (2014). Sustainable growth of shrimp aquaculture through biofloc production as alternative to fishmeal in shrimp feeds. Journal of Agricultural Science, 6 (6): 176–188.

Megahed, M.E.; Elmesiry, G.; Ellithy, A. and Khaled, M. (2018). Genetic, nutritional and pathological investigations on the effect of feeding low protein diet and biofloc on growth performance, survival and disease prevention of Indian white shrimp Fenneropenaeus indicus. Aquacult Int, 26 (2): 589 – 615. https://doi.org/10.1007/s10499-017-0231-1.

Muir, P.R.; Sutton, D.C. and Owens, L. (1991). Nitrate toxicity to *Penaeus monodon* protozoea. Marine Biology, 108: 67–71.

Nakatsuji, T. and Sonobe, H. (2004). Regulation of ecdysteroid secretion from the Y-organ by molt-inhibiting hormone in the American crayfish, *Procambarus clarkii*. General and Comparative Endocrinology, 135: 358-364.

Nakatsuji, T.; Keino, H.; Tamura, K.; Yoshimura, S.; Kawakami, T.; Aimoto, S. and Sonobe, H. (2000). Changes in the Amounts of the Molt-Inhibiting Hormone in Sinus Glands during the Molt Cycle of the American Crayfish, *Procambarus clarkii*. Zoological Science, 17: 1129-1136.

Nunes, A.J.P. and Parsons, G.J. (1998). Food handling efficiency and particle size selectivity by the Southern brown shrimp *Penaeus subtilis* feed a dry pelleted feed. Marine and Freshwater Behavior and Physiology, 31 (4): 193-213.

Prakasha, B.K.; Ramakrishna, R.P.; Karunasagar, I. and Karunasagar, I. (2007). Detection of Laem-Singh virus (LSNV) in cultured *Penaeus monodon* from India. Diseases of Aquatic Organisms, 77: 83–86.

Pratoomthai, B.; Sakaewa, W.; Sriurairatana, S.; Wongprasert, K. and Withyachumnarn-kul, B.(2008). Retinopathy in stunted black tiger shrimp *Penaeus monodon* and possible association with Laem-Singh virus (LSNV). Aquaculture, 284: 53-58.

Sittidilokratna, N.; Dangtip, S.; Sritunyalucksana, K.; Babu, R.; Pradeep, B.; Mohan, C.; Gudkovs, N. and Walker, P. (2009). Detection of Laem-Singh virus in cultured *Penaeus monodon* shrimp from several sites in the Indo-Pacific region. Diseases of Aquatic Organisms 84:195-200.

Sritunyalucksana, K.; Apisawetakan, S.; Boon-Nat, A.; Withyachumnarnkul, B. and Flegel, T.W. (2006). A new RNA virus found in black tiger shrimp *Penaeus monodon* from Thailand. Virus Research, 118: 31-38.

Teshima, S.; Kanazawa, A. and Yamashita, M.(1986). Dietary value of several proteins and supplemental amino acids for larvae of the prawn, *Penaeus japonicus*. Aquaculture, 51: 225–235.

Treerattrakool, S.; Panyim, S.; Chan, S.M.; Withyachumnarnkul, B. and Udomkit, A. (2008). Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. FEBS Journal, 275: 970-980.

Tsai Su-Jun and Jiann-Chu Chen.(2002). Acute toxicity of nitrate on *Penaeus monodon* juveniles at different salinity levels. Aquaculture, 213: 163-170.

Vargas-Albores, F.; Guzman, M.A. and Ochoa, J.L. (1993). An anticoagulant solution for haemolymph collection and prophenoloxidase studies of penaeid shrimp (*Penaeus californiensis*). Comparative Biochemistry and Physiology, 160A: 299-303.

Wyban, J.A. and Sweeney. J.N. (1989). Intensive shrimp growout trials in a round pond. Aquaculture, 76: 215-225.

Xu, W.J. and Pan, L.Q. (2012). Effects of bioflocs on growth performance, digestive enzyme activity and body composition of juvenile *Litopenaeus vannamei* in zero-water exchange tanks manipulating C/N ratio in feed. Aquaculture, 356:147–152

Zar, J.H. (1996). Biostatistical Analysis. 3rd Edition, Prentice Hall, Inc., Upper Saddle River.