CHAPTER III

MATERIAL AND METHODS

3.1 Sample collection and dissection

Live adult prawns (*M.rosenbergii*, average body weight 25 g) were obtained from a hatchery at Jelebu, Negeri Sembilan, Malaysia whereas the sub-adult prawns which were used for IHHNV challenge study (*M.rosenbergii*, average body weight 10g, Section 3.16) were obtained from a hatchery at Bandar Sri Sedayan, Negeri Sembilan, Malaysia. The adult prawns were transported in transparent plastic bags with aerated freshwater. The organs were obtained from approximately 80 adult individuals, processed in batches of 20. Organs which included hepatopancreas, gill, muscle, eyestalk and pleopod tissues were immediately dissected and wrapped with aluminum foil. Thereafter, the sampled organs were directly frozen in liquid nitrogen before being packed in zip-lock bags and stored at -80°C prior to total RNA and genomic DNA extraction (Figure 3.1). The process of dissecting, freezing and storing were done rapidly to reduce the degradation of RNAs in the sampled organs.

3.2 DNA extraction

DNA extraction using DNeasy Blood and Tissue kit (Qiagen, Germany) was performed according to the manufacturer's instruction. The frozen tissues of approximately 25 mg were weighed and pulverized with liquid nitrogen using mortar and pestle before the samples were thawed and placed into a 2 ml microcentrifuge tube.

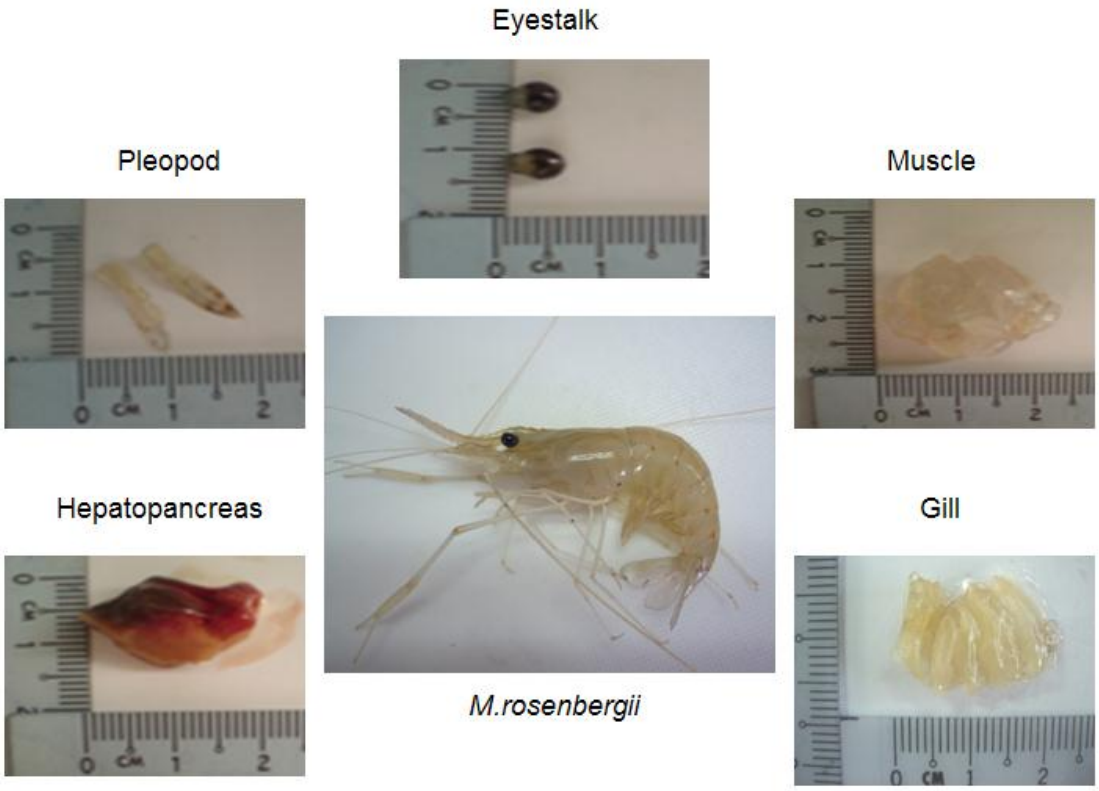

Figure 3.1 Tissue sample collection of *M.rosenbergii*

Then, 180 µL of Buffer ATL and 20 µL of proteinase K were added to the tube. The mixture was mixed well through vortexing and followed by incubation at 56°C for 3 hours. To obtain RNA-free genomic DNA, 4 µL of RNase A (100 mg/ml) was added. They were mixed and incubated at room temperature for 2 minutes. The homogenate was vortexed occasionally during incubation. Thereafter, 200 µL of Buffer AL was added and mixed thoroughly by vortexing. Then, 200 µL of ethanol (96 -100%) was added to the lysate and mixed by vortexing. The mixture was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube and was centrifuged at 6000 x g for 1 minute. The flow-through and collection tube was discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µL of Buffer AW1 was added into it and was centrifuged at 6000 x g for 1 minute. The flow-through and collection

tube was discarded. The DNeasy Mini spin column was placed again in a new 2 ml collection tube and 500 µL of Buffer AW2 was added into it and was centrifuged at 20,000 x g for 3 minutes to dry the DNeasy membrane. Later, the filtrate and collection tube were discarded. The DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µL of Buffer AE was pipetted directly onto the DNeasy membrane. Incubated at room temperature for 1 minute and followed by centrifugation for 1 minute at 6000 x g for elution. The DNA solution was stored at -20^{o}C. The purity and concentration of the DNA was measured using NanoDrop 1000 Spectrophotometer (ThermoScientific, USA) and only samples with A260/A280 ratio of 1.8 to 2.0 were used for further analysis.

3.3 Total RNA extraction

Total RNA extraction using TRIzol reagent (Invitrogen, Carlsband, CA, USA) was performed according to the manufacturer's instructions. The frozen target tissues of approximately 50 mg were weighed and pulverized with liquid nitrogen using mortar and pestle before the samples were thawed. The tissue samples were homogenized in 1mL of TRIzol reagent in a 1.5 mL microcentrifuge tube. The homogenate was vortexed for 30 seconds and was incubated at room temperature for 5 minutes before proceeding. A 200 µL aliquot of chloroform was added to the 1.5 mL microcentrifuge tube containing homogenate and the mixture was shaken vigorously for 15 seconds. Then the mixture was incubated at room temperature for 3 minutes and followed by centrifugation at 12000 x g, 4°C for 15 minutes. After that, the supernatant was transferred to a clean 1.5 ml microcentrifuge tube containing 500 µL of ice-cold isopropanol and mixed well by inverting the tube for a few times. The mixture was then incubated at room

temperature for 10 minutes and followed by centrifugation at 12000 x g, 4°C for 10 minutes where the resulting supernatant was carefully decanted. A total of 1 mL of 75% ice-cold ethanol was then added to the pellet and the tube was inverted gently several times to wash the RNA. The tube was spun at 7500 x g, 4°C for 5 minutes. The ethanol was aspirated carefully using a sequencing pipette tip. The tube was inverted on clean absorbent paper and the pellet was left to air-dry for 10 minutes. Finally, the RNA pellet was dissolved in 50 µL of DEPC-treated water and stored at -80^{o}C. The purity and concentration of the RNA was measured using NanoDrop 1000 Spectrophotometer (ThermoScientific, USA) and only samples with A260/A280 ratio of 1.8 to 2.0 were used for further analysis.

3.4 DNase treatment

RNA samples were treated with Deoxyribonuclease (DNase) I (5Prime, Germany) before being subjected to downstream processes. The amount of each reagent used for the DNase treatment is shown in Table 3.1. The mixture was then incubated at room temperature for 10 minutes. The DNase- treated RNA sample was cleaned up by an isopropanol precipitation. 200 µL of isopropanol was added to the DNase- treated RNA sample and followed by centrifugation at 15,000 x g, 4°C for 20 minutes. Supernatant was decanted carefully without disturbing the pellet. The pellet was washed with 1 ml of 75% ethanol and followed by centrifugation at 15,000 x g, 4°C for 10 minutes. The ethanol was aspirated carefully using a sequencing pipette tip. The tube was inverted on clean absorbent paper and pellet was left to air-dry for 10 minutes. Finally, the RNA pellet was dissolved in 50 µL of DEPC-treated water and stored at -80^{o}C.

Table 3.1 DNase I Digestion Mixture

Reagent/ Solution	Volume (μL)
RNA sample	80
Buffer DNase Digestion Buffer	10
DNase I stock solution	2.5
DEPC-treated water	Top up to 100 μL

3.5 Preparation of small RNA library

For preparation of small RNA library, total RNA (Section 3.3) was isolated from the gill and hepatopancreas tissues from a pool of adult *M.rosenbergii*. Thereafter, the total RNA was treated with DNase (Section 3.4). Approximately 20 μg of DNase treated total RNA representing each tissue was dissolved in DEPC-treated water at a concentration of not less than 750 ng/μl and stored at -80^{o}C prior to small RNA sequencing at the Beijing Genomic Institute, Shenzhen, China (BGI, Shenzhen). The integrity of the RNA was measured by using Bioanalyzer (Agilent Technologies, USA). Only RNA samples with high integrity (RIN >7) were used for further analysis.

3.6 Illumina deep sequencing

Two small RNA libraries of gill and hepatopancreas tissues were sequenced using standard methods at BGI, Shenzhen. 10ug of total RNA were size fractionated by Novex 15% TBE-Urea gel (Invitrogen) and RNA fragments of length between 18 to 30 nucleotides were isolated. The RNA fragments were then ligated with 5' RNA adapter (5'-GUUCAGAGUUCUACAGUCCGACGAUC-3') (Illumina, San Diego, CA, USA) and 3' RNA adapter (5'-pUCGUAUGCCGUCUUCUGCUUGUidT-3'; p: phosphate; idT: inverted deoxythymidine) (Illumina, San Diego, CA, USA) to their 5' and 3' termini. Thereafter, the RNA fragments with adapter in both ends were amplified by RT-

PCR using adaptor primers (Illumina, San Diego, CA, USA) for 17 cycles. Fragments of about 90 bp (small RNA + adaptor derived cDNA) were isolated from Novex 6% TBE gel (Invitrogen) of RT-PCR products. After quantification, the purified cDNA was used directly for cluster generation and sequencing analysis using the HiSeq 2000 Illumina Genome Analyzer (Illumina, San Diego, CA, USA). Thereafter, the image files generated by the sequencer, Illumina Genome Analyzer (Illumina, San Diego, CA, USA) were processed using open source Firecrest and Bustard applications (Illumina, San Diego, CA, USA) to produce digital-quality data (36 nucleotide).

3.7 *In silico* analysis and small RNA annotation

Each 36 nucleotide end read (raw dataset) obtained from Illumina Genome Analyzer (Illumina, San Diego, CA, USA) was processed with a bioinformatics' pipeline developed at Beijing Genome Institution, BGI (unpublished) as follows: 1) filtering low quality reads, 2) trimming of adaptor sequence at the 3' end, 3) cleaning up 5' end adaptor contaminants formed by adaptor and adaptor ligation, 4) remove sequences with poly-A tail, 5) filtering of reads smaller than 18 nucleotides to obtained high quality clean reads (18 to 30 nucleotides in length) and draw size distribution. Thereafter, the clean reads (18 to 30 nucleotide) were further subjected to computational analysis.

The clean reads (18 to 30 nucleotides in length) that mapped perfectly to the *de novo* transcriptome of *M. rosenbergii* (Transcriptome Shotgun Assembly (TSA) Sequence archive under the accession number: TSA JP351514-JP355722) which was assembled using SOAP2 (Li *et al.*, 2009) were further annotated and classified into different categories including rRNA, tRNA, snRNA, snoRNA and repeat associated

sequences by aligning the small RNAs against the known non-coding RNAs obtained from NCBI non-coding genbank database (URL:http://www.ncbi.nlm.nih.gov/genbank/) and Rfam 10.0 (Gardner *et al.,* 2009; Griffiths-Jones *et al.,* 2005; Griffiths-Jones *et al.,* 2003) with NCBI BLASTN (Altschul *et al.,* 1997) and RepeatMasker (Smit *et al.,* 1996-2010; URL: http://www.repeatmasker.org). Those sequences were discarded assuming that they represent degradation and other undesired products.

The filtered sequences were aligned with NCBI BLASTN (Altschul *et al.,* 1997) to a non-redundant reference set of all animal miRNAs from miRBase 15.0 (Griffiths-Jones, 2004; Griffiths-Jones *et al.,* 2006; Griffiths-Jones *et al.,* 2008; Kozomara and Griffiths-Jones, 2011) by allowing at most two mismatches outside of the seed region. Small RNAs that matched known miRNAs of other animal species were assumed to correspond to conserved miRNA orthologs in *M.rosenbergii.* The orthologous miRNA sequences were assigned to their respective miRNA families based on the similarity (identity score) to any existing miRNA family members. Pre-miRNAs were predicted based on the presence of hairpin structures identified by using MIREAP (Liu *et al*., 2010b) to scan through the *de novo* transcriptome of *M.rosenbergii* using default settings. The RNA secondary structures of pre-miRNA hairpin were drawn using RNAfold (Hofacker, 2009). Candidates corresponding to known miRNAs from miRBase 15.0 (Griffiths-Jones, 2004; Griffiths-Jones *et al.,* 2006; Griffiths-Jones *et al.,* 2008, Kozomara and Griffiths-Jones, 2011) and supported by at least two reads of mature sequences were considered real miRNA genes (Liu *et al*., 2010b).

In order to predict novel miRNA candidates from the two small RNA libraries, the following criteria were used: (1) small RNA annotated as orthologous miRNAs or as other classes of noncoding RNA were excluded; (2) an individual locus had to be

supported by a minimum of five independent sequence reads originating from at least one small RNA library to be considered for further analysis; (3) the loci lacking hairpin-like RNA secondary structures with the mature sequences within a stem were eliminated (Liu *et al*., 2010b).

The flanking region of the unannotated reads that perfectly mapped to the *de novo* trancriptome by SOAP2 (Li *et al*., 2009) were extracted and analyzed by MIREAP under default settings. MIREAP is a computational tool designed to identify known and novel miRNA candidates based on the prediction of hairpin structures within data from deeply sequenced small RNA libraries. This software which was downloaded from https://sourceforge.net/projects/mireap, takes into account miRNA biogenesis, sequencing depth and structural features to improve the sensitivity and specificity of miRNA identification. The RNA secondary structures of pre-miRNA hairpin were drawn using RNAfold (Hofacker, 2009). Stem-loop hairpins were considered distinctive only when they fulfilled three criteria: (1) mature miRNAs are present in one arm of the hairpin precursor; (2) absence of large internal loops or bulges ($n< 5$); (3) a minimal free energy of hybridization lower than -20 kcal/mol (Chen *et al.*, 2009). Pseudo-pre-miRNA sequences were removed by stepwise use of the following filters: NCBI BLASTX (Altschul *et al.*, 1997) against Non-redundant NCBI database (Nr) (URL: http://www.ncbi.nlm.nih.gov/genbank/) and Swiss-Prot (The UniProt Consortium, 2010) to exclude transcripts similar to any known, putative, and hypothetical proteins (E-value < 0.01); EMBOSS program getORF to exclude possible open reading frame (ORF) (Rice *et al*., 2000) (default parameters); ESTScan to detect potential coding regions based on the codon bias of coding regions derived from the *de novo* transcriptome of *M.rosenbergii* (Iseli *et al.*, 1999) (default parameters); INFERNAL program (Nawrocki

et. al., 2009) cmsearch against the Rfam 10.0 database to remove homologs of known non-coding RNA based on sequence similarity and secondary structure (Gardner *et al.,* 2009; Griffiths-Jones *et al.,* 2005; Griffiths-Jones *et al.,* 2003) (E-value <0.01) and tRNAscan-SE to remove tRNA sequences (Lowe *et al.,* 1997).

3.8 MicroRNA target gene prediction and enrichment analysis

For miRNA target prediction, a support vector machine (SVM) developed at Beijing Genomic Institute, BGI (unpublished) was trained with experimentally tested target and miRNA of *Drosophila melanogaster* and *Homo sapiens* to determine the optimal parameters to be used in RNAhybrid 2.2 (Kruger *et al*., 2006). These values were: helix constraint: 2 to 8; internal loop size: 5; bulge loop size: 5 and maximum target length: 100,000. RNAhybrid predicts potential binding sites for miRNAs in large target RNAs using the principle of finding the most energetically favourable hybridization site between two sequences (Kruger *et al*., 2006). The potential targets of the miRNA were matched to the *de novo* transcriptome of *M.rosenbergii* and were used for the following annotation: NCBI BLASTX (Altschul *et al.*, 1997) against Non-redundant NCBI database (Nr) (URL: http://www.ncbi.nlm.nih.gov/genbank/) / Swiss-Prot (The UniProt Consortium, 2010)/ Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.,* 2002, URL: http://www.genome.jp/kegg)/Clusters of Orthologous Groups of proteins (COG) (Tatutsov *et al.,* 2003; URL: http://www.ncbi.nlm.nih.gov/COG) to annotate transcripts similar to any known, putative, and hypothetical proteins (E-value < 10^{-5}). Functional annotation using gene ontology (GO) was performed using BLAST2GO (Ashburner *et al*., 2000). For quantification and evaluation of target gene expression, RPKM (reads per kilobase per

million reads) and Bejamini and Hochberg, (1995) false discovery rate (FDR< 0.001) (Wang *et al.,* 2010) were used respectively. The false discovery rate (FDR) was applied to evaluate the significance of gene expression difference. A statistical procedure known as enrichment (over-representation) analysis was used to narrow down potential miRNA targets (Gusev, 2008). The pathway enrichment analysis was based on the association of targets for individual miRNAs with pathways annotated in the KEGG database (Kanehisa *et al.,* 2002, URL: http://www.genome.jp/kegg). The Fisher's exact test with Bonferroni correction (p-value <0.05) for multiple testing was applied to determine the pathway enrichment for target genes (false discovery rate, FDR< 0.001) of each co-expressed miRNA [(fold change (log_2) >1 or fold change (log_2) >-1 and p-value <0.05)] between gill and hepatopancreas tissues. The statistical analysis of Fisher's exact test with Bonferroni correction (p-value <0.05) was computed using IDEG6 (Romualdi *et al*., 2003). The parameters considered in this test are: (1) Total number of miRNA target genes; (2) Total number of differentially expressed miRNA target genes; (3) Number of miRNA target genes associated with a specific pathway; (4) Number of differentially expressed miRNA target genes associated with a specific pathway. Inverse correlation analysis of the miRNA and their target gene (mRNA) expression profiles was carried out for each enriched pathway using the lists of differentially expressed miRNAs (fold change (log_2) >1 or fold change (log_2) >-1 and p-value <0.05) and mRNAs (false discovery rate, FDR<0.001) between gill and hepatopancreas tissues.

3.9 Stastistical tests for differential expression

3.9.1 Normalization of the expression data

The expression data for each small RNA library was standardized according to the equation below (Zhu *et al.*, 2010):

$$X_{std} = X_{expressed} / X_{total} \times 10^6 \quad (1)$$

Whereby $X_{expressed}$ indicates the total number of reads for a particular miRNA in gill and X_{total} indicates the total number of clean reads in G, where G is the small RNA library from gill tissue.

$$Y_{std} = Y_{expressed} / Y_{total} \times 10^6 \quad (2)$$

Whereby $Y_{expressed}$ indicates the total number of reads for a particular miRNA in hepatopancreas and Y_{total} indicates the total number of clean reads in H, where H is the small RNA library from hepatopancreas tissue.

3.9.2 Fold change

$$\text{Fold change} = \log_2 (Y_{std} / X_{std}) \quad (3)$$

Whereby X_{std} indicates the normalized expression for a particular miRNA in gill, which is calculated from equation (1) and Y_{std} indicates the normalized expression for a particular miRNA in hepatopancreas, which is calculated from equation (2)

3.9.3 Audic-Claverie

The statistical test of Audic-Claverie, (1997) which is based on Poisson statistics, was used to analyze data from the two different libraries. This model test has been previously applied to serial analysis of gene expression (SAGE) (Velculescu *et al.* 1995) and RNA-Seq (Sultan *et al*., 2008) expression data. The statistic is calculated according to equation below:

$$p(y \mid x) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!\,y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}} \quad (4)$$

where *x* indicates total number of reads for a particular miRNA in gill, y indicates the total number of reads for a particular miRNA in hepatopancreas. N_1 and N_2 represent the total numbers of clean reads in G and H respectively. The *p*-value indicates the probability of obtaining *y* count in H given *x* counts in G.

In this study significant signature was used as follows:

****** fold change ($\log_2$) >1 or fold change ($\log_2$) <-1 and p-value<0.01

***** fold change ($\log_2$) >1 or fold change ($\log_2$) <-1 and 0.01 <= p-value<0.05

3.10 MicroRNA dot-blot

3.10.1 Preparation of microRNA oligo dot-blot nylon membrane

The miRNA dot-blot method used in this study was modified from Wang and Cheng (2008). A library of 47 antisense oligodeoxynucleotides (50 nmol scale, 100 μM, 20 to 22 nucleotides) of *M.rosenbergii's* mature miRNA, mature-star miRNA (miRNA*)

and other small RNA sequences were synthesized (Table 3.2). Each of the antisense oligonucleotides, 10 µM was dotted in duplicate in the form of 96-well. The negative control (non probe control) used was nuclease free-water. The positive control used was antisense oligonucleotides of beta-actin gene, 5'GAG TTG TAT GTG GTC TCG TGG A 3' (22 nucleotides). The positive control was dotted on additional strip of nylon membrane in serial concentration of 10 µM, 8 µM, 6 µM, 4 µM, 2 µM and 0 µM.

Hybond –XL nylon membrane (Amersham, UK) was cut to the exact size 8.5 cm (width) × 12 cm (length), of the dot-blot manifold. The membrane was placed in wetting buffer (0.4 M Tris-HCl, pH 7.5) and soaked for 5 minutes. The wet membrane was placed on the manifold. The manifold was cleaned and rinsed with Millipore water prior to use. The oligos were diluted in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to 10 µM in a 96-well plate to make duplicate dot blots. Thereafter, equal volume (20 µl) of 2 × denature buffer was added to the diluted oligos, mixed well, and incubated for 10 minutes at room temperature. Later, 40 µl of nuclease free-water and 150 µl of dilution buffer (0.1 × SSC, 0.125 N NaOH) were added to the diluted oligos (samples) on ice. A 100 µl of samples was loaded into the manifold. A light suction was applied to the manifold until the loading buffer is drawn through the wells. A 100 µl of washing buffer (0.5 N NaCl, 0.5 M Tris-HCl, pH 7.5) was added to each well and a light suction was applied to neutralize and to wash the membrane. This washing step was carried out twice. After that, the membrane was removed from the manifold and the wet membrane was placed on a piece of whatmann paper. The oligos were fixed to the membrane by UV-crosslinking at 120 mJ/cm^2 for 1 minute. The membrane was air-dried, labeled with pencil, sandwiched with two whatmann paper and stored at 4^{o}C in a plastic bag until

needed. The dot blot apparatus was washed and rinsed with distilled water before the next run.

Table 3.2 Antisense oligonucleotides for miRNA dot-blot

Location on nylon membrane	miRNAs	miRNA antisense sequences (5' to 3')
A1; A7	miR-125_G	ACAAGAAAGGGTCTCAGGGA
A2; A8	miR-125_H	TCACAAGAAAGGGTCTCAGGGA
A3; A9	miR-750_H	AGCTGGAAGAGTTAGATCTGG
A4; A10	G-m0003	CTTAAGTAATGCCTACAGTGCA
A5; A11	G-m0005	CTGTTTCGCCGTGTTTAGTACT
A6; A12	G-m0006	TAGTACTAAACACGGCGAAACAG
B1; B7	UCR	AACATACCAGTTTGCAGCCCTG
B2; B8	UCR	CCTCCTCCTCTCCCTCTCCCT
B3; B9	G-m0009	CAACCTCCCACCGCAGACCCCA
B4; B10	G-m0011/ H-m0027	ACATACCAAATTGCAGCCCTCT
B5; B11	G-m0007	TTAAGTAATGCCTACAGTGTA
B6; B12	G-m0019	TTAAGTAATGCCTACAGTGCA
C1; C7	G-m0008/ H-m0016	AAACATACCAAATTGCAGCCCT
C2; C8	G-m0004	ACATATGGGGCTTCAACAGAGT
C3; C9	G-m0004*	CACTCCAGTCAAGCACCAAT
C4; C10	G-m0002/ H-m0009	ATGCCTGAGCACCTTGCTTGT
C5; C11	UCR	GATGCTACTGTGTCCTGTTAGA
C6; C12	UCR	CAATTCGGTGTTCGCTTCACA

D1; D7	UCR	ACTCCGTGACCGCTGGTTTGTT
D2; D8	UMR	TTGACAAAACCAGCCGAAAACGTA
D3; D9	H-m0012	CTTAAGTAATGCCTACAGTGCA
D4; D10	UMR	TCTCTCTCCACTTCTTGAACA
D5; D11	H-m0019	TATCCCTCGGATCTTAAAGACA
D6; D12	UMR	GATTGTAAAGTACAGCCTTACAGA
E1; E7	UMR	TGCCCGTGAAGACGATAATAACGA
E2; E8	H-m0020	ACATCACCCTGTCTCAATCTCCA
E3; E9	H-m0020*	ACGGCCTGTGAATTGGTGGAGGTATA
E4; E10	H-m0021	GCGAAATCCGTCCTGCTCGAAAG
E5; E11	H-m0022	GAAACTGCTGCTCTTCCGCTCGTA
E6; E12	UMR	GAACGTCACTCTCATCAGAAG
F1; F7	UCR	AAAACCCTCTCAAACCTCCTA
F2; F8	UCR	CTCAACAAGTCTTGGGTC
F3; F9	H-m0025	CAACTGTAGTCTAGTCGCGTG
F4; F10	H-m0034	AAAGAAGACCAGATTTAGAGACA
F5; F11	H-m0030	AGAAACTAAAGCTACAGGTTCT
F6; F12	H-m0041	CTTGAGTACCCCACAGCAATCCTA
G1; G7	H-m0044	CATAAACCGGCATGTTCTTA
G2; G8	UCR	ATGCCATTTCGCCACACTATGGA
G3; G9	UMR	CTTTCCAAATCATCTCTTGATA
G4; G10	UMR	AGTAAATCAACAGATAATAAACCAA
G5; G11	H-m0033	TTTCCACCAAATACAGTTCAA
G6; G12	UCR	GCTAGCCAAGAGCTCTACCCA

H1; H7	UCR	AACATACCAAATTGCAGCCCCC
H2; H8	UMR	CTAGAGGGCTGCAAATTGGTAT
H3; H9	UMR	TACCAAATTGCAGCCCTCT
H4; H10	UMR	ACATTGCTAGGTACCTAGTGCA
H5; H11	UMR	AGCCAGTGCCCCAATTTGTGCTCA
H6; H12	Non probe control	Nuclease free water

3.10.2 Total RNA extraction and enrichment of microRNA

A100 µg of total RNA (Section 3.3) with concentration of 1 µg/µl was used for enrichment of microRNA. The total RNAs were preheated at 80°C for 3 minutes and cooled on ice for 2 minutes. The RNA was then pipetted to the top of Amicon Ultra-0.5 ml centrifugal filter, 100 kDa (Milipore, USA) and followed by centrifugation at 14000 × g, 4°C for 10 minutes to obtain enriched microRNAs. Save the eluate containing the small molecular weight RNAs in the collection tube. A NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) was used to obtain the concentration of the small RNAs. The samples were stored at -80°C until needed for labeling.

3.10.3 RNA labeling

A 500 ng of enriched small RNA derived from 100 µg total RNA was labeled with 32P-αATP. At room temperature, tailing reaction reagent was added to each enriched small RNA sample in the order shown in Table 3.3 and mixture was mixed well followed by incubation at 37°C for 3 to 4 hours.

Table 3.3 RNA labeling

Components	Volume (µl)	Final concentration
5 × poly (A) polymerase reaction buffer (USB, USA)	5	1 ×
Enriched small RNA	16.5	500 ng
32P-αATP (ARC, USA)	2.5	0.05mCi
Yeast Poly(A) Polymerase (USB, USA)	0.5	600U
RNasin (Promega, USA)	0.5	20-40 U
Total	25	

3.10.4 Post-tailing clean up

A G25 Sephadex Gravity column was used for post-tailing miRNA clean-up. Pasteur pipettes plugged with glass wool was packed with silicon. The pre-packed column was stored under TE buffer in a tube. Prior to usage, the TE buffer was allowed to run through the column twice. When the TE buffer enters the column matrix, labeled probes mixed with blue dextran-orange (1:1) (Sigma Aldrich, USA) was added into the column. The blue fraction which contained the labeled probes was collected and checked with Geiger counter.

3.10.5 Prehybridzation and hybridization

The dotted nylon membrane was pre-wetted with wetting buffer before insertion into hybridization bottles. The membrane was prehybridized with 10 ml of Ultrasensitive Hybridization Buffer (Ambion, USA) at 42°C for 1 hour. Thereafter, hybridization was carried out with 10 ml of Ultrasensitive Hybridization Buffer and purified labeled probes at 42°C for 16 hours. Following hybridization, the membranes were washed with 20 ml of 2 x SSC/0.5% SDS at 42°C for 10 minutes (twice), 20 ml of 1 x SSC/0.5% SDS at 42°C for 15 minutes and 20 ml of 0.5 x SSC/0.5% SDS at 42°C for 10 minutes. The

membrane was then wrapped with cling film and inserted into a cassette. X-ray film (Kodak, Japan) was inserted into the cassette in dark room. The cassette was then stored in -80°C freezer for three days. After exposure, the X-Ray film was developed in the dark room. The developed film was hung to air-dry. Image captured by the film was scanned.

3.11 Reverse transcription

First strand cDNA was synthesized using a Reverse Transcription System kit (Promega, USA). The amount of each reverse transcription reagent used is shown in Table 3.4. The tube containing cDNA synthesis mixture was placed in a thermal cycler. The reactions were incubated at 25° for 10 minutes followed by one cycle of 42°C for 15 minutes, 95°C for 5 minutes and 4°C for 5 minutes.

Table 3.4 Reverse Transcription Mixture

Component	Final Volume	Final concentration
Magnesium chloride ($MgCl_2$), 25mM	4µL	5mM
Reverse tansciption 10× buffer	2µL	1x
dNTP mixture, 10mM	2µL	1mM
Recombinant RNasin ribonuclease inhibitor	0.5µL	1 Unit /µL
AMV reverse transciptase	0.6µL	15 Unit /µL
Random primer	1.0µL	0.5µg /µL
RNA template	20ng	-
Nuclease- free water	Top up to 20µL	

3.12 Validation of miRNA expression by semi quantitative RT-PCR and sequencing

DNase (5Prime, Germany) treated total RNA of gill and hepatopancreas was reverse-transcribed to cDNA using a Reverse Transcription System kit (Promega, USA) (Section 3.11). For each 25 µL of PCR reaction, 20 ng of cDNA or DNA was used as

template with 1.5 μL of 25 mM magnesium chloride, 5.0 μL of 5x PCR reaction buffer, 0.5 μL of 10 mM dNTP mix, 0.5 μL of each miRNA-specific forward and reverse primers (Table 3.5), 0.125 μL of GoTaq DNA polymerase (Promega, USA) and topped up to 25 μL with nuclease-free water. The reactions were incubated at 95°C for 3 minutes, followed by 29 repeated cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The final cycle was followed by extension at 72°C for 5 minutes. The positive control used was beta-actin (forward primer sequence: 5' TCC ACG AGA CCA CCT ACA AC 3'; reverse primer sequence: 5' GAG GGC AGT GAT TTC CTT CT 3') and a template free negative control of nuclease-free water was included in each PCR run. Each miRNA was analyzed in duplicate. The PCR products were separated using 2% agarose gel at 78V, 150mA for about 45 minutes and the sequence determined using a conventional DNA sequencing service (Figure 4.3).

Table 3.5 Primers for putative *M.rosenbergii* miRNA precursors

Putative *M.rosenbergii* miRNA precursors	miRNA-specific forward and reverse primer	Expected size of PCR product
mir-125_G, mir-125_H, G-m0002, H-m0009	125F: 5'-CAGAGAGCTCGCCATCCAT -3' 125R: 5'-CTGAATCAATCTGCCCATGT-3'	162bp
mir-750_H	750F: 5'-AGCTGACTCCCTGGACAAGA3' 750R: 5'-TTTCCTAAAGTCGCCTCGAA-3'	297bp

3.13 PCR product purification

NucleoSpin Extract II (Macherey-Nagel, Germany) was used for PCR clean-up according to manufacturer's instruction. One volume of PCR product was mixed with 2

volumes of Buffer NT. The mixture was carefully pipetted into the NucleoSpin Extract II column which was placed into a collection tube and was then centrifuged for 1 minute at 11,000 × g. The flow-through was discarded and the NucleoSpin Extract II column was placed back into the collection tube. After that, 700 µL of Buffer NT3 was added to the NucleoSpin Extract II column. The flow-through was discarded and the NucleoSpin Extract II column was placed back into the collection tube. The tube with the NucleoSpin Extract II column was centrifuged again at 11,000 × g for 2 minutes to remove residual of Buffer NT3. The NucleoSpin Extract II column was placed in a clean, 1.5 ml microcentrifuge tube. Thereafter, 30 µL of pre-warmed Buffer NE was pipetted directly onto the membrane and was incubated at room temperature for 1 minute. Finally, the column was centrifuged at 11,000 × g for 1 minute to collect the purified PCR products.

3.14 Validation of miRNA expression by stem-loop quantitative real-time PCR

A Custom Taqman MicroRNA Assay (Applied Biosystems, Foster City, CA, P/N: 4398987) was designed based on small RNA sequences of *M.rosenbergii*. The expression of mature miRNAs was assayed using the Custom Taqman MicroRNA Assay (Table 3.6) specific for miR-125 (Assay ID: CSS061K), miR-750 (Assay ID: CST947S), G-m0002/H-m0009 (Assay ID: CSVl3D0), G-m0005 (Assay ID: CSRR8XB), G-m0008/H-m0016 (Assay ID: CSS063J), G-m0011/H-m0027 (Assay ID: CST949R) and G-m0015 (Assay ID: CSV13FZ) on five types of tissues of *M.rosenbergii* i.e. hepatopancreas, gill, muscle, pleopod, and eye. Each sample was analyzed in triplicate. DNase (5Prime, Germany) treated total RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (P/N 4366596) with respective miRNA-specific

stem-loop RT primer (Applied Biosystems, Foster City, CA). The 15.0 µL of reverse transcription (RT) reaction included: 1.0 µL of DNase treated total RNA (50 ng/µL), 1.5 µL of 10x RT Buffer, 1.0 µL of 50 U/µL MultiScribe Reverse Transcriptase, 0.15 µL of 100mM dNTPs, 0.19 µL of 20 U/µL RNase-inhibitor, 3.0 µL of specific RT primer and 8.16 µL of nuclease-free water. The reactions were incubated at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and hold at 4°C. Real-time PCR was performed using the standard Taqman MicroRNA Assays protocol on a 7500 Real-Time PCR (Applied Biosystem, Foster City, CA). The 10 µL PCR included 0.66 µL of RT product, 5 µL of 2 x Taqman Universal PCR Master Mix, No AmpErase UNG (P/N: 4324018), 0.5 µL of 20 x Taqman Small RNA Assay and 3.84 µL of nuclease-free water. The reactions were incubated at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The level of miRNA expression was measured using Ct (threshold cycle) at fixed threshold level of 0.2. Outlier (>2 standard deviations) was discarded from subsequent analysis. Comparative Ct method was used to determine miRNA expression levels (Livak *et al*., 2001; Schmittgen *et al.,* 2008). The Delta Ct was calculated by subtracting the Ct of reference gene (snRNA) from the Ct of the miRNA of interest. The Delta Delta Ct was calculated by subtracting the Delta Ct of the calibrator (gill) from the Delta Ct of each sample. Fold change was generated using the equation 2(-Delta Delta Ct) (Livak *et al*., 2001; Schmittgen *et al.,* 2008). The Custom Taqman MicroRNA Assay for snRNA (Assay ID: CSPACl9) was used to normalize the relative abundance of miRNA.

Table 3.6 Custom taqman miRNA assay

Assay_id	miRNAs	Target sequences (5'to 3')
CSS061K	miR-125	TCCCTGAGACCCTTTCTTGT
CST947S	miR-750	CCAGATCTAACTCTTCCAGCT
CSVI3D0	G-m0002/H-m0009	ACAAGCAAGGTGCTCAGGCAT
CSRR8XB	G-m0005	AGUACUAAACACGGCGAAACAG
CSS063J	G-m0008/H-m0016	AGGGCUGCAAUUUGGUAUGUUU
CST949R	G-m0011/H-m0027	AGAGGGCUGCAAUUUGGUAUGU
CSVI3FZ	G-m0015	GAACGAGACGGCGAGAGAGUGA
CSPACI9	snRNA	TTGGAACGATACAGAGAAGATTAGCAT

3.15 Pearson correlation coefficient

A correlation of miRNA deep sequencing counts and stem-loop quantitative real-time PCR was assessed with Pearson correlation coefficient, r to determine the agreement between both platforms. A Pearson correlation scatter plot of (logarithmized) normalized sequencing counts (deep sequencing data) against –DeltaCt values (qRT-PCR) was drawn. The Ct (cycle threshold) is defined as the PCR cycle at which the fluorescent signal cross the threshold. Hence, Ct values are inversely related to the logarithmized amount of target nucleic acid in the sample. Conversion of the Ct values by multiplication with (-1) results in negative Ct values which is proportional to the logarithmized amount of target nucleic acid.

3.16 IHHNV challenge test on *M.rosenbergii* and qRT-PCR of the miRNA expression

The sub-adult prawns (average body weight 10 g) were obtained from Bandar Sri Sedayan, Negeri Sembilan, Malaysia. The prawns were maintained in flat-bottomed glass tanks with aerated and filtered water at 28°C +/- 1°C in the laboratory (4 sub-adult prawns per tank). All prawns (72 sub-adult prawns) were acclimatized for a week before

challenged with infectious hypodermal and hematopoietic necrosis virus (IHHNV). The IHHNV infected prawn pleopod tissues were homogenized in PBS solution. Each prawn was injected with crude extract of virus (IHHNV) in PBS solution (100 µL per 10 g prawn). The tissues of the sub-adult prawn were collected before injection, 0 hrs (control) and post-injection of 3 hrs, 6 hrs, 9hrs, 24 hrs, 48 hrs. These samples were frozen in liquid nitrogen and stored at -80°C. Total RNA were extracted from the hepatopancreas tissues using Trizol (Section 3.3) for each time point. The expression of mature miRNAs at various time point of infection was assayed using the Custom Taqman MicroRNA Assay (Table 3.6) (Applied Biosystems, Foster City, CA, P/N: 4398987) specific for miR-125 (Assay ID: CSS061K), miR-750 (Assay ID: CST947S), G-m0002/H-m0009 (Assay ID: CSVl3D0), G-m0005 (Assay ID: CSRR8XB), G-m0008/H-m0016 (Assay ID: CSS063J), G-m0011/H-m0027 (Assay ID: CST949R) and G-m0015 (Assay ID: CSV13FZ). Each sample was analyzed in triplicate. DNase (5Prime, Germany) treated total RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (P/N 4366596) with respective miRNA-specific stem-loop RT primer (Applied Biosystems, Foster City, CA). The 15.0 µL of reverse transcription (RT) reaction included: 1.0 µL of DNase treated total RNA (50 ng/µL), 1.5 µL of 10x RT Buffer, 1.0 µL of 50 U/µL MultiScribe Reverse Transcriptase, 0.15 µL of 100mM dNTPs, 0.19 µL of 20 U/µL RNase-inhibitor, 3.0 µL of specific RT primer and 8.16 µL of nuclease-free water. The reactions were incubated at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and hold at 4°C. Real-time PCR was performed using the standard Taqman MicroRNA Assays protocol on a 7500 Real-Time PCR (Applied Biosystem, Foster City, CA). The 10 µL PCR included 0.66 µL of RT product, 5 µL of 2 x Taqman Universal PCR Master Mix, No AmpErase UNG (P/N: 4324018), 0.5 µL of

20 x Taqman Small RNA Assay and 3.84 µL of nuclease-free water. The reactions were incubated at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The level of miRNA expression was measured using Ct (threshold cycle) at fixed threshold level of 0.2. Outlier (>2 standard deviations) was discarded from subsequent analysis. Comparative Ct method was used to determine miRNA expression levels (Livak *et al*., 2001; Schmittgen *et al.,* 2008). Fold change was generated using the equation 2^(-Delta Delta Ct) = [Ct(miRNA) – Ct(reference gene)] $_{\text{time x}}$ – [Ct(miRNA) – Ct(reference gene)] $_{\text{time 0}}$ (Livak *et al*., 2001; Schmittgen *et al.,* 2008). The Custom Taqman MicroRNA Assay for snRNA (Assay ID: CSPACl9) was used to normalize the relative abundance of miRNA.