GENE-ENVIRONMENT INTERACTIONS IN FRESHWATER PRAWNS *Macrobrachium rosenbergii*

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GENE-ENVIRONMENT INTERACTIONS IN FRESHWATER PRAWNS *Macrobrachium rosenbergii*

ABSTRACT

Temperature is an essential factor in establishing growth, reproduction and distribution of organisms. In the current fluctuating climate circumstances, especially one as humid as Malaysia's aquatic organisms' survival is dependent on their adaptability to their environment's thermal changes. These adaptive characteristics can be either physiological or genetic. Temperature also plays an important role in the innate immune response to prawns and could alter the functional expression of immune genes. This study investigated the impact of temperature, as n contributor to environmental stress on immune gene expression in freshwater prawn, *Macrobrachium rosenbergii*, and on the histological and physiological changes of three organs, hepatopancreas, hemolymph and muscle. The expression of 14 genes (AK1, Lec1, Lec2, Lec3, Lec4, Ta-Lec, Ppo, PpoAIII, Hmo, HSP70, Chp, ALF2, NfkBI-α2, LAPs 2), representing five different pathways (metabolite, prophenoloxiade (PPO), lectin, apoptosis and chaperon) was studied at four different temperatures, 24 °C, 28 °C, 32 °C, 36 °C, at 6 hours and 12 hours post thermal stress, in these organs. The weight, body colour, hemocyte colour, count and viability. Hepatopancreas Somatic Index (HIS), phenoloxidase activity, total protein quantification of each prawn was observed at each time point. The experiments were carried out under three conditions: controlled laboratory conditions, good farm management and poor farm management. The gene expression pattern observed was similar in all three conditions. Higher gene expressions were observed at elevated temperatures (32 °C and 36 °C) in hemolymph at both timepoints. The chaperon (HSP70, Chp, Hmo) and PPO (Ppo, PpoAIII) genes showed a higher expression than the metabolite (AK1, ALF2), apoptosis (NfkBI- α2, IAPs 2) and lectin (Lec1, Lec2, Lec3, Lec4, Ta-Lec) genes. In contrast. Lower expressions were observed at 24 °C and 36 °C in muscle and hepatopancreas. The chaperon, PPO and lectin genes showed higher expression levels in comparison with the metabolite and lectin genes at both six- and twelve-hours post-stress in muscle and hepatopancreas. At higher temperatures, discoloration of hemocyte, reduction in hemocyte count, reduction in hemocyte viability, decrease in total protein, reduction in weight, reduction in HIS, reduction in PPO activity and body discoloration were observed. Histological examinations revealed formation of abnormal lumen in hepatopancreas, together with tubule rupture, muscle necrosis and degeneration, at elevated temperatures. The histological and physiological changes observed, in comparison with the gene expression observed, suggest that the freshwater

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prawn can adapt genetically in order to attempt to survive under different thermal conditions, however, this is impossible physiologically.

Keywords: *Macrobrachium rosenbergii*, gene expression, temperature, giant freshwater prawn, gene-environment interaction.

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GEN DAN INTERAKSI REREKITARAN FRESHWATER PRAWNS *Macrobrachium rosenbergii*

ABSTRAK

Suhu adalah faktor penting dalam merangsang pertumbuhan, pembiakan, dan taburan organisma. Dalam keadaan fluktuasi cuaca kini, contohnya seperti Malaysia yang beriklim lembap, kemandirian organisma akuatik bergantung pada penyesuaian terhadap perubahan suhu persekitaran. Ciri-ciri adaptif ini merujuk kepada ciri-ciri fisiologi atau genetik. Suhu juga memainkan peranan yang penting dalam sistem imun semula jadi udang, dan boleh menukar ungkapan gen imun fungsi. Kajian ini dijalankan untuk menyiasat kesan suhu sebagai penyumbang kepada tekanan persekitaran, terhadap ungkapan gen imun dalam udang galah. *Macrobrachium rosenbergii*, termasuk perubahan histologi dan fisiologi pada organ seperti hepatopankreas, hemolimfa, dan otot. Ungkapan 14 gen (AK1, Lec1, Lec2, Lec3, Lec4, Ta-Lec, Ppo, PpoAIII, Hmo, HSP70, Chp, ALF2, NfkBI-α2, LAPs 2), mewakili lima laluan berbeza (metabolite prophenoloxidase (PPO), lectin , apoptosis dan chaperon) telah dikaji pada empat suhu berbeza, 24 °C, 28 °C, 32 °C, 36 °C, pada jam ke-6 dan jam ke-12 selepas tekanan suhu, dalam organ yang dinyatakan. Jisim dan warna tubuh udang, warna, bilangan, dan kebolehhidupan hemosit, Hepatopancreas Somatic Index (HSI), aktiviti phenoloxidase dan kuantifikasi jumlah protein setiap udang telah dicerap pada setiap titik masa. Eksperimen telah dijilankan dalam tiga kondisi, iaitu keadaan makmal terkawal, pengurusan ladang ternakan yang teratur, dan pengurusan ladang ternakan yang tidak teratur. Hasilnya, pola ungkapan gen yang dicerap menunjukkan persamaan pada setiap kondisi. Ungkapan gen yang tinggi terlihat pada suhu yang tinggi (32°C dan 36°C) dalam hemolimfa pada kedua-dua titik masa. Gen chaperon (HSP70, Chp, Hmo) dan gen PPO (Ppo, PpoAIII) menunjukkan ungkapan yang tinggi berbanding dengan gen metabolite (AK1, ALF2), gen apoptosis (NfkBI- α2, IAPs 2) dan gen lectin (Lec1, Lec2, Lec3, Lec4, Ta-Lec). Sebaliknya, ungkapan gen yang rendah dicatat pada suhu 24°C dan 36°C di otot dan hepatopankreas. Gen chaperon, PPO dan lectin menunjukkan ungkapan gen yang lebih tinggi berbanding dengan gen metabolite dan lectin pada kedua-dua titik masa di otot dan hepatopankreas. Pada suhu yang tinggi, hemosit menunjukkan kepudaran warna, pengurangan terhadap bilangan sel, kebolehhidupan dan jumlah kandungan protein. Penurunan jisim udang, HSI, dan aktiviti PPO, dan kepudaran warna tubuh juga dapat dilihat. Pemeriksaan histologi membuktikan pembentukan lumen yang tidak normal di hepatopankreas, termasuk ruptur tubul, nekrosis dan penyerosotan otot pada suhu yang

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tinggi. Perubahan pada histologi dan fisiologi udang, berbanding dengan ungkapan gen yang dicerap, mencadangkan bahawa udang galah boleh mengadaptasi secara genetik untuk hidup dalam pelbagai kondisi suhu, walaupun, ianya tidak mungkin berlaku secara fisiologi.

Kata kunci: *Macrobrachium rosenbergii*, ungkapan gen, suhu, udang galah, interaksi gen-persekitatan

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I want to dedicate this research to human rights advocates, especially women rights activists, and environmental activists. Those life lovers that are fighting dedicatedly for justice, freedom, and the improvement of people's lives.

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LIST OF SYMBOLS AND ABBREVIATIONS

(-)	:	Negative
(+)	:	Positive
<	:	Is less than
>	:	Is greater than
ºC	:	Degree Celsius
Mg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar
10X	:	Ten times
1X	:	One time
AMOVA	:	Analyses of molecular variance
bp(s)	:	Basepair
cDNA	:	Complementary DNA
dATP	:	deoxyadenosine triphosphate
dCTP	:	deoxycytidine triphosphate
ddH2O	:	Double distilled water
dGTP	:	deoxyguanosine triphosphate
DNA	:	Deoxyribonuclei acid
dNTP	:	deoxyribonucleotide triphosphate
dTTP	:	deoxythymidine triphosphate
E. coli	:	*Escherichia coli*
EST	:	Expressed Sequence Tags
g(S)	:	Gram(s)
h or hr or H	:	Hour

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HSI	:	Hepato somatic Index
IHC	:	Immunohistochemistry
IHHNV	:	Infectious hypodermal hematopoietic necrosis
m	:	Meters
M	:	Molar
MANOVA	:	Multiple Analysis f Variance
MgCL2	:	Magnesium Chloride
mM	:	Milimolar
mRNA	:	Messenger RNA
mtDNA	:	Mitochondrial DNA
NCBI	:	National Centre for Biotechnology Information
nmole	:	Nanomole
PCR	:	Polymerase Chain Reaction
PL	:	Post-larva
ppm	:	Parts per million
ppt	:	Parts per thousand
qRT-PCR	:	Quantitative real time PCR
RNA	:	Ribonuclei Acid
rRNA	:	Ribosomal RNA
RT-PCR	:	Real time PCR
Std. Dev.	:	Standard Deviation
Ta	:	Annealing temperature
TVC	:	Total Vibrio spp. count
V	:	Volt
WSSV	:	White Spot Syndrome virus

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CHAPTER 1: INTRODUCTION

Temperature is a critical abiotic factor that affects organisms thoroughly from the ecological macro-environment to the fundamental cellular and internal molecular environments. Generally, all microorganisms experience temperature fluctuations during day and night and also by seasonal changes in their natural habitat. There is a strong monitoring evidence that global temperature has been increasing in the past decades. The evidence also confirms that it will continue to increase in the future as well (Hornbach et al., 2016). The question of how organisms can be affected by global warming and climate changes has been argued since 1999, and it is still heavily concerning to humankind (Hughes et al., 2003; Sagarin et al., 1999; Stillman, 2003).

Species distributions borders are expected to change in response to global warming. For example, in Monterey, California, southern species in the intertidal zone shifted northwards in the time span of 60 years (Sagarin et al., 1999). Environmental temperature is swiftly increasing both in domestic or aquatic habitats therefore understanding the aspects of thermal tolerance physiology is essential. In the order to describe the impact that global warming and climate change may have on an organism (Stillman, 2003). It has been known that the upper and lower thermal tolerance limit (CTmax and CTmin) of an organism can be modified with acclimatizing to its surrounding temperature (Cuculescu et al., 1998). Thermal tolerance often depends on the thermal scale availability related to the conditions of the natural habitat. Stillman in 2003 used comparative approaches which demonstrated that during in created temperature, warm adapted species are less able to select their CTmax intolerance while cold-adapted species are the opposite (Stillman, 2003). The geographical and latitudinal study may also be helpful for assessing the ecological consequences of climate change. It might have proper evidence in explaining the bases of an organism's distribution borders. A critical aspect

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of this study is the impact of global warming effects on species population in a latitude dependent approach. This aspect is assessed by thermal stress levels and the organisms, response in their natural habitat. Seasonal temperature conditions influence a thermal phenotype (Hopkin et al., 2006). It would be insightful to know the level of gradual acclimatization between seasons in natural habitats. There is evidence which shows that the thermal phenotype varies at both molecular and physiological levels (Dietz, 1994). For instance, in crab *C. maenaas* and *C. pagurus*, the maximum thermal tolerance was significantly lower in winter than it was in summer (Cuculescu et al., 1998).

Providing the molecular response repertoire derived from environmental changes is essential to develop strategies to prevent mortalities related to climate parameter in the natural environment and in aquaculture farms. In this study, through the essential pathway of gene expression analysis, we tried to identify the resistant-related gene expression signatures in prawn succumbing water temperature changes. To understand the mechanisms that underlie resistance in prawn, some studies have been conducted to identify gene regulations by environmental changes.

1.1 Problem statement

The immune signaling pathways which lead to regulation of immune gene expression under thermal environmental stress are an important concern in the innate immune responses of crustaceans.

Firstly, environmental parameters affect gene expression and these effects cannot be controlled.

Secondly, direct observations of interfering effects on other pathway mechanisms are regularly not possible. Thirdly, pathways have different gene expressions in different situations and in different tissues. This study focuses on the effect of temperature, the

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quantitative genes, the innate immune response in *M. rosenbergii* in controlled laboratory settings and testing to see whether similar responses can be seen in the prawns in habituating the farm.

Malaysia is a South Asian tropical country with high humidity and an average of 27 °C temperature (T. J. Conway et al., 1994b). Crustaceans are popular in this country. In order to culture prawns in Malaysia's fluctuating environment, the animal needs to adapt rapidly to the environmental circumstances (Dall et al., 1990). Aquaculture animals have limited tolerance range to water quality and temperature. Critical temperatures for crustacean and invertebrate species have been observed in different species by studying different parameters such as the innate immune system, growth rate, fertilization, ventilation rate, accumulation of heavy metals, heart rate and etc (Banu et al., 2016). The best possible survival rate and growth factor are observed at their optimal environmental circumstances, and each water parameter can directly affect their survival rate. As an aquatic animal, *M. rosenbergiis's* survival and growth depends upon the water environmental circumstances. So, it's important for Malaysian farmers to know about *M. rosenbergii's* optimal conditions for better growth and survival rate (Pauly & Kinne, 2010). Having a good biological underestandig of *M. rosenbergii's* tolerance limits optimal survival, and growth condition will help farmers increase their culture yield which will encourage them to increase their farming and develop their broodstock. This study was therefore, conducted to investigate the innate immune system's susceptibility to water temperature and to further examine the adaptations range of this animal. In this study, three different tissues of *M. rosenbergii* were genetically and physiologically screened in respect to their physiological importance in the animal's survival and adaptation to the advert environmental circumstances.

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In this study, we developed a method to address these problems which are specific to immune gene responses to environmental thermal stress which either leads to the survival or mortality of the species in the aquaculture farms in Malaysia.

1.2 Objectives of the study

This study is done to fulfill the following objectives:

1.2.1 Objective one:

To determine the performance of essential pathway analysis and identification on *M. rosenbergii* organ tolerance by screening the hemolymph, hepatopancreas and muscle tissue, which one experimentally challenged by different temperatures in the laboratory.

The initial concern in this study was to obtain insight into different tissue tolerance in adverse environmental circumstances. To achieve this purpose, we screened three different tissue based on three different gene clusters under three different environmental circumstances. Hemolymph, Muscle, and Hepatopancreas tissues were studied through metabolite, innate immune-related and environmental controller genes. So, the hypotheses of this study are:

H_0: There is no significant difference among the pathways in different organs (hemolymph, hepatopancreas, and muscle tissues) of *M. rosenbergii* and its tolerance against different temperatures in lab settings.

H_a: There is a significant difference among the pathways in different organs (hemolymph, hepatopancreas and muscle tissues) of *M. rosenbergii* and its tolerance against different temperatures in lab settings.

H_0: Different tissue will have a same expression across different temperature.

H_a: Different tissue will have a different expression across the different temperature.

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H_0: Different gene clusters will have the same expression across different tissue.

H_a: Different gene clusters will have the same expression across tissue.

1.2.2 Objective two:

To identify candidate immune related-genes that are involved in the survival of the animal in high temperature which can provide a better insight into the host-environment interaction between *M. rosenbergii* and its habitat in Malaysia.

H_0: There is significant candidate immune related-gene involved in survival of the animal in high temperatures which can provide a better tolerance of the host-environment interaction between *M. rosenbergii* and its habitat in Malaysia.

H_a: There is no significant candidate immune related-genes involved in survival of the animal in high temperature which can provide a better tolerance of the host-environment interaction between *M. rosenbergii* and its habitat in Malaysia.

H_0: There is a presence of non-genetic adaptation to thermal tolerance.

H_a: There is no presence of non-genetic adaptation to thermal tolerance.

H_0: *M. rosenbergii* will adapt physiologically in line with temperature changes.

H_a: *M. rosenbergii* will not adapt physiologically in line with temperature changes.

1.2.3 Objective three:

To understand the Gene-environment interactions in *Macrobrachium rosenbergii* in two different farm environments.

H_0: There is no significant effect of farm management on gene-environment interactions in *M. rosenbergii.*

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H_a: There is a significant effect of farm management on gene-environment interactions in *M. rosenbergii.*

H_0: Different tissues will have the same gene expression across different temperatures in the well-managed farm.

H_a: Different tissues will not have the samemgene expression across different temperatures in the well-managed farm.

H_0: Different tissue will have the same gene expression across different tempratures in the poorly-managed farm.

H_a: Different tissue will not have the same gene expression across different tempratures in the poorly-managed farm.

H_0: Different tissues will have the same gene expression across the same temprature between the Laboratory and the poorly-managed farm.

H_a: Different tissue will not behave the same gene expression across the same temprature between Laboratory and non -managed farm.

1.3 Research frame

This study sets to get insight in the response to heat stress, especially at the molecular level in *M. rosenbergii,* in its natural habitat in Malaysia. It is initially necessary to establish a standard and controlled habitat and establish a panel of gene expression level which changes in a fast time and temperature frame. Then the study proceeded to the organism's natural habitat. The of commercially monitored farms whit standard husbandry according to FAO (Moffitt & Cajas-Cano, 2014), and inappropriate management farm without standard criteria were both monitored. The gene expression level in three different environments was analyzed. The central question of the present

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study, the biochemical reactions of the heat stress response in *M. rosenberii,* is regarding the biochem.

Chapter 2 describes the background of the study. It contains general information about gene signaling, innate immune system, and *M. rosenbergii* and its standard habitat condition and biochemical characterization.

Chapter 3 of this thesis describes the construction from methodology to experimental design in both the laboratory-controlled condition and the farm's natural environment. It details the study of biochemical pathways and physiology study in three different tissues including hemolymph, hepatopancreas, and muscle at different temperatures and time frames.

Chapter 4 of this study describes the construction and annotation of five different biochemical pathways through fifteen coding genes in three different tissues including hemolymph, hepatopancreas, and muscle to varying temperatures in the laboratory condition and the natural habitat. It also, describes the impact of the macro-environmental management on the microenvironmental water quality, and finally describes the internal level of molecular interactions.

Chapter 5 of this thesis describes the knowledge gained from the experimental work done. This information may also set the stage for elucidating fundamental molecular bases behind thermal tolerance.

Efforts to link the impact of global warming on organismal molecular physiology may pave the way towards developing our understanding of *M.rosenbergii's* biology.

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CHAPTER 2: LITERATURE REVIEW

2.1 Genes and genome sequencing

The achievements of the recent genomic study technique genome sequencing, caused the identification of nearly most of the responsible genes for the biological complexity of numerous organisms (Merrill et al., 2006). These genes do not work in an integrated way, so their allocation is the subsequent important task to clear the function of each of these genes. They are linked in an extremely organized network in which information flows through the cell (Azeloglu & Iyengar, 2015). Inference of the genes in cellular networks under environmental effects is the main subject of this thesis.

Gene expression is an extremely organized process. During these complicated processes a cell can provide a suitable response to external signals. This is the fundamental adaption procedure to environmental change. In eukaryotic cell gene expression, initially a signal reaches the cell membrane, then, it will be recognized by a specific transmembrane receptor. Then, the receptor will bind to a ligand that initiate and creates an intracellular response. Each specific receptor has a specific and important role in cellular mechanism and signal transduction (Cortés et al., 2015). The initial warning sign will activate a transcription factor protein in the cytoplasm. The activated transcription factor protein will then enter in to the cell nucleus. This activated protein will activate the promoter region of a gene. The promoter region is an important genomic region which contains the information that turns the gene on or off. Depending on the function of the promoter the transcription factor may activate or inactivates the gene expression. The process of gene expression activation is called, started transcription. In transcription, RNA polymerase II (RNAP II) protein, will start to copy the genetical information from the subjected gene into the messenger RNA (mRNA) (Vogel et al., 2018).

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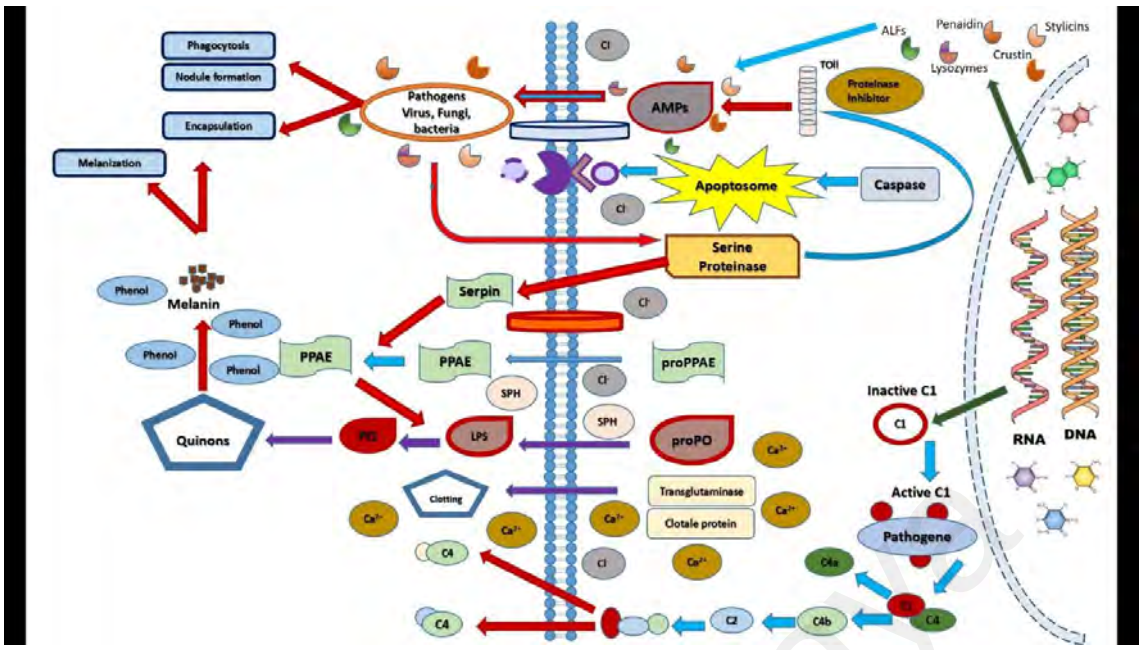

Figure 2.1: Demonstrating the signal transduction and gene regulation of the innate immune system of an invertebrate.

2.2 Signal transduction, gene regulation, and transcriptional regulatory networks

The nuclear mRNA is located in the nucleus. This mRNA consists of two genomic regions, exons and introns. Exon regions are transferred from the nucleus as coding fragments of the messenger RNA (mRNA). Noncoding intron region will be removed from the mRNA through the process of splicing process. Finally, the spliced nuclear mRNA will be transported from the nucleus into the cytoplasm. In the cytoplasm, It will be translated into a polypeptide sequence, that later on folds into a two, three and four-dimensional structure as an active protein structure (Lundin et al., 2013).

The expression of a single gene might have some influence on the activation and expression of other genes and proteins. Gene regulation happens at all levels such as transcription, signal propagation, translation or even protein degradation. At each level, several regulatory processes may correspond. For example, a transcription factor might

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be transcriptionally or non-transcriptionally regulated. The mechanism of controlling the transcription factor in mRNA is called transcriptional regulation. Non-transcriptional regulation is the process which controls the level of protein modification such as ligand binding or phosphorylation or the dissociation of an inhibitor protein or the activity of transcriptional factor protein or any other mechanisms that cause protein activations (Aaskov et al., 2006). All these mechanisms will create transcriptional regulatory networks. If, the produced protein is a transcription factor protein, this network process can be repeated. The transcription factor protein can re-enter the nucleus and activate or inhibit the expression of other gene. The combination of the networks of transcription factors and transcription targets are called, gene regulatory networks or transcriptional regulatory networks. Regulatory networks predictions and reconstructions were subsequent of the field of bioinformatics. This is generally because of the availability of gene expression of genome-wide measurements through different genome study methods such as microarrays, real-time gene expression and recently fluidigm method which provide a deep view of cellular gene activity. It also promises new visions into regulatory relationships (Perez & Groisman, 2009).

2.3 Signal transduction pathways

The second significant process is signal transduction pathways which It is prominent from the receptor to the activated form of a transcription factor protein. Signal transduction activation epitomizes a complex biochemical signal that characterizes Signal transduction pathways. This activation will connect the external signals to the transcriptional response (Lundin et al., 2013).

Protein interactions and modifications and post-translational modifications are the main steps in signal propagation, which are not active on a transcriptional level. Here we will elucidate important parts of the innate immune system signaling pathways by

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emphasis on the immune deficiency pathway (Imd). Imd, initially, manages the defense responses against thermal shock in *Macrobrachium rosenbergii*.

Relish is a transcriptome factor. It is important because the immune induction of genes encoding immune protein peptides like Nf-Kappa B depend on Relish. This protein has inhibitory repeated domain sequences in its inactive form. Initially, Relish should be phosphorylated, then dispart from its inhibitory domains to be activated. This protein doesn't have expression on the transcriptional level, it has a conversion from an inactive into an active form, and its total protein amount remains the same before and after activation. The biological principle of this reaction confirmed that the cells have quick response pathways to the external impacts. There are many pathway mechanisms intermediating between the specific receptor located in the cell membrane. However, the exact roles of these pathway mechanisms are often unknown (López-Maury et al., 2008).

Cellular signaling pathways normalize vital mechanisms in living cells. In several study cases, abnormal variations of cellular signaling mechanisms will cause severe diseases up to mortality. Understanding the mechanisms of signaling pathways is, therefore, a primary problem in current biology (Cao et al., 2017).

2.4 Regulation of gene expression

The diversity of cell phenotypes which are produced from identical genomes is primarily due to differences in gene expression, whether between different cell types in a multicellular organism or as a result of diverse gene expression responses between different physiological situations or developmental stages (Song Li et al., 2012). The balance of existing proteins in the cell is the product of a series of complex mechanisms that produce proteins from encoded genes in DNA. Gene expression happens when DNA is transcribed into RNA and follows by RNA translation into protein. The rate of production of functional proteins in the cell is regulated at many stages of gene

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expression, primarily at the level of transcription but also at posttranscriptional levels (Stern & Orgogozo, 2008).

It is convenient to describe gene expression as a series of sequential steps, from transcription to post-translational protein modifications; however, many transcriptional and post-transcriptional mechanisms of gene expression are interdependent and simultaneous, and the extent of this interdependence is not fully understood (Orphanides & Reinberg, 2002).

2.5 Transcriptional regulation of gene expression

Initiation of transcription requires the assembly of the pre-initiation complex at the transcription start site. RNA polymerase is recruited and moves along the DNA producing an RNA transcript (elongation). Transcription is terminated, and the RNA transcript is post-transcriptionally modified. RNA polymerase II transcribes the majority of eukaryotic protein-coding genes. A rate-limiting step for transcription is the recruitment of RNA polymerase II to the core promoter. RNA polymerase II stalling, in which the polymerase pauses on the gene during transcript elongation, has been observed in vivo in mammalian cells and on Drosophila heat-shock genes. The aggregation of RNA polymerase II close to the transcription start site appears to be a widespread mechanism for stress response regulation and recovery (Orphanides & Reinberg, 2002).

2.6 Chromatin structure and chromatin remodeling

Transcriptionally active chromosomal regions have been correlated with chromatin structure and associated chromatin modifications. Transcriptional activation and repression by specific transcription factors and transcription initiation involving the general transcription factors require that proteins are bound to specific regions of DNA. Nuclear DNA in eukaryotic cells exists as highly structured chromatin, of which the basic

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structure is the nucleosome consisting of DNA wrapped around a central core of eight histone proteins (Orphanides & Reinberg, 2002).

Chromatin structure is dynamically altered by the presence of histone variants and by several covalent modifications of histone tails, including phosphorylation, methylation, and acetylation of specific residues. Histone tail modifications act dynamically and in combination to alter the local chromatin structure, either opening up regions of the chromatin or therefore permitting a higher rate of binding by transcriptional activators or condensing chromatin into a transcriptionally inactive state. For transcription to be initiated, regulatory and general transcription factors must bind to DNA in that promoter regions and around the transcription start site. Regions of open chromatin are more likely to be available for binding and therefore to be more transcriptionally active than regions of densely compacted chromatin (Reeves, 1984). Histone modifications and nucleosome positioning have been correlated with transcriptionally active and inactive chromosomal regions, such as the *S. cerevisiae*, the human and mouse cell lines, and during the human heart cell development (McDonald et al., 1999; Orphanides & Reinberg, 2002). Such correlations are consistent both with chromatin modifications causing transcriptional activation and with existing transcriptionally active regions being marked as such and promoting transcription of an already active region. In multicellular organisms, regions of chromatin are condensed to the point of being transcriptionally silent. Chromatin modifications and associated DNA methylation can be inherited across cell divisions, contributing to the maintenance of differentiated cell lines in multicellular organisms and the differential expression of genes between different cell types and disease states (Orphanides & Reinberg, 2002). Recent studies using chromosome conformation capture technology indicate that the location of chromosomal regions in the nucleus is highly predictable and is reproduced across the successive cell cycle. Given the apparent importance of relative chromosomal positions in the nucleus, a resulting hypothesis is

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that the arrangement of transcribed regions on the chromosome may be constrained by the regulation of transcription (Dekker, 2014).

2.7 Co-transcriptional and post-transcriptional regulation of gene expression

Concurrently with transcription, nascent pre-mRNA transcripts are modified by the addition of a 5' m7G cap structure, and following transcript termination, the 3' end of the transcript is polyadenylated. The 5' cap structure and 3' poly-A tail are important binding targets for proteins involved in mRNA stability, translation initiation, and nuclear export. Pre-mRNA contains introns which are removed by the spliceosome complex at consensus sequences at exon-intron boundaries to produce mature mRNA (Santos et al., 2011). Alternative splicing contributes significantly to the diversity of proteins in higher eukaryotes. Functional mature mRNA is exported from the nucleus where it is translated in association with ribosomes. mRNA transcript stability is regulated by RNA-binding proteins and small RNA molecules. Families of RNA-binding proteins, such as the (AU-rich element) ARE-binding proteins, bind specifically to mRNA sequence motifs or mRNA structure motifs. RNA-binding protein sequence motifs are often but not always positioned in the 3' untranslated region (UTR). Small interfering RNAs (siRNAs) and microRNAs (miRNAs) are two kinds of small RNAs which have been implicated in the regulation of mRNA stability and translation. Post-transcriptional regulation of gene expression is studied with an emphasis on the regulation of translation and mRNA stability (Clark et al., 2003).

2.8 The effect of the temperature on the molecular response of a eukaryotic

Temperature is a dangerous abiotic factor which affects organisms on its ecological level through infiltrating it's the molecular and cellular levels.

The environmental temperature might have many fluctuations for many creatures throughout their lives. This effect may occur yearly, seasonally, or daily and usually does

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not remain constant. There is a question to ponder on, and that is that how an organism struggles with long-term or severe temperature changes (P. W. Hochachka & G. N. Somero, 2002). Initial heat shock research investigates the mechanisms behind the response to critical heat stress by observing the heat shock in Drosophila. In this study, puffs characteristics had been studied in the salivary gland chromosomes of Drosophila (Ritossa, 1962). Later, it was understood that these studied chromosomal puffs were associated with RNA synthesis and heat shock protein expression. Since 1974 up till now, it is well known that heat shock protein family including (e.g, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small heat shock protein families) are regulated under "heat shock response" mechanisms in almost all organisms (Lindquist, 1986). The heat shock proteins are grouped by size or categorised by function. They are also famed as molecular chaperones because they are involved in protein folding. It has also been reported that they are preventing the formation of protein accumulation inside the cell (Parsell & Lindquist, 1993). Additionally, it have been reported that they have functions in ATP-independent` (small heat shock proteins) ATP-dependent (Hsp60, Hsp70) mechanisms (Fink, 1999). Initial studies revealed that the binding of the heat shock factor (HSF) to cis-regulatory heat shock element (HSE) regions induced heat shock regulatory networks. Furthermore, interaction between Hsp70 with HSF and can cause heat shock auto regulation or block it (Morimoto & Santoro, 1998).

Researchers have approved that the heat stress response is a complicated mechanism. Research has also confirmed that the heat shock response has a variation in the initiation or termination timing or in the stress intensity. It furthermore depends on different types of Hsps in different organisms. It also may comprise from the induction of many other non Hsps genes (Brokordt et al., 2015; Junprung et al., 2017; Lindquist, 1986; Madeira, 2014; Ravaux et al., 2016).

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The comparison between Drosophila and mussel *Mytilus trossulus* showed that heat in the Drosophila shock protein is synthesized during heat stress, but it will only be expressed after stress in mussel *Mytilus trossulus*. A range of HSPs proteins involved in response to heat were detected by faint SDS-PAGE bands in marine snails, yeast, and mussels. Hsps protein also maintains a fundamental influence for thermos-tolerance in yeast (Hoffmann, 2003).

Moreover, recent work on Drosophila and yeast has exposed no limitation in the binding target of the HSF to heat shock proteins. This confirmed that almost 3% of genomic loci are available as binding target (Hahn et al., 2004). The recent interest in gene and mechanisms characterizations has risen by the development of genomic tools. The accessibility to the heat shock response studies by gene expression monitoring on a wide-ranging scale by high throughput technology and quantitative PCR has increased (Birch-Machin et al., 2005). Post research has shown that the level of change in gene and protein expression are not essentially correlated. Recent researches have confirmed that by some protein regulations. For instance (e.g., elongation factors) showed that these proteins are expressed at the translational level, but not in protein level or vice versa. This means that an increased in this gene expression generally does correlate with an increase in their protein expression (Suzuki et al., 2006). Moreover, additional research on Drosophila confirmed a parallel linkage between genomic and metabolomic profiles resulting in heat stress.

Previously, most studies had focused on simple modeled organisms such as prokaryotes, Drosophila, and yeast. Recent studies; however, focused on the heat shock response in higher organisms (Hoffmann, 2003). Heat stress can cause many types of changes, fluctuations or variations in higher organisms or animals?

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Higher aquatic organisms that are living in an aquatic habitat are widely exposed to a range of biophysical challenges. They are widely exposed to temperature fluctuations. These creatures may counteract these challenges by increasing or decreasing their body temperatures (Hofmann et al., 2005).

A noteworthy example is the *T. funerals* which was monitored in a study for 26 days from March-April. Nearly after half of the 26 days, the water temperature was midday low, but the body temperature was 27℃. Its body temperature was high enough to induce Hsp70 and Hsp90 gene expressions (Tomanek & Somero, 1999).

Fascinatingly, in the similar heat stress condition, before midday (2.5h at 30℃), Hsp70, Hsp38, Hsp90, and Hsp77 expression was increased and after 6h, 30 min., 6h, and 14h it dropped to normal levels, this showed that *T.funebrails* can tolerate the stress response during the two low-temperature waves (Tomanek & Somero, 1999). In addition, the up-regulated HSPs levels that contribute to survival with reversible protein denaturation also exist following heat stress in the studied area. Reversible protein denaturation is the mechanism that handle the reversible denaturation through protein degradation (Hofmann et al., 2005).

There are some studies on the recovery from temperature fluctuation of intertidal specious such as mussel *M. trossulus* and crab, *Petrolisthes cinctipes*. Studies on the heat shock response in these species have confirmed the immersion of Hsp70 and Hsp90 in reversible protein denaturation (Stillman, 2003). In this stage, a question arises that, what other mechanisms or genes either than Hsps family genes might be up or down-regulated in response to the thermal stress? And how do these changes may vary over time? And what is the immune system response?

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2.9 Innate immune system in invertebrates

Invertebrates and other arthropods share similar defense responses without the presence of immunoglobulins. Despite the absence of antigen-antibody specificity, the innate system possesses good identification and responds swiftly to incapacitate and eradicate pathogens. Primarily, the exoskeleton that shields the body structure is a natural physical barrier towards many types of pathogenic microorganisms (Johnson et al., 2012). The presence of the cuticle serves as a lining for the foregut and hindgut of a prawn and its body surface. Moreover, gills could allow any exchange due to a lack of epicuticle; whereas a gut present without an outer lipid layer could allow the permeability. Crustaceans are enclosed with an open circulatory system that transports oxygen, hormones, nutrients, and cells via the hemolymph (Vazquez et al., 2009). The association of humoral and cellular defense mechanisms is observed during the hemocyte flow and helps integration with plasma cells. Humoral defense system involves the production and release of lectins, antimicrobial peptides (AMP) and prophenoloxidases (proPO) while cellular defense system includes hemocyte mediated responses for encapsulation and phagocytosis. The circulating fluids known as crustacean hemolymph or blood comprise of hemocytes that include fluids or plasma. The defense mechanism is stimulated based on diverse arrangements of pathogen characteristics which are on the surface level and involved in various immune responses. Upon the intrusion of pathogens into the tissue, proteolytic pathways will be activated immediately in order to exterminate the pathogens (Ratcliffe et al., 1985). Firstly, it will be recognized and various effector cascades will be triggered such as antimicrobial proteins (Underhill & Ozinsky, 2002), clotting proteins (Morita et al., 1985), encapsulation and phagocytosis (Bogdan et al., 2000). The proPO cascade could help activate other immune cascades such as the assembly of melanization, phagocytosis, encapsulation and nodule formation that are mediated through pathogen-associated molecular patterns (PAMPs) by crustacean proteins. The main question is that

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how, when and why a specific immune defense mechanism get chosen in crustaceans among Pattern recognition receptors (PRRs), or pattern recognition proteins (PRPs), Apoptosis pathway, Melanization, Pro-PO activating system, Plasma Clotting protein, and Lectin Pathway (Akira et al., 2006). In crustaceans, the physiological barricade is the first hindrance to any invading particles or pathogenic micro-organisms. When any damage, particles or pathogenic micro-organisms are recognized, proteolytic pathways immediately become active (Roth et al., 2009). This instantly activated pathway will eliminate the invaded particles, the second hindrance is when, and the coagulation cascade gets activated in order to avoid the loss of hemolymph. Then the proPO system will join the immune arena by stimulation of oxidative metabolites and melanin production. Prophenoloxidase is the most effective protein. Its activation will stimulate other important procedures in the immune response. Phagocytosis, encapsulation and nodule formation will be activated by prophenoloxidase incitement (Labbe & Little, 2009; Maliwat et al., 2017).

2.9.1 Pattern recognition receptors (PRRs)

In this innate immune system mechanism, the invading microorganism would be recognized by germline-encoded PRRs (Akira et al., 2006). The pattern recognition receptors will bind to the first available pathogen-associated molecular patterns (PAMPs) on the surface of the microorganism. Each PRRs specifically recognizes specific antimicrobial peptides AMPs and activates signaling pathways of the immune responses. It will activate phagocytosis, encapsulation, nodule formation depending on the PAMPs or may activate AMPs synthesis (Medzhitov & Janeway Jr, 2000), (Figure 2.1).

2.9.2 Antimicrobial peptide (APMs)

AMPs could neutralize and/or destroy various types of invading pathogens including Gram-positive, Gram-negative, fungal, viral and protozoal. It is reported in numerous

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species ranging from plants, invertebrates to vertebrates. It is present as a small cationic protein, functioning as an immunomodalator, which has features of antimicrobial response and enhanced immunity. It was also best described as a candidate for the development of novel antibiotics and also in the development of therapeutic agents. In shrimp, numerous types of AMPs have been characterized such as crustins, anti-lipopolysaccharide factors penaeidins, hemocyanin, histones, and many more. The two well-known AMP is well described in *M. rosenbergii* which is known as cousin and anti-lipopolysaccharide factors (Rosa & Barracco, 2010).

Crustin is one of the antimicrobial peptides that is classified as a cationic cysteine-rich antibacterial polypeptide in crustaceans. Crustin which is the homolog of 'caring' was first identified in the shore crab, *Carcinus maenas* as an 11.5kDa peptide (Relf et al., 1999). Crustins were isolated from a broad range of crustaceans which include *Litopenaeus vannamei*, *Litopenaues setiferus* (Bartlett et al., 2002), *Panulirus argu* (Stoss et al., 2004), *Penaues monodon* (Supungul et al., 2004), *Marsupenaeus japonicas* (Rattanachai et al., 2004), *Homarus gammarus* (Hauton et al., 2006), *Fenneropenaeus chinensis* (Yi-Chen et al., 2007), *Carcinus maenas* (Brockton et al., 2007), *Pacifastacus leniusculus* (Pikul Jiravanichpaisal et al., 2007), *Litopenaeus schmitti*, *Farfantepenaeus brasiliensis* (Rosa et al., 2007), *Farfantepenaeus subtilis* (Rosa et al., 2007), *Fenneropenaeus indicus* (Antony et al., 2011), *Macrobrachium rosenbergii* (Arockiaraj et al., 2013).

The term crustin was first coined to define the mRNA transcript found in *L. vannamei* and *L. setiferus* (Bartlett et al., 2002). Crustins are cationic, cysteine-rich AMPs with an isoelectric point ranging from 7.0 to 8.7 with a molecular weight ranging between 7 and 14 kDa with one whey acidic protein (WAP) domain at the carboxyl terminus (Bartlett et al., 2002). This domain consists of tightly packed 8 cysteine residues

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in a conserved architecture which is known as a four-disulfide core (4 DSC). The WAP domain containing proteins are widely distributed from invertebrates to vertebrates. The term 'WAP' is given to a family of proteins that are derived from the whey fraction of mammalian milk, and it comprises of two WAP domains with 50 amino acids (Subramanian et al., 1999). The WAP domain in mammals includes trapping, elafin, and antileukoproteinase. It is one of the conserved features among many species and few crustins mainly in shrimp (Bartlett et al., 2002).

The molecular assemblies between the signal sequence and WAP domain vary in crustin, yet it is substantiated by its unique molecular architecture. The cysteine rich region lies in between the signal sequence (N-terminus) and the WAP domain (C-terminus) for Type I crustins which are present in crabs (Brockton et al., 2007) crayfish (Pikul Jiravanichpaisal et al., 2007), shrimp (Qin et al., 2010), freshwater prawn (ZhaoZhiYing et al., 2009) and lobsters (Christie et al., 2007) and other crustins in the Pleocyemata family. This type of crustin was categorized under Type I since it resembles similar domain organizations as a carcinogen (Relf et al., 1999). Likewise, Type II crustin exists with both long glycine-rich and cysteine regions within the signal sequence (N-terminus) and a WAP domain (C-terminus) which are present mainly in crayfish and shrimp. The Type III crustin is also known as a single whey domain (SWD), chelonianin-like protein or antileukoproteinase-like protein. It is a peptide that lacks both cysteine and glycine-rich regions, but it is substituted with proline or arginine region between the signal sequence and the WAP domain. So far only a few Type III crustins were identified in crayfish (Pikul Jiravanichpaisal et al., 2007) and shrimp (Rattanachai et al., 2004).

M. rosenbergii's crustin carries a signal sequence and WAP domain along with proline and arginine-rich regions. It was also classified as a type III crustin. It also has 12 conserved cysteine residues that were previously observed in many other crustins as well

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as antimicrobial peptides like a carcinogen (Amparyup et al., 2008). The conserved residues were predicted to form six disulphide bonds in the tertiary structure. This WAP domain shows a wide range of functions including antiproteinase and antimicrobial activities. Moreover, the amino acid residue of protease inhibitors, methionine, which is located near to the second cysteine in WAP domain is replaced by cationic and hydrophobic amino acids in crustin's domain. This alteration turns the protein to be amphipathic and permits insertion of the protein into the outer layer of microbes (Antony et al., 2011).

Crustin as an AMP effector was reported to carry antibacterial activities especially in Gram-positive bacteria. *M. rosenbergii's* crustin could exhibit its response towards *infectious hypodermal and hematopoietic necrosis virus* (IHHNV) and *white spot syndrome virus* (WSSV) and the gram-negative bacteria *Aeromonas hydrophila* and *Enterococcus faecium* (Gram-positive). The recombinant crustin also shows a great response towards both Gram positive and Gram negative bacteria by distinguishing the pathogens (Arockiaraj et al., 2013). The first identified crustin from *C. maenas* has the ability to encounter the growth of Gram-positive bacteria that include *Aerococcus viridans var tomato*, two strains of marine *Planococcus* spp. and a salt-tolerant strain of *Micrococcus luteus* (Relf et al., 1999). The recombinant crustin of *F. chinesis* also aims Gram-positive bacteria that include *Staphylococcus aureus*, *M. luteus* and 3 other bacilli (Yi-Chen et al., 2007). One of the recombinant crustins of *P. monodon* highly inhibits the activity of *Streptococcus iniae* and *S. aureus,* but not towards *A. viridans var homari* and *M. luteus* (Amparyup et al., 2008). Nevertheless, the Type II crustin identified from *P. monodon* indicates robust antibacterial activity against both Gram-positive and Gram-negative bacteria that include *A. viridans var homari*, *Escherichia coli 363* and *Vibrio harveyi*.

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On the other hand, anti-lipopolysaccharides factor (ALF) is one of the important conserved AMP found in crustaceans. Initially, ALF was known as anti-lipopolysaccharide LPS factor as it was recognized as an inhibitor factor of LPS-mediated hemolymph coagulation in *Limulus polyphemus* and *Tachypleus tridentatus* (Morita et al., 1985). ALF was further identified and cloned in many other crustacean species such as *Litopenaues setiferus* (Bartlett et al., 2002; Supungul et al., 2002) , *Penaeus monodon* (Supungul et al., 2002), *Macrobrachium olfersi* (Barbieri et al., 2016), *Farfantepenaeus paulensis* and *Litopenaeus schmitti* (Capparelli et al., 2012), *Macrobrachium rosenbergii* (Arockiaraj et al., 2012a), *Macrobrachium nipponense* (Tzeng et al., 2015), *Fenneropenaeus chinesis* (Yi-Chen et al., 2007), *Litopenaeus vannamei* (Wang et al., 2011), *Marsupenaeus japonicas* (Nagoshi et al., 2006), *Pacifastacus leniusculus* (H. Liu et al., 2006), and *Scylla paramamosain* (Imjongjirak et al., 2007), *Eriocheir sinensis* (ZhaoDaxian et al., 2009), *Scylla serrata* (Ko et al., 2007), *Procambarus clarkii* (Li et al., 2009) and *Portunus trituberculatus* (Yue et al., 2010).

Moreover, multiple isoforms have also been found in the many other types of crustacean species. To date, seven forms of ALF genes were identified from *M. rosenbergii* (Arockiaraj et al., 2011). Five forms of ALF genes were reported from the two genomic loci from *P. monodon* that are actively involved in defense mechanisms against various types of pathogen infections (Antony et al., 2011). *Portunus trituberculatus* has four types of ALF genes (Yue et al., 2010).

ALF is classified as a single domain AMP that has an LPS domain with a signal peptide. The LPS has two conserved cysteine residues that form the disulfide bonds. The deduced ALF from *M. rosenbergii* shows the conserved structure of the signal peptide, LPS binding domain and two cysteine residues (Arockiaraj et al., 2014). According to (Morita et al., 1985), the second and third sheet of β-sheet is enveloped by the LPS domain

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along with the four antiparallel β-sheets. The structure of *Macrobrachium rosenbergii* showed 5 α-helices and 4 β-sheet along with two conserved cysteine residues in β-sheet that forms disulfide bonds in a hairpin loop (Conserva & Jesu Costa Ferreira, 2012). This similar pattern was also observed in ALF from *E. sinensis* (Zhang et al., 2011*)*. The pI value of ALF and LPS binding domain of *M. rosenbergii* falls in a range of 5.00 to 10.00 among crustaceans (Ren et al., 2012) . Basically, ALF is a basic protein that is responsible for binding and neutralizing LPS. The basic ALF shows antibacterial activity, while the acidic ALF may show antiviral or antifungal activity. Similar results were obtained for *E. chinesis*, *M. japonicas*, *Litopenaeus stylirostris* (Yang et al., 2010).

ALFs were reported to be involved in immune defense mechanisms in a wide range of pathogenic infections such as those including bacteria, virus, fungi or yeast. Generally, ALF can bind to the liposaccharides (LPS) on the pathogens (Imjongjirak et al., 2007). *Mr*ALF shows its response against white spot syndrome baculovirus (WSBV), white spot syndrome virus (WSSV), *Aeromonas hydrophila*, *Escherichia coli*, *Vibrio anguillarum*, and *Staphylococcus aureus*. It is reported that the cationic and hydrophobic properties of AMPs allow it's interaction and insertion into the anionic cell walls and phospholipid membranes of the pathogens and ultimately leads to a antimicrobial action (Morita et al., 1985). The ALF from *Portunus trituberculatus* increases upon an infective challenge from *Vibrio alginolyticus*. In *Scylla paramamosain* an ALF had demonstrated antimicrobial activity against *Vibrio* (Imjongjirak et al., 2007). While, the ALF in *Pacifastacus leniusculus* shows antiviral activity upon an attack from the white spot syndrome virus (WSSV). The ALF from *Penaues monodon* was shown to be up-regulated upon an interactive challenge from *Vibrio harveyi* challenge (Tharntada et al., 2008) .

The functional property of ALF was indicated by recombinant ALF with LPS domain through various types of Gram-negative and Gram-positive bacteria (Zhang et al., 2011).

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The recombinant ALF from *M. rosenbergii* could show antibacterial activity towards *Aeromonas hydrophila*, *Vibrio cholera,* and *Escherichia coli*. Moreover, it could also bind to both bacterial types that were studied including LPS of *E. coli*, and lipoteichoic acids of *B. subtilis* and *S. faecalis*. Meanwhile, reported that *Mr*ALF shows a high antimicrobial response to almost more than five types of bacteria which were tested. Moreover, the bactericidal test after 24 hours also indicates that *Mr*ALF could kill all the bacteria (Arockiaraj et al., 2014). A similar type of activity was also shown by recombinant ALF protein from *Scylla paramamosain*, *Penaeus monodon* and *Pacifastacus leniusculus*. The recombinant ALF from *Penaeus monodon* has successfully decreased the replication of WSSV. This indicates the participation of *Mr*ALF as a part of the defense mechanism during a pathogen attack (Somboonwiwat et al., 2008).

2.9.3 Pro-PO activating system

The prophenoloxidase protein is the active form of pro-Po system and is the main domain protein of Pro-PO activating system. Pro-PO is synthesized and localized in crustacean's granulitic hemocyte. Then it triggers the pattern recognition protein and migrates to plasma by exocytosis (Amparyup et al., 2013). The pro-Po system contains several protein complexes that will participate in melanin formation, cytotoxic reaction, cell adhesion, encapsulation, Prophenoloxidase, andphagocytosis (Söderhäll & Cerenius, 1998). Each crustacean species has a specific composition of the zymogens of either the protease cascade, pattern recognition proteins or serine protease and pro-PO. The complex of pro-PO and LPS will bind to the microbial cell wall components and induce activation of serine protease zymogens in the pro-PO system (Amparyup et al., 2013). The most triggering molecule to pro-Po is the LPS binding protein which is found in the crayfish pro-PO system. In an in vitro study, the cleavage of pro-PO activating serine proteases showed antimicrobial activities which suggest that pro-PO activation with serine protease may have a dual function (Gupta et al., 2005).

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2.9.4 Prophenoloxidase Pathway

A humoral response that plays a key role in antigen recognition and neutralization in a wide range of host models is known as prophenoloxidase (proPO) (Cerenius et al., 2008). The proPO system was also identified as a center of the defense mechanism characterized in many organisms including arthropods, ascidians, cephalochordate, molluscs, and annelids (Theopold et al., 2004). A segment of a complex enzyme cascade could activate PO along with Ca^{2+} ions that oxidize phenols into quinone and further catalyzes it into melanin cascade for encapsulation, wound healing and for antimicrobial activity (Arockiaraj et al., 2012b). Various types of microorganisms or parasites may activate the proPO cascade, especially the large size pathogen. This type of pathogen encapsulation and further melanization appears on the host system by the action of phenoloxidase. The proPO involves mainly in defense reactions, wound healing and cuticular hardening processes. The activation system of proPO can also be stimulated by very low quantities of microbial cell components such as β-1,3-glucans, lipopolysaccharide (LPS), and peptidoglycan (PG). These intruders could indirectly or directly induce the granulocytes via enzyme activating cascades to produce phenoloxidase (PO) (Arockiaraj et al., 2012b).

The first cloned and purified proPO was from *Pacifastacus leniusculus*, a freshwater signal crayfish (Aspan et al., 1995). Following that many other proPO genes were isolated and characterized from a wide range of species including *Drosophila melanogaster* (Fujimoto et al., 1995), *Manduca sexta* (Hall et al., 1995), *Anopheles gambiae* (Christophides et al., 2002), *Scylla serrata* (Ko et al., 2007), *Anopheles stephensi* (Cui et al., 2000), *Aedes aegypti* (Taft et al., 2001), *Bombyx mori* (Asano et al., 2001), *Armigeres subalbatus* (Huang et al., 2001), *Litopenaeus vannamei* (Liu et al., 2004), *Penaeus monodon* (Nayak et al., 2010), *Homarus Gammarus* (Hauton et al., 2006), *Macrobrachium rosenbergii* (Arockiaraj et al., 2012b).

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Upon infection, one of the major enzymes that are stimulated is PO via the proPO activation system. This takes place upon stimulation by components of the pathogen-associated molecular pattern (PAMP). The activation of proPO cascade may involve serine protease breaks, to trigger the zymogen into active PO. The PO is a copper-dependent enzyme that acts as a terminal enzyme with two functions. PO can catalyze monophenols into o-hydroxylation (cresolase activity) and oxidation of phenols into quinones (Nellaiappan & Sugumaran, 1996). Therefore, PO can convert tyrosine to dihydroxyphenylalanine (DOPA) and DOPA to DOPA-quinone along with non-specific pathways between neighboring molecules for melanin formation. Moreover, the PO is also able to regulate the negative stimulus to the host through reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (Cerenius & Söderhäll, 2004; Ko et al., 2007).

The first *Mr*proPO (*Mr*proPO1) was cloned by with six histidine residues and a thiol ester like motif. This peptide has similar structural formation as *Penaues monodon*, *P. leniusculus,* and *H. gammarus*. Furthermore, also had reported proPO (MrproPO2) from *M. rosenbergii* with six histidine residues within two copper binding sites, thiol esters like motif, a proteolytic activation site, and a conserved c-terminal region (Lu et al., 2006). The proPO activating enzyme III (*Mr*proPO3) is a conserved copper-containing enzyme that was isolated and characterized in *M. rosenbergii*. The amino acid sequence has a characteristic feature of the clip-serine protease (clip-SP) that comprises of histidine active sites at N- and C- terminal, a signal peptide and a domain from serine proteases trypsin family (Arockiaraj et al., 2012b). This clip also shows a conserved catalytic amino acid that is important for the stabilization of the catalytic site in the three-dimensional structure. The clip-SP is an important molecule that plays important roles in immune regulation and embryonic development. This sequence characteristic was also found in another proPO activating enzyme such as *C. sapidus* and *P. monodon*. In addition, another

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*Mr*proPO (*Mr*proPO4) was also found by with two copper binding sites (CuA and CuB) that have hemocyanin features, a signal sequence that is important in secretion at site of the endoplasmic reticulum and a thiol-ester like motif. Hemocyanin has a wide range of physiological processes such as osmoregulation, molt cycle, exoskeleton arrangement and protein storage. Similar structural study was also reported in *Manduca sexta* (Y. Li et al., 2009). The tertiary structure also indicates tyrosine signature that is postulated to bind to proPO and activate the melanin synthesis (Y. Li et al., 2009). In addition, the copper binding sites with conserved histidine residues were found in most insects and crustaceans that are responsible for melanization, encapsulation and sclerotization (Hughes & Austin, 1999).

*Mr*proPO1 gene expression and PO activity were measured using CpG oligodeoxynucleotide. This has clearly shown that the proPO system can be activated through CpG oligodeoxynucleotide via protein kinase C signaling pathway. This supports their previous study as the challenge was carried out with *Aeromonas veronii* and *Lactococcus garvieae*. The proPO system can be triggered by the stimulation of bacterial challenge, bacterin or oligodeoxynucleotide stimulation. Meanwhile, the expression of *Mr*proPO2 was studied based on five different molting stages of *M. rosenbergii* The gene expression of proPO in the postmoult stage showed a gradual increase and further reached the higher expression levels during the later stage of the postmoult stage (Lu et al., 2006). The proPO expression declined sharply in the intermoult stage, and the lowest level was observed in the premoult stage. Therefore, it was concluded to be an immunomodulatory system. *Mr*proPO3 shows its responses against IHHNV while *Mr*proPO4 shows responses towards a wide range of pathogens including WSBV, *Mr*NV, *A. hydrophila* and *V. Harvey*. This indicates that *Mr*proPO is actively involved in the defense mechanism of innate immunity in an invertebrate (Arockiaraj et al., 2012a). The PO activity of *Mr*proPO3 is not similar to gene expression study that might be because of its

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complex activity at different phases of the host-pathogen interaction. *Mr*proPO4 shows a robust association between gene expression and enzymatic activity that might be because of the involvement of various phenoloxidases. A similar mechanism was also suggested by (Adachi et al., 2003).

2.9.5 Melanization Pathway

Melanin is synthesized by the proPO-activating system and is an enzymatic cascade which is involved in several enzymes (Gorman et al., 2008). This protein is the main substance for melanization which an important immune defense pathway in crustaceans is. The melanization is a prominent immune response which involves the synthesis of melanin to encapsulate pathogens. The process of melanization is a complex process which starts from proPO-activating system binding to microbial cell wall components. This complex will activate the stimulation of the SP cascade which promotes proteolysis to convert proPO into active PO. To date, several genes associated with the shrimp pro-PO activating system are identified and characterized (Jang et al., 2011).

2.9.6 Lectin Pathway

Invertebrates have a well-developed defense mechanism to detect and exterminate any opportunistic microorganisms or potential pathogens. Anciently diverse conserved molecules establish their non-self recognition system through pathogen-associated molecular patterns (PAMPs). In response to this architecture, a family of proteins that were termed as pattern recognition proteins (PRPs) will be exerted by the immune system to initiate the downstream mechanisms. A well-studied protein, the lectin is a functional precursor of antibodies which recognizes the signature molecules of non-self-particles via PAMP (Kilpatrick, 2002). Lectins are organized in a broad spectrum of protein units, and every architecture shows different molecular mass depending on the species and the molecular assemblies. Lectins are proteins or glycoproteins. They normally do not have

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catalytic activity. They are mostly recognized due to the absence of covalent binding with sugar moities. Thus, they agglutinate cells by binding to glycoproteins or glycoconjugates to the cell surface (Agundis et al., 2000). Therefore, they are considered as significant pattern recognition proteins in innate immunity of the crustaceans. They also play important roles in non-self-recognition and clearance of invading microorganisms. They are existing in circulating fluids, either as cell surface receptors or soluble proteins (Marques & Barracco, 2000). Different types of lectins have been diagnosed in a crustacean. Lectin1, lectin2, lectin3, l3ctin4 and C-type lectins are the most recognized lectins in crustaceans. C-type lectins are the largest group of immune function that is mostly found in crustacean hepatopancreas (Zhang et al., 2011). Many lectins have been purified and characterized from crustacean hemolymph. In crustaceans, lectins have been reported to contribute to innate immune responses, including prophenoloxidase activation, encapsulation1.nodule formation of hemocyte, opsonin formation, antibacterial activity, and antifungal activity and may also be contributing to injury healing (Smith et al., 2003).

It is reported that lectin serves a decisive role for both physiological and pathological processes with exclusive interactions between intricate carbohydrates that include glycoproteins, glycolipids, polysaccharides or proteoglycans (Vázquez-Mendoza et al., 2013). Likewise, some lectins respond only to certain types of carbohydrate forms that encompass the whole sugar molecule, a portion of the molecule or the glycosidic linkages (Lis & Sharon, 1998). Generally, lectins are described to be activated based on the conserved sugar binding activity towards the N- or O-acetylated sugar residues, such as N-acetyl- glucosamine (GlcNAc), N-acetyl- galactosamine (GalNAc), N-acetyl-neuraminic acid, or O-acetylated sialic acid in decapod (Vazquez et al., 2009). Several activities take place during these multifaceted interactions such as the embryonic development, intracellular trafficking, cell-cell and cell-matrix recognition, cell homing,

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protein synthesis and transport, signal transduction, endocytosis, phagocytosis and inflammation (McGreal et al., 2004).

Up till today, many lectins are recognized and studied in their molecular configuration. As non-self- recognition molecules, lectins show a high abundance of activity in domain assemblies, sugar substrates, tissue distribution, and even expression upon being challenged by pathogen in order to describe the potential functional roles in immunological assays. Lectins are highly heterogeneous molecules that are categorized based on their structures, motifs, and functions. The motifs are known as carbohydrate recognition domains (CRD) and show the disparities between the structures. The Animal Lectins Homepage (http://www.imperial.ac.uk/ research/animal lectins/default) declared that animal lectins are termed into 13 different groups namely the C-type, F-type, M-type, L-type, P-type, I-type, R-type, F-box lectins, chitinase-like lectins, ficolins, calnexin, galectins, and intelectins. The intracellular lectins were reported in protein sorting and trafficking whereas extracellular lectins mainly were shown to function in cell signaling and pathogen recognition (Kilpatrick, 2002). Even though repertoires of lectin show a large divergence, almost 7 types of lectins were identified in the GenBank that are known as C-type, M-type, L-type, P-type, fibrinogen-like domain lectins, galectins and calnexin/calreticulin. Sequence distinction in CRD indicates diverse interactions between sugars that are known as mannose-binding lectins, fucose binding lectins, rhamnose binding lectins, galactose-binding lectins, GlcNAc-specific lectin, and GalNAc-specific lectins. C-type and mannose-binding lectins were discovered in *M. rosenbergii*.

C-type lectin is one of the proteins that is well studied in many invertebrates and constitutes common structural features such as a carbohydrate recognition domain (CRD), disulfide bond sites and calcium binding sites. The CTL reported in *M. rosenbergii* has a single or double CRD, a signal peptide that may act like a secretory

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protein that participates in transporting mature proteins outside the cell, a cysteine residue that forms disulfide bridges, and calcium binding sites. The CRD structure is composed of a double loop composed of four conserved cysteine residues and stabilized by two disulfide bridges. However, some CTL is present without signal peptides that may act as receptors or calcium-independent binding sites. The position of the hydrogen bond from the donor and acceptors binding sites on Ca2+ regulates the binding of carbohydrate. The CRD fold is formed by a double loop structure that is stabilized by two conserved disulfide bridges along with hydrophobic and polar interactions. CRD that has a Glu-Pro-Asn (EPN) motif that binds to mannose whereas CRD that bears at Gln-Pro-Asp (QPD) motif binds to galactose. The Trp-Asn-Asp (WND) motif shows a connection with monosaccharide residues in the presence of the Ca2+ binding site. This variation shows CRD binding capability towards different sugar moieties on invading pathogens (Zelensky & Gready, 2005).

CTL found in higher invertebrates has diverse functionality, and it is also highly conserved in vertebrates. Many types of CTL's were found in *Drosophila melanogaster*, *Caenorhabditis elegans*, also in *Manduca sexta* that help in activation of other immune-related genes likely prophenoloxidase, encapsulation, and melanization. CTL is also found in many different penaeid shrimp that carry antimicrobial activities with a single CRD, whereas the double CRD may act as a PRR via binding to bacteria for further neutralization. The knockdown of CTL *Mr's* CTL knockdown shows its association with the activation of other types of innate immune systems such as anti-lipopolysaccharide factor (ALF) and lysosomes. It has also been shown that CTL can initiate multiple signal transduction cascades upon being challenged by a pathogen resulting in the stimulation of other immune molecules (Antony et al., 2011).

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CTL has even shown its robust activity towards many types of pathogens like bacteria and viruses. *M. rosenbergii* exhibits agglutination activity towards both Gram positive and Gram-negative bacteria which indicates the possible association of lectin and carbohydrate components on the surface of pathogens. Mannose-binding lectin that was categorized as an AMP was also found in *M. rosenbergii*. Many mannose-binding lectins were identified and characterized, ranging from plants to vertebrates, to invertebrates (Marques & Barracco, 2000). Insects and shrimp which indicate the evolution and diversity of the mannose-binding lectin gene. *Mr.* MBL is also predicted to have a signal peptide region, a CRD domain, a cysteine region and a calcium binding site similar to CTL. These cysteine residues are highly conserved and important in forming disulfide bonds. It even acts as a calcium-dependent lectin with four calcium binding sites and is composed of highly conserved 'specific carbohydrate recognition motifs' at the Tyr-Ser-Asn (YSN) and Gly-Asp- Leu (GDL) sites. The tertiary structure of this molecule is composed of α-helix, coil regions, and β-sheets (Vázquez-Mendoza et al., 2013).

Antimicrobial activities of MBL were also observed in response to different bacteria such as *A. hydrophila, E. coli*, *V. parahaemolyticus, V. alginolyticus, V. harveyi, B. subtilis, B. licheniformis, B. coagulans, S. pyogenes, M. luteus and L. monocytogenes* (Rosa & Barracco, 2010). Among which, the Gram-negative bacteria show a better binding tendency compared to Gram-positive bacteria which may be due to the binding capacity of the mannose and the carbohydrate from both bacteria. A similar result was observed in Mr since LPS is the main pathogen associated with molecular patterns in Gram-negative bacteria (Tharntada et al., 2008). Gene expression studies show that mannose-binding lectin can be regulated upon WSSV, MrNV, A. hydrophila and V. harveyi infection (Tharntada et al., 2009). Previous reports regarding Mr and Fc show similar results for the lectin response of *M. rosenbergii* and *Fenneropenaues chinesis* upon being challenged by Vibrio. Moreover, the bactericidal efficiency test shows the

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possibility of efficiently eradiating viral and bacterial colonies. This shows that MrMBL also takes part in the defense mechanism for pathogen eradication.

2.9.7 Apoptosis Pathway

Apoptosis is genetically controlled by programmed cell death. This pathway will eliminate harmful cells (such as a viral cell) and damaged cells. This pathway is highly critical in some normal processes such as the development of the embryo, metamorphosis, and most importantly in immune defense (Wen et al., 2012). Apoptosis plays a key role in the immune defense of crustacean against viral attacks. Many viruses during viral infections, exhibit various strategies and utilize to inhibit apoptosis in host cells. The virus will try to delay the host cell's capabilities until it has produced sufficient viral offspring (Wang et al., 2008). Viruses generally try to inhibit apoptosis, but some exceptional viruses try to utilize apoptosis in order to spread their viral progeny to other host cells. The most important molecule which mediate apoptosis is caspase (Elmore, 2007). Caspase overexpression was diagnosed in *M,japonicas* under WSSV infection (Leu & Lo, 2011).

2.9.8 Plasma clotting protein

Blood loose prevention is an essential immune process in case of injury. This mechanism is variant in different crustacean species. Lipoprotein is another clotting protein which is found in freshwater crayfish. Each subunit of lipoprotein has lysine and glutamine side chain. Then the injured tissue or hemocyte will release transglutaminase which will cause the chains to covalently cross-link to each other. All of these reactions will happen in the presence of Ca2+ as shown in (Figure 2.1).

In some species such as crayfish, the hemolymph clotting is based on the direct transglutaminase-mediated cross-linkage of specific plasma proteins. These proteina are ahomologous to the female-specific protein vitellogenin (Hall et al., 1999). An interesting

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fact about the clotting protein in both sexes which constitutes a separate group of clotting factors is its sequence similarity with the female-specific protein, vitellogenin, Therefore, crustacean clotting proteins are evolutionarily related to vitellogenins, but they have entirely different functions and are expressed in both sexes. So, they should not be considered as true vitellogenins (Lee et al., 2008).

In conclusion, the innate immune system is the primordial immune system which has been evolving up until today in every living system. According to a recent study, crustaceans were classified as the ancestors of insects in the group of invertebrates since they could resist and survive wide range of environmental effects. Hence, *M. rosenbergii* which possesses innate immunity as one of its defense systems was discussed in this review. To get a virtuous view of an important defense mechanism of *M. rosenbergii* from the context of a humoral peptide, this review focused mainly on important antimicrobial peptides, lectin, and prophenoloxidase that are being studied to date. The discovery of these genes in *M. rosenbergii* could help further research in applications to develop natural antibiotics or therapeutic agents which could further ensure healthy prawn cultivation and ensure food security for sustainable human consumption (Sharabi et al., 2013).

2.10 Regulation of genome-wide gene expression under temperature perturbation

Among physiological changes, temperature has been one of the fundamental influences in the history of living organisms. Even a small change in temperature will alter the growth of different microbial species. The physiological effects of temperature involve cell cycle progression, metabolic activity, cell wall and membrane dynamics, protein aggregation, and sequestration. For example, in the budding yeast, the temperature increase may lead to a transient arrest in the G1 phase, reduced metabolic

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activity, and protein misfolding. In addition to physiological effects, heat shock evokes a signaling cascade that activates a transcriptional stress respond.

Temperature change disrupts the normal protein folding process and causes the accumulation of denatured proteins, which in turn activates two independent stress responses. First, the heat shock transcription factor Hsfl induces the transcription of downstream heat shock proteins to block the expression of cyclins and result in transient arrest. Hsfl target genes contain multiple copies of a 5-bp sequence of nGAAn called "heat shock elements," or HSEs. Simultaneously, the general stress response Msn2/4 mediates the transcription of genes with "stress responsive elements" (STREs), 5-nucleotide sequences of CCCCT or AGGGG. The Msn2/4 complex has two functional proteins, Msn2 and Msn4, in which Msn4 expression is stress induced. Msn2/4 is a more general stress response that responds to a variety of stresses in addition to temperature increase.

Nevertheless, the findings of Gasch et al. have also revealed that transient expression responses disappeared when the indication of stress was extended. Moreover, the steady-state expression at the heat shock temperature resembled the steady-state expression at the normal temperature rather than the transient expression at the heat shock temperature. Whereas the transient programmed responses represent the necessary protective mechanisms, the steady-state programmed responses allow cells to rearrange their regulatory systems and continue their growth. Therefore, understanding homeostatic programmed responses is necessary to examine how biological systems are modulated by environmental signals in order to promote progressive changes in developmental programs such as the cell division cycle.

In eukaryotes, transcription regulation is modulated by the combinatorial effects of the structural state of DNA (i.e., chromatin formation) and regulatory proteins called

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transcription factors. The DNA structural state regulates transcription by limiting the accessibility of transcriptional machinery to the promoter regions, representing global transcriptional regulation. Transcription factors bind to specific DNA sequences in the promoter regions and activate the transcription of target genes, representing specific transcriptional regulation. The combination of global and specific transcriptional regulation constitutes a coordinated regulatory network that dynamically governs cellular functions. In unicellular eukaryotes such as the budding yeast, the cell-division cycle process involves a network of ~800 genes; only small numbers of key regulators are responsible for the control of this complex process. While the transient response to heat shock stress involves two specific regulatory pathways (Hsf1 and Msn2/4), the steady-state response likely relies on global transcriptional regulation to drive cellular functions under temperature perturbations. Theoretical studies on the yeast cell-division-cycle network claimed that such networks are stable and robust against perturbations to biological systems. Two recent experimental-computational studies using the network modeling of gene expression have revealed that global transcriptional machinery is likely the main coordinator of gene expression during cell growth transitions. Experimental studies on fission yeast also indicated the presence of a coordinated global regulation of transcription that controls cell growth. Preliminary evidence; therefore, supports the fact that global transcriptional regulation plays a key role in guiding level programs to guide developmental programs, and specific transcriptional regulation plays a complementary role as a protective mechanism against environmental perturbation.

2.11 Environmental categorization, homeostasis, and regulatory mechanisms

The area surrounding any organism is called its environment. The environment has a lot of different categories in each field of science. In molecular science; scientists deal with the biological system of organisms. Cells are the primary agents of organisms. They will work under optimal conditions which are usually in a well-defined specific range.

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Factors such as temperature, the concentration of pH, nutrients, salt and mineral ions and waste materials have direct effects on cell functions. So, the environment will be categorized by the cell as an index. The fluid environment inside the cell is called its intracellular environment. The fluid outside the cell which is surrounding the cell is called the internal environment (Weihrauch et al., 2001).

Between unicellular and multicellular organisms, unicellular organisms are more susceptible to their environmental conditions. Unicellular organisms either tolerate an abnormal condition or die. On the other hand, a multicellular organism that has a multi-environment has a garter chance for adaptation and survival chance. Their internal environment is different from their external environment. They have a complex of mechanisms that enable them to tolerate the abnormal conditions of the external environment. Their specialized structures allow them to control the features of their internal environment. These structures can be either their specialized tissues or their specific complex systems (Weihrauch et al., 2001).

Homeostasis is the mechanism by which the internal conditions of an organism are sustained at a stable level. Among aquatic species those animals, with the body fluid concentration similar to external environment are called Osmoconformers (Halberg, 2012).

Those animals with a body fluid concentration that is different from the external environment are called osmoregulators.

Osmoconformers use less energy than osmoregulaters. Because the concentration of their body fluids varies with the concentration of the surrounding water, their internal environments are always under the various form of stress. Osmoregulators use lots of energy in guaranteeing persistent internal conditions. The benefit of this energy usage is

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that the internal conditions are always ideal for cellular mechanisms. The circulatory system sustains homeostasis by keeping the levels of nutrients, oxygen, hormones, nitrogenous waste, carbon dioxide, and last but not the least, critical temperature levels. The benefit of all of these complex mechanisms is that the internal conditions of osmoregulators are continuously ideal for cellular processes. For instance, Enzymes can be efficiently activated in a specific temperature range. A continuous internal temperature means that the enzyme efficiency is at an optimum level despite external environmental temperature conditions (Halberg, 2012).

In an aquaculture study, there is a very different environment as opposed to the surrounding environment. The environment inside the animal as the internal-environment and the surrounding environment as the micro-environment and the external environment as the macro-environment had been termed. These environments are different in terms of temperature, the amount of water, salts and minerals concentrations and other environmental factors. All living creatures consist of internal environments which are very different from their external environments. The internal environment is a lot more stable than the external environment (Chen et al., 2017). This is because multicellular living things are able to tolerate extreme changes in factors such as temperature and water availability. If these factors change to drastically, the chemical reactions within living tissues that are necessary for the maintenance of life will be disrupted. This disruption will result in the concluding cell death of the organism. The substances concentration in the tissues is a very important factor that must be kept constant in the internal environment of living organisms. For instance, if the water concentration in an organism's body be more than the natural limits, it may cause cells to lyse (which means "burst"). On the other hand, abnormal low water concentration will increase the salt and other mineral concentration in the tissue and might results in cell-shriveling. In order to maintain the substance concentration in normal range in living cells, the living organism body must

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find the way to keep substance balance. It means when there in inadequate or extera substance it must have the exchange substance reactions.

2.12 Problem of environmental effects and its control

Both partial and full culture business have the risk of financial losses due to water environmental pressures such as water quality, water hardness, salt, oxygen concentration and temperature fluctuations and water borne diseases. This financial loose may happen either through mortality or deteriorating meat quality, which will result in reduce the profit margins. Both of this will be resulting in the profit margins reductions. There is a wide-ranging of reports for thirty years back that shows the problems of disease in cultured shellfish, but unfortunately, nobody focused on the aquatic environment as the most effective reason of all of these changes, which will have significant effects on the quaculture industry economic costs. In the last few years, many aqua farms around the world had breaking experience because of the pathogenic epidemics of White Spot and Taura or Yellow Head Virus. It is notoriously difficult to estimate the effects of shrimp disease since a common strategy is to organize emergency harvests at the first sign of disease.

A large quantity of aquaculture production might have wild-caught that may be gotten pre-existing bacterial or viral infections from the wild stock. Aquaculturist may increase outbreak risk because they kept the animals under stressful conditions of overcrowding, high food levels, elevated water temperature and poor water quality. In the stressful environments bacterial related diseases, such as *Vibrio* spp. or *Pseudomonas* spp can widespread quickly. The potential for a disease outbreak is a permanent threat to any aquaculture industry. To some extent, good husbandry and proper environmental control can help to avoid wide spreading the pathogens through the farm (Smith et al., 2003).(Halberg, 2012)

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2.13 Water quality and temperature and the criteria for establishing a standard farm

Selecting a suitable site to establish a standard farm, which is a combination of Macro and Micro-environments will play a critical role in aquaculture farming. Pre-studies on topography, ecosystem, metrology, and socioeconomics, as the macro-environment criteria, are required to design a farm that offers species compatibility and economic viability. Elements such as water quality, tidal fluctuation, soil, topography, vegetation, the source of seed, and accessibility are to be considered (FAO, 2011a; Moffitt & Cajas-Cano, 2014).

In this part, the criteria for appraising each element to be considered when judging a site's suitability is described. The optimal rules to establish a standard farm are also briefly described. All these factors have direct or indirect effects on the water temperature which is the parameter in focus for this review. Water quality refers to the micro-environment which entails all the inherent physio-chemical and microbiological characteristics of water pH, dissolved oxygen saturation, and salinity. These factors are frequently measured (Niu et al., 2003). The optimal level of these elements is shown in Table 1. The water content contains the primary food source including zooplankton and macroalgae. The water should be preferably rich in microorganisms and must not be thick and bedraggled. High-quality water which has an optimal level of acidity, temperature, dissolved oxygen rate and salinity has the highest density to inhibit valuable food source for a crustacean. Eventhough, in the absence of natural food resources, standard commercial foods can be the best alternative new foods, it is still more beneficial to have a natural food source for the animals. The most undeniable effect of water temperature on the chemical content is oxygen reduction, which will cause a deficiency in zooplankton, macroalgae, the total viable count of the bacteria, yeast, and the Total Load of *Vibrio*-like organisms, (TVLO) density as the animals' food source. In muddy waters,

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low temperature, due to a lack of light penetration, will reduce the photosynthetic activity; and the light limitation will reduce the growth of the phytoplankton (Burford, 1997; MacGibbon, 2008).

Crustaceans adjust their body temperature in accordance with the temperature of their habitat, which is water. The water temperature exerts a direct effect on internal reactions, such as biochemical, biological and chemical contents, and the survival process of the ectothermic organisms in the water. This method will affect many other essential variables such as the animals' feeding rates, animal growth factors, and animal oxygen consumption. Hence, hypoxia, or decreasing water oxygen level, will reduce the feeding activity in animals and will cause a decrease in the farm yield (FAO, 2011a).

The water temperature is not controlled at all in many types of farms. In a study on the effect of heat on the feeding rate of *M. rosenbergii*, it was reported that the feeding rate of the animals increased corresponding to the rise in temperature. However, in that study, the oxygen was supplied artificially. Also, it was reported that the animals hatched their eggs in 28℃ water temperature (New & Nair, 2012).

The knowledge of the tidal characteristics of the proposed site is essential. This information is important for the pond-bottom evaluation of the dikes, the dragging system design, and the slope ratio determination. The ponds' best tidal ratio is measured according to the pond depth and the water flow. The tidal coefficient would determine the highest and the lowest level of water which controls the water flow in the pond and prevents flooding and will be useful in monitoring water temperature. In addition, the soil type and texture are two of the critical parameters which should be analyzed before farm construction. The acidity, the amount of the organic load, the level of fertility, and the physical composition must be measured through physical and chemical tests. The chosen site area must avoid any industrial or agricultural pollutants, such as pesticides. The

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preferable soil contains enough amount of clay to hold the water. A recommended soil type is sandy clay or sandy loam texture which quickly gets hard and compact, which is helpful to keep the temperature rate invariant (Banu & Christianus, 2016). The coastal soil contains accumulated pyrites (Iron sulphide), which cause acidic sulphate problems during the first few years of farm establishment. In such farms, the low-level PH of the water will affect the water quality and will correspondingly reduce the natural productivity. Another contributing agent to the soil type is the vegetation of the area. Plants can be indicators for soil productivity or acidity. For example, *Avicenna* species improves the soil productivity, and *Rhizosphere* species will increase the acidity of the soil. The soil type has a corresponding effect on the ponds' water quality, which affects the yield of the farm and the meat quality.

The topography details of the pond are essential to design the layout of water filling and draining. In areas where the slope is towards the sea, natural management is applicable. However, in the non-slope areas, using mechanical pumps will cover the forfeit.

The accessibility of the pond is a considerable advantage. And, its proximity to the animal stock collection site is a plus because it will reduce the shock of the transport and handle the stress. There are many other factors that will help to increase the animals' health and the yield of the farm, correspondingly. Factors such as technical assistance, electricity and water supply, facilities, and the quality and availability of the labor all have direct effects on the water temperature.

All the above factors, which are categorized as Macro-environmental factors, are useful factors in the micro-environment and the internal environment, and finally, the molecular-environment which controls the immune system of the crustaceans.

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Understanding the immune system of the crustaceans and their molecular pathways is crucial in getting more insight regarding the environmental effect on the immune system of the crustaceans. In the next section, we further elaborate on the immune system and its molecular pathways (Liu et al., 2012).

Crustaceans and shellfish production have high economic value worldwide, and culture farm areas have increased rapidly since the 1980s up to the level that their worldwide annual production is over 8 million metric tons. Over half of this production is made up of different species of prawns and shrimp. Beside prawns and shrimp, production of other species such as crayfish and lobster are also increasing. Among all farmed shellfish, farming marine shrimp is around 40% of the total aquatic farming. The majority of marine shrimp farms are located in Asia, mainly Malaysia and Thailand (Banu & Christianus, 2016). The shellfish culture in tropical and subtropical areas has been established and developing stage, other countries such as Spain, Italy and Cyprus have also established molluscan aquacultural farms. The UK and Ireland have established hatcheries for Homarid lobsters. Since there is a growing interest to establish crustacean culture farms, there is emergent need to control, prevent or minimize the distressing effects of environmental changes in the crustacean culturing. Actions have been taken to improve the lifetime and life quality in crustaceans. Scientists are becoming more interested in developing in an environment that advise protection and improve immune reactivity against environmental changes in shellfish. The main environmental changes which are currently under inspection for crustaceans include temperature, salinity, and oxygen levels that have a direct effect on specific genes and cellular pathways.

A number of papers that are now appearing in the literature claim to demonstrate their environmental stress impact on aquaculture immunity and disease resistance, but none of them demonstrate the effect of water temperature as the most important agent which

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affects immune responses. This research explores problems resulting from standard farm establishment to water temperature and their direct effect on the immune system of crustacean's immune system.

2.14 Shellfish, crustacean and their production

Shellfish and crustacean production are high-value activities worldwide and their annual world production is over 8 million metric tons. More than half of the aquacultures' is shrimp and prawns and their proportion farms has increased rapidly since the 1980s. Recent estimations show that the level of farmed shrimp and prawn's is around 40% of the total. The majority of shrimp and prawn farming activity is in Asia, particularly Thailand and Malaysia. Even though the vast growths in prawn aquaculture is placed in tropical and subtropical areas, some countries suc as Spain, Cyprus, and Italy have some limited aquaculture farming (Moffitt & Cajas-Cano, 2014).

There is a growing need to control, prevent or minimize the devastating effects of environmental changes in crustacean culture, without recourse to water temperature. In keeping with approaches to lifetime control in crustacean, interest is developing in an environment that confer protection and/or enhance immune reactivity to environmental changes in shellfish (Moffitt & Cajas-Cano, 2014). The main environmental changes currently under scrutiny for crustaceans include temperature, salinity, and oxygen levels that have a direct effect on specific genes and cellular pathways. These genes are thought to act as 'immunostimulants' because of their known effects on the crustacean's immune system in vitro. A number of papers that are now appearing in the literature claim to demonstrate their positive impact on immunity and disease resistance, but none of them demonstrate the effect of water temperature as the most important agent which effects the immune responses. This article considers the problems resulting from standard farm establishment to water temperature and their direct effect on crustacean immune system

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while describing the types of immunostimulatory genes which are claimed to have positive effects; and evaluating their merit in enhancing immune capability in cultured species (Escobedo- Bonilla et al., 2008). Based on the results of many of the published studies questions are raised about the value of these Genes for the control of culture stability and even infection in aquaculture, especially for long-lasting protection in both adults and juveniles. This research further discusses the potential risks to the well-being of the stock animals from non-standard culture and makes a case for rigorous testing of the putative environment, at the gene and functional levels, as well as for the need to consider alternative strategies and approaches to animal health control.

2.15 Biology of *Macrobrachium rosenbergii*

Macrobrachium rosenbergii or giant river prawn, known as the giant Malaysian freshwater prawn, the Malaysian prawn belongs to the family: Palaemonidae, order: Decapoda, class: Arthropoda, phylum: Animalia. *M. rosenbergii,* an important species in the Malaysian economy has been illustrated as a scientifically recognized prawn since 1705. This species, as well as other *Macrobrachium spp* (e.g., *M. nipponesis*), is commercially important because of its edible value. The basic biology of *M.rosenbergii* was studied in Penang, Malaysia (Ling & Merican, 1961) and *M. rosenbergii and M. nipponesis* are the only species of the freshwater prawn involved in the Malaysian culture.

2.16 Real-time PCR

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Since its discovery (Kb, 1987), the PCR technique has undergone significant improvements in methodology and has revolutionized molecular biology techniques from the conventional techniques. It is a basic technique that can amplify a small amount of template DNA or reverse transcribed RNA, also called complementary DNA (cDNA), in large quantities in a few hours. However, conventional end-point PCR has a drawback. It lacks reliable quantification and does not easily yield reproducible

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results. This limitation was resolved twenty years ago, with the invention of real-time PCR by Higuchi et al. A novel "real-time" quantitative PCR (qPCR) method with greater specificity was also invented by Higuchi (Higuchi et al., 1992). The method detects the increased expression of DNA or cDNA template while the amplification is still progressing. Real-time qPCR has been used in many research applications mostly in gene expression quantification.

qPCR has two basic quantification methods, Absolute quantification, and relative quantification. These two methods are suitable for different applications.

Absolute quantification usually is used to determine the absolute/exact quantity of a genomic DNA or RNA template within an unknown sample. In this method standard curve is a prepared from a dilution series of control templates of the known concentration. The relative quantification method is usually used to determine the relative concentration of template (or target gene) in unknown samples. In this method, data normalization was done according to a stably expressed reference gene, and compared relatively with a calibrator sample (for example time zero, or untreated sample) (Dorak, 2006).

Real-time PCR applies different fluorescent detection technologies to detect PCR products. One of the most used ones is SYBR Green. This fluorescent dye is used for the quantification of double-stranded DNA (dsDNA) PCR products. It must be added to the reaction mixture that consists of template cDNA (or genomic DNA), gene-specific primers (forward and reverse), and buffers. After the annealing of the primers, a few fluorescent dye molecules will bind to the double-stranded DNA. The result is a significant increase of the number of the molecules emisting upon excitation. Upon each cycle, more fluorescent dye molecules bind with existing synthesized DNA.

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During the initial cycles of real-time PCR, the signal level can be denoted by the baseline of the reaction. Generally, there is little change in fluorescent signal within cycles of 3 to 15. The background or the noise of the reaction can be linked with the low-level signal of the baseline (Bustin et al., 2009).

To determine the early cycles, it is necessary to take into consideration enough cycles when determining the baseline in the real-time PCR. However, the cycles in which the amplification signal begins to rise above the background should be avoided. The baseline value should be set in a way that the threshold cycle (Ct) can be determined accurately.

The threshold is an important parameter in the real-time PCR reaction. The threshold is the level of signal that reveals a statistically significant increase in the signal calculated reference line. It is set to differentiate related amplification signals from experience. The threshold cycle (Ct) is the cycle number at which the fluorescent signal of the reaction crosses the threshold line. The Ct value is used to calculate the initial cDNA copy number because the Ct value is inversely related to the amount of the starting template.

A melting curve is another important factor of Real-time PCR that shows a single amplified product of a gene. It is rarely possible to have a more amplified product from the primer-dimer and genomic DNA that will result in multiple peaks. The melting curve analysis confirms the specificity of a quantitative reverse transcription PCR reaction. The melting curve can be found when dsDNA starts to melt by heating. As the temperature is raised, double-stranded DNA becomes a single-stranded DNA, and the dye (SYBR Green-based) dissociates from the DNA. Prior to dissociation, agarose gel electrophoresis can be performed (prior to dissociation) to estimate the size of the amplified product (after amplification with gene-specific primers).

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2.17 Biomark™ HD

Biomark HD is a new real time PCR technology which has the ability to adjust from 12 to 192 sample throughputs per run without changing technologies. This technology is affordable and cut costs. It decreases the number of runs and reagent volumes by more than 95% compared to using plates. It has the flexibility to run multiple applications and can easily change markers over time. It has also been confirmed to have trusted performance. Biomark™ HD sets a new standard for high-throughput real-time PCR, endpoint PCR and digital PCR, with benefits that are impossible to reproduce using other conventional PCR systems. Integrated fluidic circuit (IFC) technology saves time and money by performing thousands of reactions in nano liter volumes on a device that is the size of a conventional 96-well plate. Biomark HD, together with IFCs and the IFC Controller for loading samples and assays, streamlines workflows for applications demanding sensitivity and dynamic range at extremely high throughput, including genotyping, gene expression, copy number variation detection and digital PCR.

2.17.1 Gene expression analysis with real-time PCR

The protocol for RNA isolation and cDNA synthesis was modified in gene expression analysis. In this modified method, Promega product for DNase treatment and Roche kits were used instead of Qiagen product. DNase treatment in RNA isolation and in cDNA synthesis procedures are the important parts in these methods.

2.18 RNA isolation

In gene expression analysis having a high-quality RNA is very important. Any kinds of RNA contamination will affect the result of the study. RNA can be contaminated by genomic DNA. To reduce contaminating genomic DNA from RNA, proper RNA isolation and cDNA synthesis procedures need to be followed.

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A good RNA must have high concentration and purity. Purity is assessed by calculating the A260/A280 ratio. A260/A280 ratio values should be between 1.8 and 2.0. This RNA will be transformed to cDNA as template for gene expression study.

2.18.1 Primer design and efficiency

Fifteen candidate Immune reference genes and a gene of interest (HSP70) were considered for gene expression analysis in *M. rosenbergii* under heat shock. The primers were designed by using Primer 3 (Version 0.4.0 http://frodo.wi.mit.edu) based on the available transcriptome sequences of *M.rosenbergii* in animal genetics and genome evolutionary laboratory, University Malaya (D'haene, 2010).

A standard curve was constructed to calculate the gene-specific PCR efficiencies from a 10-fold series dilution of cDNA template for each primer pair. The correlation coefficients (R2) and slope values can be obtained from the standard curve, and the efficiency (E) of PCR was calculated according to the equation E = 10-1/slope. The dilution series was made of pooled cDNA samples from *M. rosenbergii* cDNA, diluted in steps of tenfold over five dilution points. Real-time PCR was performed in triplicate as described above, except that 2 ul template was used instead of one. The confirmed gene expressions proceeded to fluidigm high trough put gene expression for all the genes.

2.19 Network analyst

Comparable differential gene expression analysis and differential gene network analysis involves multiple subtraction of interactions that have been mapped in different experimental conditions. The subtractive process filters out the expressed genes with housekeeping' interactions. Housekeeping gene is a common gene to all static conditions of gene of interest. Finaly, by extracting the selective interactions that are relevant to the studied condition or phenotype, the typical complexity of static gene networks will be reduced.

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Today, pathway analysis is a routine analysis in genomic laboratories. There are many software or web services available that accept and analyze different genomics data, transcriptomics data anlysis, proteomics sequencing data with protein–protein interactions data, and lastely metabolomics data. All these kinds of data are considered highthrouput data. The recent available methods and tools that have been used to visualize and analyze these main types of the high-throughput data have been reviewed by Gehlenborg (Gehlenborg et al., 2010). Moreover, a decade ago different genetic variation data, such as single-nucleotide polymorphism (SNP) originating from analyses of array-based genome-wide association studies (GWAS), commenced to be combined into pathway analysis (J. Wang et al., 2007). Since then, the method was applied to other types of studies involving SNPs such as: epigenome-wide association study (EWAS) (Shimada-Sugimoto et al., 2017) or sequencing-based GWAS (Yi & Zhi, 2011). Even though genetic connotation research is move forward to advancing rapidly, especially GWAS studies are frequently performed for the genotype–phenotype studies, biological understanding of those data remains a challenge yet; particularly when understanding concerns linking genetic findings with identified biological processes (Manolio, 2013).

2.20 Conclusions and perspective

Asian Coastal Countries are mostly attracted to aquaculture farming industry including Malaysia. Among all aquatic animals, prawn (udang) has found an important superior attention in comparison with other aquatic animals in Malaysia. Among all prawn species, *Macrobrachium rosenbergii* has been introduced as a highly-efficient species to the Malaysian Aquaculture Industry. This aquatic animal is currently one of the most economically important farmed freshwater crustacean species.

M.rossenbergii's farming cultured a lots of job opportunities and over a hundred thousand employees are working in the farming section, food production industry and

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export industry. Unfortunately, during the last decade, environmental effects such as temperature, salinity, and different pathogenic diseases caused severe economic outbreaks in the prawn's reproduction farm. It also affected local and national economies and even threatened the prawn industry in some producing countries. However, during the last decade, Malaysian prawn farmers have gained good progress in *M. rossenbergii's* implementation strategies to combat prawn viral disease, but the neglecting on environmental effects are still an important issue.

Many factors can influence individual growth rate in *M. rosenbergii*. In this aquatic animal's gene-environment interaction studies has specific concerns. The information of the identification and characterization of the specific gene that contributes to immune resistance to environmental changes can be applied to speed up genetic improvement programs and increase productivity through genomic selection or genome-wide selection (GS) and immune gene related improvement. Currently there is only limited genomic information available for any crustacean species including GFP, several putative candidates genes have been identified or implicated in growth and muscle development and innate immune response in other crustacean species.

Variations of the environmental factors in prawn culture ponds usually cause the different kinds of an outbreak such as pathogenic outbreaks including viral and bacterial infection. This study is designed to get more insight in the molecular mechanisms of immunological outbreaks caused by temperature variations and the biological changes of the prawns at the early stage of environmental heat stress. High throughput Gene expression technology has been used to analyses the gene expression in prawns with 4 different temperatures at the two-time-points under a controlled laboratory condition. Temperature variation starts from 24 °C and rises to 40 °C. The experiment was included another prawn group as a control. The control group didn't suffer any stress. Furthermore,

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studies have been done in an environmental situation by screening two farms in terms of environment management. Gene expression of the animals from two different farms with poor and well management were screened. The environmental study was conducted in different times and temperatures during day light in aquatic natural habitat. Later the output of the gene expression from poor and good management farms were compared with standard lab condition.

The environment's parameters are rapidly changing. Currently there is abundant evidence for species distribution transferences in response to the local climate change. It is becoming increasingly clear that the environment plays an important role in the dissemination of genes which are controlling the immune system. There is insufficient information available to reach a final conclusion on the significance and impact of the environmental influence on immunological responses in a crustacean. As the usage of harmful chemicals and greenhouse gasses continue to increase. Ecological fluctuations also continue to increase mostly due to temperature. Huge local environmental changes are caused by pollution, deforestation, runoff and erosion, toxicity in water, with further possible effects on local climate changes in temperature levels. The resistance of a species against harmful environmental changes depends on its vulnerability to these environmental changes. These tolerances are determined by the animal's genetic structure, physiologicals and immunological tolerance. In the face of a lack of information about the whole genomic sequence, several immune-related molecules of crustaceans have been identified. Attaining the whole genomic data, will allow many more numbers of genes and their contribution in the immune defense mechanisms to be unveiled. At present study, functional analysis of some putative immunological and metabolical controller genes such as AMPs, Ppos, anti-apoptotic proteins, PRPs, Lectins, caspase, etc. have shown the importance of these molecules in reactions against the prawn's environmental heat stress.

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Current knowledge in prawn immunity against heat stress has been summarized, and the outline picture has been illustrated here in (Figure 1). These studies provide insigh into the innate immune system of prawns against one of the most important environmental factors which is water temperature. This study eventually sought to improving the immune system in order to gain immunologic control against out breaks, and ultimately lead to the development of disease resistant shrimp which would positively impact the aquaculture industry.

Hence, establishing aqua farms according to standardized criteria will reduce the uncontrollable harmful effects of fluctuating temperature. There is an ever-increasing hope gratitude that genetic changes would happen rapidly and would lead to evolution in response to climate change. The evolution of thermal responses associated with immunostimulant genes are also likely to involve changes in genome structure, allelic structure, gene expression and gene duplication. For example, Heat shock proteins are considered as chaperone molecules that can protect other macromolecules from degradation. Even though some of the proteins from this family have other functions. In some organisms such as fish and bacteria the expression patterns of the heat shock protein genes have been linked to adaptation to thermal environments. These examples prove that there are many cases of environmental impacts on gene expression. However, it is important to keep in mind that there is a very complex interaction between genes and the environment which describes individual phenotype and characteristics.

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CHAPTER 3: METHODOLOGY

3.1 Experimental animal

3.1.1 In laboratory sampling

Healthy 270 male juvenile freshwater prawns (90 days) of the same genetic background with a body weight of 25 ± 1 g and a body length of 11.2 ± 1.2 cm were collected from a local prawn farm, Malaysia. The prawns were acclimated in the aerated pond water at 25 ± 1 °C for seven days in the lab before the experiment. Forty prawns were randomly collected at 6 and 12 h separately during the heat shock treatment at each 28 ± 1 °C, 32 ± 1 °C, 36 ± 1 °C temperatures for gene expression, histopathology, morphological and immunohistochemistry study. The detailed methodology of experimental design is shown in (Figure 3.1).

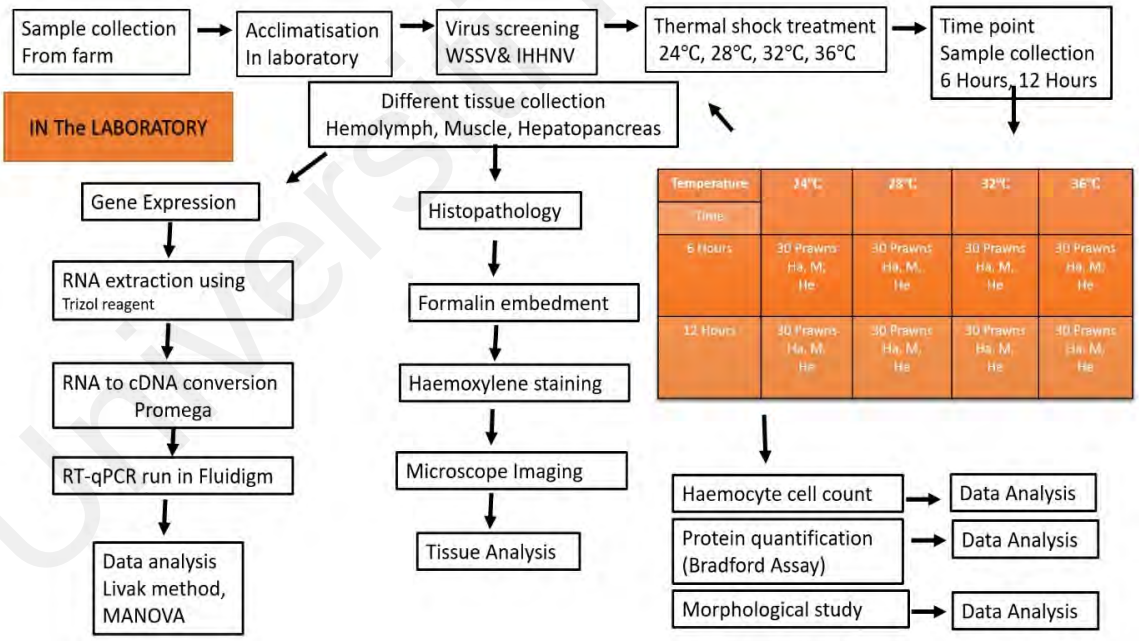

Figure 3.1: Schematic representation of the experimental workflow

In order to study the gene expression response of prawns to heat shock stress, ten juvenile prawns were randomly selected as control at 0 hours samples before heat shock

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treatment. The twenty shrimps were placed in a heated container with pond water maintained at each temperature of 24 ± 1 °C, 28 ± 1 °C, 32 ± 1 °C, 36 ± 1 °C. Ten prawns were removed for six hours, and another 10 prawns continued in the same condition for twelve hours sampling for a continuous post-stress study.

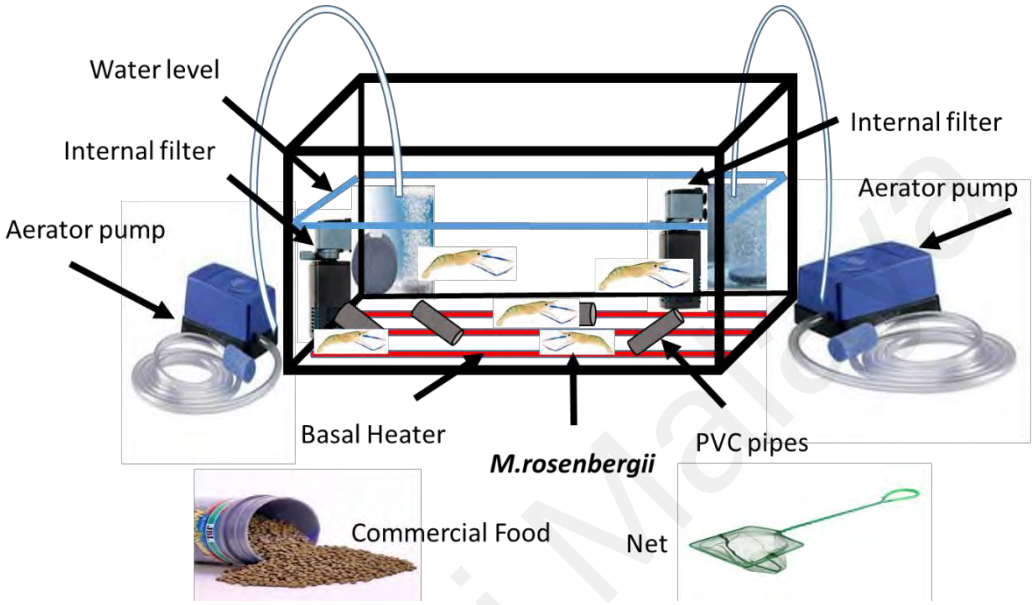

Figure 3.2 Schematic representation of the experimental workflow

The hemolymph was withdrawn from the ventral sinus located at the first abdominal segment using an equal volume of modified Elsevier solution anticoagulant (Figure 3.3).

Figure 3.3 Hemolymph Collection by cardiac-puncture technique, using a 2ml syringe.

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Hemocyte pellets often prawns were immediately used for RNA extraction and hemocyte cell count and prophenoloxidase activity. After the hemolymph was collected, different tissues including hepatopancreas and muscle often prawns were dissected out and preserved in liquid nitrogen for later RNA extraction for gene expression analysis. Also, hepatopancreas and muscle tissue often prawns were dissected out and preserved in 10% formalin for histopathology analysis. No mortality was observed during the experiment 24 ± 1 °C, 28 ± 1 °C, 32 ± 1 °C but no mortality was observed in 6 hours post-stress at 36 ± 1 °C and no mortality was observed in 12 hours post-stress at 36 ± 1 °C.

3.1.2 Farm sampling

Farm detections

Two farms 0.5 ha with the stocking density of 25 pcs per square meter, were chosen according to the management system for sampling purpose. The management criteria such as water circulation, feeding system, and husbandry were considered as macro-environment (Figure 3.4, 3.5, 3.6). The parameters such as water temperature (ºC), transparency (cm), DO (mg/l), pH, alkalinity, NH_3-N (mg/L), NO_3-N (mg/L) were measured during the sampling days as micro-environment elements. The farm with good water circulation and transparency (cm), high level of oxygen (DO (mg/l)), normal pH, low alkalinity, low NH_3-N (mg/L), low NO_3-N (mg/L) (Table 3.1) considered as a well-management farm. The farm with low-quality water circulation and transparency (cm), low oxygen (DO (mg/l)) level, abnormal pH, high alkalinity, high NH_3-N (mg/L), low NO_3-N (mg/L) (Table 3.1) considered as a poor management farm.

Water sampling methodology

Water samples were collected from the selected farms using 500 ml plastic bottles between 09-11AM. After collection of the samples, dissolved oxygen (DO) was measured immediately in the sampling site. Other physicochemical parameters of the water samples

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such as water pH, ammonia, nitrate, nitrite, alkalinity, total phosphorous and total hardness were measured by using HACH test Kit (Model FF-1A Cat. No. 2430-02). Temperature and salinity were recorded by using mercury thermometer and refract meter, respectively. For the measurement of soil quality parameters, soil samples were collected from different prawn farms. Following air drying, the soil samples were analyzed in the Soil Science Laboratory. One-way analysis of variance (ANOVA) is carried out to test the significant difference between the two farm locations (i.e., treatments) for water and soil quality parameters. Correlation analysis was done to determine the positive or negative relationships between water quality, and soil quality parameters of the farms. Statistical tests were performed using computer-based statistical software SPSS (Statistical Package for Social Science) version 24 IBM.

Figure 3.4: An overall view of a well-maintained farm with good water circulation, water transparency and commercial feeding in Negeri Sembilan.

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Figure 3.5: An overall view of a well-maintained farm with good water circulation, water transparency, and commercial feeding.

Figure 3.6: An overall view of a non-maintained farm without water circulation, pond structure water transparency, and low-quality feeding.

Table 3.1: Water Quality Criteria**.**

Water Quality Criteria of standard farm, laboratory condition, farm1 and farm2					
Water Quality Characteristic of Concern	Ecological or Health Effect	Standard	In-Lab	Farm1	Farm2
Temperature	Aquatic suffer metabolic stress at high temperatures		28	29.58±0.121	30.40±0.033
Transparency (cm)				21.06± 0.166^{c}	32.13±1.179^{a}
Dissolved Oxygen	High levels of dissolved oxygen are necessary for aquatic respiration.	5.0 mg/l average 4.0 mg/l minimum	8	6.70 ± 0.139^{a}	5.99±0.536^{ab}
pH	affects the solubility of other water quality contaminants	7.0 - 9.5	8	8.51 ± 0.084^{a}	6.58± 0.384^{b}
Alkalinity		140±1	140	145.598.48^{a}	124.65±4.92 ^{b}
NH_3-N (mg/L)		00.05±0.1	0.05	0.08±0.005	0.13 ± 0.042
NO_3-N (mg/L)		1.08±0.01	1.08	1.08 ± 0.017	1.22 ± 0.029

Healthy juvenile male freshwater prawn, GMP (n= 480) with an average body weight of 25 ± 1 g and average body length of 11.2 ± 1.2 cm were collected from each local prawn farm, Malaysia. The prawns were dissected immediately after collection from the pond.

Hemolymph, muscle, and hepatopancreas were stored in liquid nitrogen for gene expression purpose. A number of twenty randomly collected hepatopancreas and muscle were fixed in 10% neutrally buffered formalin for histopathology study. The hemolymph was withdrawn from the ventral sinus located at the first abdominal segment using an equal volume of modified Elsevier solution anticoagulant and subsequently centrifuged for 10 min at 800×g, 4 °C. Hemocyte pellets were immediately used for RNA extraction and hemocyte cell count and prophenoloxidase activity. After the hemolymph was collected, different tissues including hepatopancreas muscle were dissected out and preserved in liquid nitrogen for later RNA extraction for gene expression analysis. No mortality was observed during the experiment.

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3.2 Screening for IHHNV and WSSV

Virus screening experiment was conducted to diagnose infected prawns. Briefly, DNA was extracted from swimming leg using NucleoSpin Tissue, DNA extraction kite. A Nested PCR technique was applied to identify infected prawn using specific primer no 309. The primer sequence used for PCR detection is shown in Table 3.2.

Table 3.2: Designation Sequence Expected.

Primer sequence for IHHNV and WSSV detection		
Amplicon		Size
IHHNV309F	5'-TCCAACACTTAGTCAAAACCAA3'	309
IHHNV309R	5'-TGTCTGCTACGATGATTATCCA-3'	
WSSV 143F	5'-TGC-CTT-ATC-AGCTNT-CGA-TTG-TAG-3'	848
WSSV 145R	5'-TTC-AGN-TTT-GCA-ACC-ATA- TT CCC-3'	

Crude IHHNV virus was obtained from IHHNV infected *Macrobrachium rosenbergii* prawn, and it was used throughout this experimental work as positive confirmation. Pleopods which are the abdominal appendages are often used for swimming used as the viral suspected DNA source.

The first step PCR reaction mixture was prepared as 5µl of 5X buffer, 2 µl of 25mM MgCl2, 2 µl dNTPs,2 Primer F 389,2 µl of primer R 389 0.25 µl DNA polymerase, 1 µl DNA template and topped up with 13.75 µl dH2O to a final volume of 25 µl. After preparing the PCR mixture, the tube was placed in a PCR machine. A program was created based on the protocol from the manufacturer (Promega). It started with incubating the reaction mixture at 95 ºC for 10 minutes. Then, followed by 95 ºC for 2 minutes and decreased temperature to 55 ºC for 5 minutes then increased the temperature 72 °C 1min (15 cycles) then continued the same condition for 5 min to disassociate the complementary DNA and RNA template. Finally, it was held at 4 °C for 5 minutes.

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In the second step (Nested PCR (Promega) for IHHNV Detection) the mixture was 5µl of 5X buffer, 1 µl of 25mM $Mgcl_2$, 2 µl dNTPs,0.5 Primer F 389,0.5 µl of primer R 389 0.25 µl DNA polymerase, 1 µl DNA template and topped up with 14.75 µl dH2O made the final volume of 25 µl. After preparing the PCR mixture, the tube was placed in a PCR machine. A program was created based on the protocol from the manufacturer (Promega). It started with incubating the reaction mixture at 95 ºC for 2 minutes. Then, followed by 95 ºC for 30 seconds and decreased temperature to 55 ºC for 30 seconds then increased the temperature 46 to 72 ºC (30 cycles) then continued with the same condition for 5 min to disassociate the complementary DNA and RNA template. Finally, it was held at 4 °C for 5 minutes.

3.3 Real-Time Polymerase Chain Reaction (RT-PCR)

3.3.1 RNA extraction and reverse transcription

Total RNA was extracted from hemocyte, muscle, and hepatopancreas tissue with TRIzol Reagent (Invitrogen, USA) as described in the manufacturer's protocol. The samples were homogenized with Trizol. Homogenization was necessary to reduce the "viscosity" of the lysates. Then, the disrupted samples were loaded onto a Spin Column and spun down. RNA quality was assessed by electrophoresis on 2% agarose gel and nono-drop UV/V spectrophotometry. Finally, the RNA samples proceeded to cDNA synthesis immediately for reverse transcription. cDNA samples were stored in -20 freezers till they were used for real-time PCR.

The cDNA was synthesized in a 25-µl reaction volume containing 2 µg of DNase I-treated total RNA, 1× Moloney murine leukemia virus (MMLV) buffer, 0.5 mM deoxyribonucleotide triphosphate (dNTP), 0.4 mM oligo-dT, 20 U of RNase inhibitor (Promega), and 200 U of MMLV reverse transcriptase (Promega). The cDNA was then diluted five times, and one µl of the dilution was used for each RT-PCR reaction.

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3.3.2 Determination of RNA Quantity and Quality:

Spectrophotometrically by Nanodrop, Native Agarose Gel Electrophoresis of RNA, and Agilent 2100 Bioanalyzer

RNA high purity (no contaminants) and high integrity (not degraded) is the most critical criteria in the RT-PCR experiments. The purity and quality of RNA samples reduce the variability between biological replicates. Following RNA elution from the Spin Column, the concentration (ng/µl) and purity of the RNA samples were measured spectrophotometrically by Nanodrop. RNA content was determined by measuring the absorption at the OD 260/280 and 260/230 ratio in a pH neutral buffer. A desired OD 260/280 of 1.8–2.0, and 260/230 of 2.0–2.2 indicated good quality RNA that is devoid of protein and other contaminations including salt, carbohydrates, peptides, and phenol (Figure 3.7).

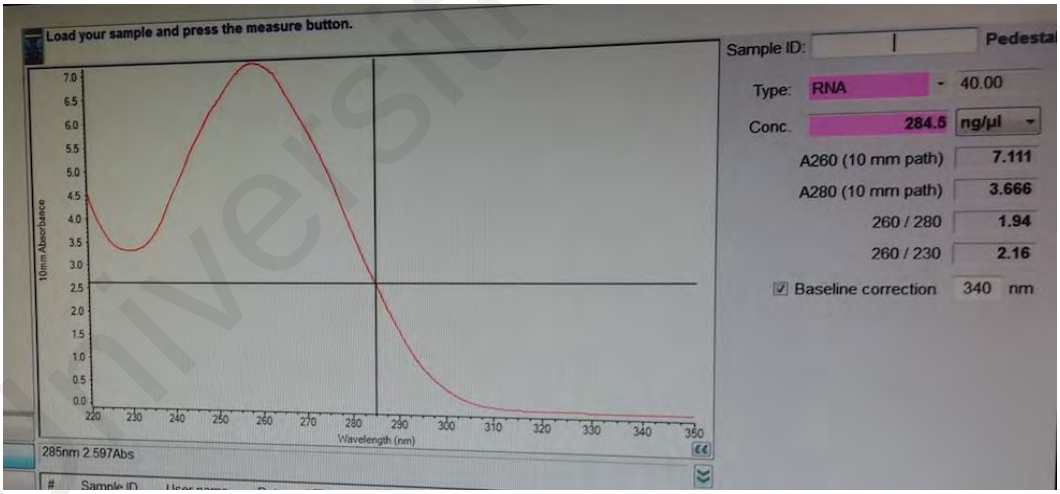

Figure 3.7: RNA integrity

In addition, RNA quality and integrity were assessed using conventional methods such as 1% native agarose gel electrophoresis in the presence of a fluorescent dye such as ethidium bromide and also Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Briefly, 1µg of isolated RNA was loaded onto a 1% agarose gel in TBE (89 mM

Tris-HCl pH 7.8, 89 mM borate, 2 mM EDTA) with 0.5 µg/ml ethidium bromide added to the gel, and electrophoresis (Bio-Rad) was performed for 80 min at 60V. Fluorescence of the RNA was visualized with a gel doc imaging system (UVP). Native agarose gel electrophoresis was sufficient to judge the integrity and overall quality of a total RNA preparation by inspection of the 28S and 18S rRNA bands (Figure 3.8).

Moreover, the quality and integrity of RNA extracted from the brain were verified using Agilent 2100 Bioanalyzer instrument according to the manufacturers' protocols. All samples showed high-quality RNA (RNA Integrity Number (RIN) =8) (Figure 3.9). In this study, agarose gel electrophoresis and bioanalyzer were utilized for checking the quality of one sample, selected randomly from each group as a representative.

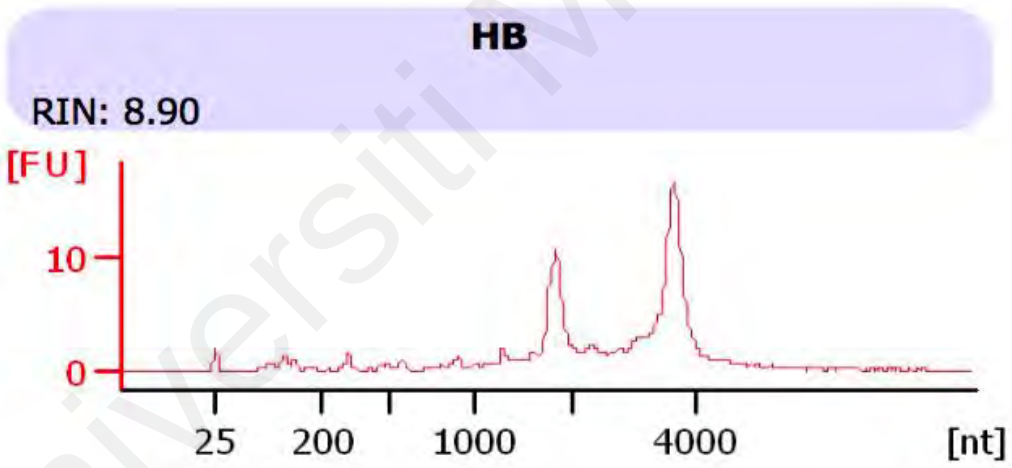

Figure 3.8: RNA integrity

3.3.3 Reverse Transcription PCR

Complementary DNA (cDNA) was generated by reverse transcription of 1000 ng of total RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). The reaction consisted of the following reagents named in 3.3. The 2 X RT master mix was placed on ice and mixed gently.

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Table 3.3: Reagents used for generation of Complementary DNA (cDNA).

Component	**Volume (µl)/Reaction Kit**
10X RT Buffer	2.0
25X dNTP Mix (100mM)	0.8
10X RT Random Primers	2.0
MultiScribe ^{TM} Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H_2O	3.2
Total per Reaction	**10**

cDNA preparation for Reverse Transcription reactions, 10 µl of 2 X RT master mix was pipetted into RNase free PCR tubes. Then the sample was pipetted into each tube and pipetted up and down twice to mix. The lid was closed gently, and the tube was centrifuged briefly to spin down the contents and to eliminate any air bubbles. All tubes were placed on ice, and then all were loaded in a thermal cycler (Biometra). Conditions for the thermal cycler are mentioned in Table 3.2.

Table 3.4: Cycling protocol for reverse transcription into cDNA.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25 ºC	37 ºC	85 ºC	4 ºC
Time	10 min	120 min	5min	∞

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3.3.4 Primer Designing by Bioinformatics Tools

Choosing specific and appropriate primers is probably a crucial early step and the most important factor affecting the Real Time-Polymerase Chain Reaction (RT-PCR). Specific amplification of the intended target required that primers match to specific targets in certain orientations and do not allow undesired amplification.

In designing specific primers, several available and useful programs were used to search against an appropriate nucleotide sequence database, such as Primer-BLAST, Primer3, and MFE primer-2.0 at various melting temperatures at good target coverage and followed by a careful examination to avoid primer dimerization. In addition, in real-time PCR experiments, to normalize the data reference genes were used as controls. A perfect reference gene is, therefore, one that does not exhibit changes in expression between samples from various experimental conditions or time points. In this study, Elongation factor gene was chosen as reference genes. All the primers with their sequences are mentioned in Table 3.5.

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Table 3.5: List of primer sequences used for real-time RT-PCR assay.

	Gene		Gene ID	Forward Primer Sequence	Reverse Primer Sequence
1	Elongation Factor1-alpha	EF1-A	EL609261.1	ACTGCGCTGTGTTGATTGTAGCT	ACAACAGTACGTGTTCACGGGTC
2	Arginine Kinase1	AK1	HQ191218	GTCTGGTGATCGCAACCTTCA	GTAGATACCGCGGCCTTCAG
3	Anti-lipopolysaccharide factor	ALF	JQ364961.1	TGAAGCTCAGGGTTGGGAAGT	ATACCATTTGGTCGTCCACCC
4	Inhibitor of Apoptosis protein	IAPs	HQ668090.1	GAGCAGATCCAGCGATTCTTCA	AACACACACAGCTAAACAAGATACGA
5	Small Heat Shock 70	HSP 70	AY466445.1	GAAAACTGCGAGGCGTCA	ATTTACCATTTGGTCGTCCACCC
6	Chaperonin	Chap	HQ668094.1	GAGCAGATCCAGCGATTCTTA	AACACACACAGCTAAACAAGATACGA
7	Lectin1	Lec1	JQ349147.1	GGCGTGAAAGGGTCAGGTT	CGGCAACTTTGTCGTTCTTAGGA
8	Lectin2	Lec2	JQ349148.1	ACAACTGCGAGGCGTCA	CGCTGTTGCCGGTAGCA
9	Lectin3	Lec3	JQ349149.1	GCTCATCTGTGAACAATACCACTTC	TCCGTCCTACTGATGTCTGCTT
10	Lectin4	Lec4	JQ349150.1	GTCGAAGAGAGCGTTGACGAA	GACATCCACAAAGAAAACAGGACAT
11	Tachylectin	TaLec	N.A	ACAACTGCGAGGCGTCATT	GACATCCACAAAGAAAACAGGACAT
12	Prophenoloxidase 1	PPO1	HF570111.1	GATCATCACGTGACTCTTCCATC	CCGCCTCCAATTCCCTAAATGT
13	Prophenoloxidase activating enzyme III	PpoAIII	HQ668087	GCCGTCATTGACAATCTGTACGT	TGTTGCACAGAGTATCCCATGT
14	Hemocyanin	Hem	111863819	GTCGATTCGGGATCACAGAC	CACGAGTCTCTTCCTCTTCGTT
15	NF-kappa B inhibitor alpha	NfkBI-α	AET34918	GGTGGACTGAGATCAAAGCGATATT	ACTGTCTTTCTAAATCACATTCAACGGT

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3.3.5 Primer Synthesis and Optimization

After designing the primers, they all were sent to the First Base Company for synthesis. qPCR optimization is usually performed in order to obtain maximum yield and specificity. A critical and important step in a qPCR was to get the optimal range of primer annealing temperature (Ta) to its target sequences by testing identical reactions containing a fixed primer concentration, across a range of annealing temperatures. This ensured that the reaction was performed at the right temperature for the primers to anneal efficiently to their targets while preventing non-specific annealing and primer–dimer formation. In our study, for determination of the optimal annealing temperature, a Bio-Rad real-time PCR machine that was equipped with a temperature gradient feature was used for the wet lab validation. For confirming the specificity of primer annealing, a melting temperature (Tm) was set, ranging from 65-95 ºC for each primer separately. In addition, a range of annealing temperatures (Ta) from 52-64 ºC was tested, which were close to the calculated Tm of the primers. The optimal Ta for our primers was 60ºC. Generally, an annealing temperature of about 5 °C below the lowest Tm of the pair of primers was used.

3.3.6 Determination of Primer Efficiency by Standard Curve

A common method for determining reaction efficiency is the construction of a standard curve. The slope of a standard curve was used to estimate the PCR amplification efficiency of the real-time PCR reaction, the primer efficiency for all the primers was determined by the standard curve.

3.3.7 BioMark™ Systems and high-throughput RT-qPCR for Genetic Analysis

The BioMark™ Systems include the optical, thermal cycling, and software components necessary to perform real-time quantitative PCR (qPCR) analysis on Dynamic Array™ IFCs. The BioMark™ Systems provide orders of magnitude higher

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throughput for real-time qPCR compared to conventional platforms due to its Dynamic Array™ IFCs — nanofluidic chips that contain fluidic networks that automatically combine sets of samples with sets of assays. This innovative solution for real-time qPCR provides reaction densities far beyond what is possible with microtiter plates and significantly reduces the number of liquid-handling steps and the volume per reaction. A total of 15 multi expressed genes of broad relevance to immune system, metabolite pathways, and environmental changes were chosen, including antimicrobial proteins/ peptides (e.g anti-lipopolysaccharide factors, Lectin 1,2,3,4, Tachylectin)and , prophenoloxidase system-related proteins (e.g. prophenoloxidase, prophenoloxidase-activating enzymes), metabolites and signaling molecules (e.g., arginine kinase1, Elongation factor), homeostasis and coagulation (haemocyanin), , apoptosis and autophagy (apoptosis inhibitor) and other immune-related genes (e.g. HSP70), NF-Kappa inhibitor, Chaperonin. The expression levels of the selected genes were evaluated by using the high-throughput microfluidic RT-qPCR platform BioMark™ (Fluidigm 96.96 dynamic array systems). The sample reaction mixtures were performed in a final volume of 5 µl containing 1.25 µl of preamplified cDNA (diluted 1:5), 2.5 µl of 2 TaqMan Gene Expression Master Mix (Applied Biosystems), 0.25 µl of 20 DNA Binding Dye Sample Loading Reagent (Fluidigm), 0.25 µl of 20 EvaGreen (Biotium) and 0.75 µl of TE buffer (Table 3.6). Primer reaction mixtures were made in the same volume of 5 µl containing 2.5 µl of 2 Assay Loading Reagent (Fluidigm), 1.25 µl of 20 µl of forward and reverse primer mix and 1.25 µl of TE buffer. Both sample and primer reaction mixtures were loaded into the dynamic array chip that was subsequently placed on the NanoFlex™ 4-IFC Controller for loading and mixing. After approximately 50 min, the chip was transferred to the BioMark™ Real-Time PCR System (Table 3.7).

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The cycling program used consisted of 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 1 min at 60 °C. Melting curves analysis was performed after completed RT-qPCR collecting fluorescence between 60–95 °C at 0.5 C increments.

Table 3.6: Reagents required for Biomark HD System procedures**.**

Exonuclease I (New England Biolabs, PN M0293L)	20X DNA Binding Dye Sample Loading Reagent (Fluidigm, PN 100-3738)	TE Buffer (10 mM Tris, pH 8.0, 1.0 mM EDTA) (TEKnova, PN T0224)
SsoFast™ EvaGreen Supermix with Low Rox (Bio-Rad Laboratories, PN 172-5211)	2X Assay Loading Reagent (Fluidigm, PN 85000736)	DNA Suspension Buffer (TEKnova, PN T0021)
PreAmp Master Mix (Fluidigm, PN 100-5580,100-5581)	100 µM each Forward and Reverse Primer Stock Mixture for each assay of interest	PCR Certified Water (TEKnova, PN W3330)

Table 3.7: Equipment's required for Biomark HD System procedures.

BioMark HD System
IFC Controller HX (for 96.96 Dynamic Array IFC)
Standard 96-well Thermal cycler

First, in a microcentrifuge tube, 1 µl of each 100 µM stock primers (Forward and Reverse) were combined (pooled primers), up to 96 assays. After that, DNA Suspension Buffer was added (10 mM Tris, pH 8.0, 0.1 mM EDTA; TEKnova, PN T0221) to make the final volume 200 µl (Table 3.8).

Table 3.8: Pre-Master Mix required for the pre-amplification reaction

Component	**Volume/Reaction (µl)**
PreAmp Master Mix	76.8
Pooled Primers	38.4
Water	172.8
Total Volume	288

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Pre-Master Mix above was shaken well followed by a short spin. It was aliquoted in 8-tube strip (34.5 µl to each tube). Then, 3.75 µl of Pre-Master Mix was aliquoted for each sample in a PCR plate. 1.25 µl of cDNA was added to each well containing Pre-Master Mix and making a total volume of 5 µl. The reaction was mixed by brief vortex, followed by centrifuge. The PCR plate was placed in thermal cycler and cycle using the following table as a guide in Table 3.9.

Table 3.9: Thermal cycling conditions for the pre-amplification reaction.

Condition	**Hold**	**Cycle (10 cycles)** **Denaturation**	**Annealing/Extension**	**Hold**
Temperature	95 ºC	95 ºC	60 ºC	4 ºC
Time	2 min	15 s	4 min	∞

Exonuclease I treatment used to remove unincorporated primers. The Exonuclease I was diluted 4 U/µl as shown below in Table 3.10:

Table 3.10: Reagents required for dilution of the Exonuclease I.

Component	**96 samples with Overage (µl)**
Water	117.6
Exonuclease I Reaction Buffer	16.8
Exonuclease I at 20 Units/µl	33.6
Total Volume	168

The amount of 20 µl of Exonuclease I dilution was aliquoted to 8-tube strip, vortex, and short spin. When the PCR reaction of PCR plate which mentioned above was finished, 2 µl of diluted Exonuclease I at 4 U/µl from 8-tube strip was added to each pre-amplification reaction, vortex, centrifuge. Finally, it was placed in a thermal cycler, and was incubated with the following program in Table 3.11.

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Table 3.11: Thermal cycling conditions for pre-amplification reaction (after Exonuclease I treatment).

Condition	Digest	Inactivate	Hold
Temperature	37 ºC	80 ºC	4 ºC
Time	30 min	15 min	∞

Final products were diluted 5 times (5-fold dilution) with TE buffer 1% (10 mM Tris-HCl, 1.0 mM EDTA, TEKnova, PN T0224) as shown in the table below Table 3.12.

Table 3.12: Final product dilution.

The volume of Pre-amplification Reaction + Exonuclease I dilution	The volume of TE Buffer to Add
7 µl	18 µl
5-fold dilution	

Diluted reaction products can either be assayed immediately or stored at -20 ºC for later use (Table 3.13). Diluted reaction products should be stable for at least one week.

Table 3.13: 10X Assays mix preparation.

Assay mix Component	Volume for 30 µl Stock
100 µM each primer (Forward & Reverse)	1.5 µl (Forward) + 1.5 µl (Reverse)
1X DNA Suspension Buffer	12 µl
2X Assay Loading Reagent (PN 85000736)	15 µl
Total Volume	30 µl

3.3.8 Preparing Sample Pre-Mix and Samples

Sample mix was prepared with reagents below:

First of all, 600 µl of the SsoFast Eva Green Supermix was combined with 60 µl DELTA gene Sample Reagent. The sample Pre-amplification Mix was vortexed and spun down. Then, 80 µl of this solution was aliquoted in 8-tube strip. Next, 2.75 µl of the solution in the strip was added to each well of PCR plate. In addition, 2.25 µl of cDNA

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was added to the individual well. Samples were vortexed and centrifuged prior to adding the samples to the IFC.

Loading the chip:

1. 3 µl of each sample from the PCR plate was pipetted into the respective inlets on the chip.
2. After that, 3 µl of each primer (Forward & Reverse) was pipetted into the respective inlets on the chip.
3. 150 µl of Actuation Fluid (PN 100-6250) was pipetted into the P1 well on the chip.
4. 150 µl of Pressure Fluid (PN 100-6249) was pipetted into the p2 and p3 wells on the chip.
5. 20 µl of Actuation Fluid was pipetted into the p4 and p5 wells on the chip.
6. All the bubbles were removed by very thin sterile needles that could affect the chip run.

The chip (Figure 3.9) was placed in the IFC Controller RX. The IFC Controller RX software was used, the Load Mix (169 ×) script was run to lead the samples and assay into the chip. When the Load Mix (169 ×) script was finished, the loaded chip was removed from IFC Controller RX. Any dust particles or debris were removed from the chip surface. Finally, the chip was placed in the BioMark™ HD System. This technology is designed for the allelic discrimination 5′ nuclease assay (Figure 3.9). This platform is one of the most novel approaches, which is used for RNA assay recently. The Fluidigm microfluidic technology (Fluidigm's Dynamic Array™) uses integrated fluidic circuits (IFC) to move molecules of biological samples and reagents in a variety of patterns. IFCs reduce a qPCR reaction from the routine microlitre volume to a nanolitre scale, and also the number of liquid handling steps during the experiment, making it possible to perform routine qPCR analysis for thousands of reactions in a single run. This technology has been used in genotyping, mutation detection, absolute quantization of nucleic-acid sequences, and gene expression for humans and animals. When the running program was

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finished, all the data from the system was collected and put all in data collection software for analyzing.

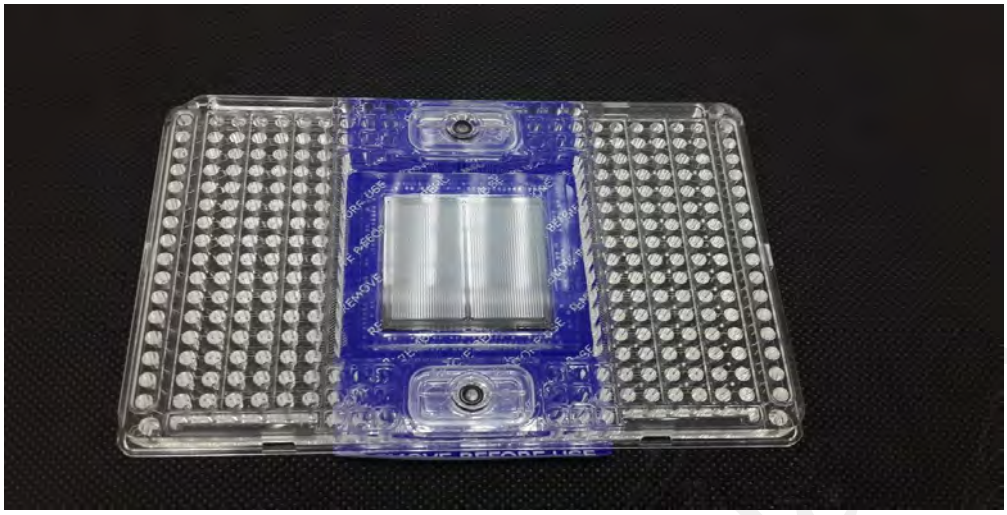

Figure 3.9: 192.24 Dynamic Array™ IFC - Fluidigm chip used by the BioMark™ HD System. Solutions were loaded on this chip and placed in the BioMark™ HD System

3.9 Morphological studies

The morphology of control and treated groups were observed based on (The giant freshwater prawns of the *Macrobrachium rosenbergii* species group (Crustacea: Decapoda: caridea: Palaemonidae) report. The weight of all individuals was measured after the heat shock treats. Body color, abdomen and cephalothorax changes between control and treated groups were observed as per standard methods (Figure 3.10).

Figure 3.10: *Macrobrachium rosenbergii*

3.10 Assessment of Hepatopancreas Somatic Index

The (Hepato Somatic Index) was calculated according to the procedure defined by (Jiliang et al., 2007). The HIS calculation is done by the below formula;

HSI= Liver weight (g) /Total body weight (g)×100

3.11 Tissue preparation for histological study

Hepatopancreas and muscle were removed from treated prawns. Both organs were immediately fixed in 10% neutrally buffered formalin for 36 hours and prepared for histological analysis following (Humason, 1979) as the standard technique. The samples were dehydrated in rising concentrations of ethanol, cleared in xylene and infiltrated with rising concentrations of liquid paraffin wax at 58 °C and later embedded in paraffin blocks. The sections were cut at 5 µm with a Rotary microtome and stained using hematoxylin and eosin (H&E). Stained sections were observed by light microscopy.

As the thickness of each section was 5 µm, we had 20 sections per Hepatopancreas (100 / 5 = 20 total sections). Since the whole section set is generally too large to investigate exhaustively, a subset of samples must be chosen. Thus, sections were randomly selected by systematic random sampling (SRS). The sampling starts by selecting a section from the serial sectioning randomly and then every kth section in the serial was selected, where k, n, and N are the sampling interval, sample size (random-selected sections), and population size (total sections), respectively (Black, 2004). This is calculated as:

k=N/n ; 20 =140/n ; n=7

Eventually, all the histological slides were examined through a light microscope (Nikon); then suitable slides were picked and captured at fields of 10× magnification at the same locations The histoarchitecture of prawn's hepatopancreas resulted in the

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number of B-cells (Blasenzellen cells), R-cells (Restzellen cells), E-cells (Embryonic cells), F-cells (Fibrillenzellen cells), the interstitial sinus was filled with abnormal infiltrated hemocytes (AIH), and the epithelial tubule shows ruptured basal laminae (RBL). To avoid any bias in cell counting, this part was performed blindly by an experienced independent observer.

3.12 Histopathology

Following the fixation of the muscle and hepatopancreas, the samples were routinely embedded in paraffin wax (Leica Biosystems, Germany) and placed in embedding blocks. Three embedded tissue blocks were selected randomly using the Excel "RAND" function by an investigator blinded to the treatment groups for the following processes. The selected embedded tissue blocks were sectioned at 5 µm thicknesses. The excision was placed on glass slides and stained by hemoxylene. Hemoxylene staining method is recommended to stain prawn tissues.

3.13 Histological analysis

Histological changes were examined semiquantitatively according to Mishra and Mohanty (2008). The histopathological parameters were categorized as normal histological structure (0%), mild changes (<10%), moderate changes (10–50%), severe changes (50–70%), and extended severe changes (>70%). These parameters helped to assess an overall histological change. Data were represented as the mean of selected best 25 slides (each organ), 75 slides (3 organs) for each prawn.

3.14 Measurement of immune parameters

The amount of 300µl hemolymph samples from the prawn's ventral sinus were extracted at each time point and temperature using a 3-mL sterile syringe which was rinsed with the anticoagulant. The hemolymph-anticoagulant mixture was then subjected to the phenoloxidase activity and total hemocyte count (THC).

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3.14.1 Total hemocyte count

The amount of 300 µl of the hemolymph and anticoagulant mixture was stained with 14 µl of trypan blue staining solution. Ten µl of the stained hemolymph was dropped on to a hemocytometer and viewed under the microscope. The stained cells which observed dark blue stain considered non-viable and were not counted. The unstained cells were counted as viable cells. The viable cells in the four squares were then counted, and the mean of the viable cells per each corner square was multiplied into a dilution factor of 1, and to 104 the cell count per mL was determined.

3.14.2 Phenoloxidase activity

In order to measure the Phenoloxidase activity, 500 µl of hemolymph was centrifuged at 8000 rpm at 4℃ for three minutes. The supernatant was removed, and the pellet was raised and suspended in 500µl cacodylate-citrate buffer and centrifuged at 8000 rpm at 4℃ for three minutes. The supernatant was discharged, and then the pellet was suspended in 100µl of cacodylate buffer. 50 µl of this suspension were placed in microcentrifuge plate. 25 µl of Zymosan was added after 10 minutes incubation followed by 5 minutes incubation with 25µl.

L-dihydroxyphenylalanine (L-DOPA) and 400µl of cacodylate buffer. Prophenoloxidase activity was then measured in the wavelength of 490 nm using microplate reader.

3.15 Protein quantification

3.15.1 Protein Determination

Quantitative estimation of serum protein was conducted using the dye-binding technique of Bradford (1976) with modifications. The Bradford reagent was prepared by dissolving l00mg Coomassie Brilliant Blue G in 50ml of 95% ethanol. 100ml of 85 % (w/v) phosphoric acid was then added to the mixture. The mixture was diluted to a final

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volume of 1 liter with deionized water. The mixture was then stirred overnight followed by filtration and stored in a dark bottle. 0.1 mg/ml of Bovine Serum Albumin (BSA) was prepared and used for protein standard curve. In this study, micro Bradford assays were used where the content of BSA contains 0 to 10u.g of protein. 1ml of Bradford reagent was added to loci of each crude serum extract (mg/ml) and the 6 standard solutions. The mixture was then incubated at room temperature for 20 minutes. The absorbance of the protein was measured at 595nm by using a Shimadzu UV-160 spectrophotometer. The protein content of the samples was determined from the standard curve. Protein concentration was determined between 2000, and 4000 times dilution. The amount of the loaded protein is calculated using the following formula C1V1=C2V2

3.15.2 Preparation of Bovine Serum Albumin Protein Assay Standards

In order to measure and plot a standard curve of protein concentration against absorbance at 595 nm, a series of dilutions of the BSA protein standard stock solution must first be prepared. The easiest way to calculate the volume of protein stock solution required for each dilution is by using the C1V1 = C2V2. The value of C1 is the concentration of the protein stock solution, V1 is the volume of the stock solution required, C2 is the concentration of the diluted sample, and V2 is the volume of the diluted sample. The concentration of the stock solution, C1, is 100µg/ml, the concentration of the diluted sample is C2, and the volume of the diluted sample is fixed at 200 µl. Therefore, the volume of stock solution required would be:

V1=C2V2/C1

The protein standards should be prepared in the same buffer as the samples to be assayed. A simple standard curve can be made using bovine serum albumin with concentrations of 0, 10, 20, 30, 40, 50 µg/ml for the microassay (extinction coefficient of BSA is 0.667).

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3.15.3 Bradford Reagent

The Bradford reagent can be made by dissolving 100 mg of Coomassie Blue G-250 in 50 ml of 95% ethanol; 100 ml 85% (w/v) phosphoric acid must also be added to the solution. Finally, distilled water should be added to the mixture to achieve a final total volume of 1 liter.

3.15.4 Procedure

Prepare a 10-fold dilution of a 1 mg/ml BSA sample by adding 100 µl of 1 mg/ml BSA to 900 µl of distilled water to make 100µg/ml BSA. Generate test samples for the reference cell, blank, BSA standards and the protein sample to be tested according to Table 3.2 in disposable cuvettes. Note that a dilution of the protein sample may be required for the resulting absorbance to fall within the linear range of the assay.

Incubate each sample at room temperature for 5 minutes. Measure the absorbance of each sample at 595 nm using a UV-visible spectrophotometer. Allow the instrument to warm up for at least 15 minutes prior to use. Plot the absorbance of each BSA standard as a function of its theoretical concentration. The plot should be linear. Determine the best fit of the data to a straight line in the form of the equation

"y= mx + b."

Where y is the absorbance at 595 nm and x is the protein concentration. Use this equation to calculate the concentration of the protein sample based on the measured absorbance. If the absorbance of the test sample is not within the absorbance range of the standards, the assay must be repeated with more appropriate dilution. The linear range of the assay and most spectrophotometers is 0.2 - 0.8 O.D. units. For protein quantification measurement, 500 µl of hemolymph was centrifuged at 8000 rpm at 4°C for three minutes. The supernatant was removed and suspended in 500µl Bradford Coomassie brilliant blue

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assay. The mixture was incubated ten minutes in dark and room temperature. The protein quantity measured by a microplate reader at an absorbance at 590 nm.

3.16 Statistical analysis

Median gene expression intensity data was imported into the MAANOVA method under SPSS version 24 and ($P<0.05$). Significant gene expression differences between control and treatment group, control and farm samples, according to gene expression variation to calculate a gene-specific variance. Genes that were statistically significant in expression between thermal stress and control groups in at least one-time point were grouped according to the fold expression by the formula

$M= \log_2$ (treatment expression / control)

$M= \log_2$ (heat) – $\log_2$ (control)

3.17 Correlations study

To study the correlation between the studied genes data was imported into the JMP statistical SAS software. Significant genes positive and negative correlations between control gene in control group and genes from each treatment group include laboratory, poorly-managed farm and well-managed farm samples has been done according to the gene expression value. Genes that were statistically correlate between thermal stress and control groups in at least one-time point were grouped according to the expression rate.

3.18 Gene Network prediction

To demonstrate the gene network prediction according to the gene expression value and correlation studies, a program has been written by using Microsoft .NET Framework. Gene expression data were imported through excel file according to the correlations between the genes. The graphs were created by using the computests.Net evaluated license. The configuration sheet to data entry is created in MSEXCEL.

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The procedure is as blow;

The control gene and control condition and treatment (Timar), are programmed according to the presented data by researcher. The next excel sheet shows the correlation data which confirms the correlations between all of the introduced genes. The correlation data would be extracted from correlation studies, using a statistical software. The correlation software in this study are SPSS. 24 and JMP analytical sotware. The correlation input is coded by numbers 0, 1, 2, according too the significancy of the gene's expression relationship in each treatment. An example is given in the following picture (Figure 3.11);

	AK1	**Lec1**	**Lec2**	**Lec3**	**Lec4**	**Ta-Lec**
AK1	1.0000	0.3728	0.4815	0.0135	-0.0229	0.2652
Lec1	0.3728	1.0000	0.7032	0.6717	-0.2365	0.3429
Lec2	0.4815	0.7032	1.0000	0.4213	-0.1224	0.1677
Lec3	0.0135	0.6717	0.4213	1.0000	-0.2692	0.6507
Lec4	-0.0229	-0.2365	-0.1224	-0.2692	1.0000	-0.3190
Ta-Lec	0.2652	0.3429	0.1677	0.6507	-0.3190	1.0000
PPO1	0.2417	0.2727	0.6309	0.1883	-0.0124	0.0994
PpoAIII	-0.2387	0.3350	0.1143	0.4193	-0.5254	0.3088
Hmo	-0.4690	-0.4731	-0.2675	-0.1323	-0.4380	-0.1373
HSP70	-0.1865	-0.0611	-0.0353	-0.1708	-0.0046	-0.3162
Chp	0.3226	0.3663	0.4245	0.2041	-0.1166	0.3035
ALF 2	-0.5252	-0.3801	-0.3903	-0.2720	-0.1850	-0.2610
NfkBI-α 2	-0.6182	-0.6115	-0.4899	-0.3401	-0.0435	-0.4494
IAPs 2	-0.4713	-0.5034	-0.4567	-0.4546	-0.1859	-0.4092

Figure 3.11: Genes correlation values

By processing the inputed data according to the following matrix formula has shown in (Figure 3.12);

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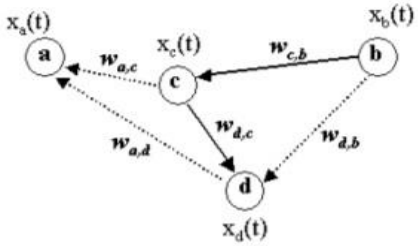

$$x_a(t+1) = x_a(t) + w_{a,c}x_c(t) + w_{a,d}x_d(t)$$
$$x_b(t+1) = x_b(t)$$
$$x_c(t+1) = x_c(t) + w_{c,b}x_b(t)$$
$$x_d(t+1) = x_d(t) + w_{d,c}x_c(t) + w_{d,b}x_b(t)$$

a) Linear Additive Model

$w_{i,j}$	a	b	c	d
a	0	0	-	-
b	0	0	0	0
c	0	+	0	0
d	0	-	+	0

b) Weight Matrix

Figure 3.12: The gene correlation network formula

The gene correlation network would be demonstrating as bellow (Figure 3.13).

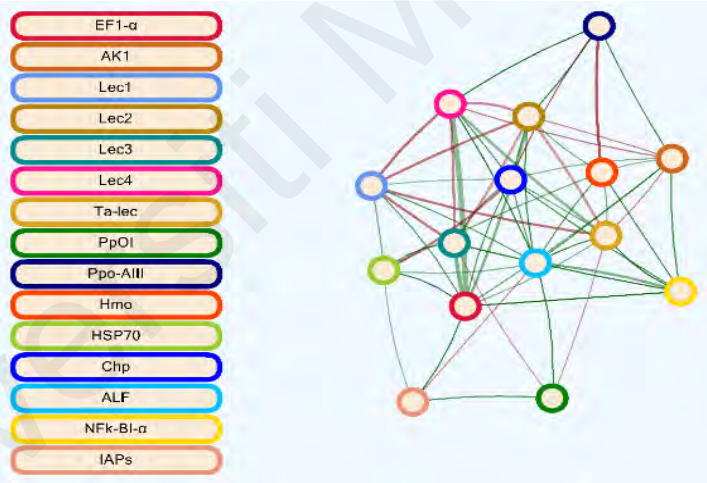

Figure 3.13: The gene correlation network demonstration

This is an initial pioneer Gene Network prediction demonstration which is designed, written and produced by the author and the coding source refrences is available in appendix III.

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CHAPTER 4: RESULT

Adaptation is an undeniable phenomenon in organisms living performances. The history of the animal's survivals since the ice age, under different temperature fluctuations, provides remarkable evidence of animal's capacity to tolerate and compensate for the unbearable environmental changes by enlisting their functional adjustment in molecular and structural level. This environmental adjustment entails increasing in the animal's performance efficiency and their survival under the environmental fluctuations. This increased capacity would confirm base on indicators criteria such as survival rates, growth factors, metabolisms performance, behavioral activity and lastly, reproduction rate and activity. However, evaluation of the adaptive values is difficult, but if the animals could combat the adverse environmental changes and shows good performance on the indicators criteria, it will be called "Adapted" to the current environmental circumstances.

Adaptation to new environmental circumstances is consist of genetically and non-genetically adaptation.

Non- genetic adaptation which is also known as acclimatization is the individuals respond to the current environmental circumstances. This response is temporary and will not be passed to the next generation. This type of adaptation may consist of structural changes such as cellular changes or tissue and organ structure changes.

Genetic adaptions cause the genotypic changes. These changes may consist of the qualitative and quantitative changes. It will affect at basic genomic structure, primary protein structure and will result in biochemical and physiological process changes. This response is permanently and will be passed to the next generation. In better words, it represents the principle of the evolution.

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In the competitive environment under adverse circumstances, the species which have better non-genetic adaption would have more survival and reproductive chance. Therefore, such this species has more existence chance than others.

Genetic adaption may happen slowly through generations. Therefore, young generations reflect the environmental changes may be a clue to the ancestor's adaption process. There are only a few genetic adaptions studies available which they work directly on a specious genomic reflects the environmental changes. One of the major problems in environmental studies is that environmental parameters are also in interaction with each other. Besides all, the environment is a complex of different criteria's which made animal's habitat. Also, in a habitat, the environmental factors such as temperature, salinity, oxygen demand and other factors can modify each other. Studies on a species reflect the environmental changes contain a complex data platform which consist of genetic and non-genetic adaptions simultaneously. This complicated complex makes it difficult to evaluate. So, to obtain a valid evaluation between, both genetic and non-genetic adaption, a long-term environmental observation under different acclimatization and genomic studies in both controlled condition and natural habitat are needed. The result of such these studies would confirm that the animal tolerance to the adverse environmental circumstances will cause the animal's stability or increase the homeostasis level which leads to the organism survival independency.

In this study, adaption is studied from the perspective of genomic and non-genomic adaption. Here, the temperature is considered as a master ecological factor in the adaption of the animal. We studied the **capacity adaption** of the invertebrate animal adjustment to the environmental changes through innate immune gene expression, changes in immunohistochemistry factors, and environmental observatory parameters on the

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survival of the *M. rosenbergii*. And **resistance adaptions** trough upper and lower temperature limits and short and long-term acclimatization.

In this study, both genetic and non-genetic adaption, a long-term environmental observation under three different environmental circumstances. The initial environment is a laboratory-controlled condition the other two environments were a well-managed farm with controlled standard animals husbandry and non-managed farm with non-controlled management and animal husbandry. So, genetic and non-genetic adaption studies have been conducted in both controlled condition and natural habitat.

The initial concern in this study was to obtain insights into different tissues tolerance in adverse environmental circumstances. To achieve this purpose, we screened three different tissues based on three different gene clusters under three different environmental circumstances. Hemolymph, muscle, and hepatopancreas tissues were studied through metabolite, innate immune-related and environmental controller genes. So, the initial hypothesis is:

H_0: Different tissue will have the same expression across the different temperature.

H_a: Different tissue will have the different expression across the different temperature.

Therefore, genetic adaption study has been conducted by the comparative genomic study among three studied tissues (hemolymph, muscle, hepatopancreas) through gene expression study. The animals were succumbing to different water temperatures and acclimatized under controlled conditions in animal genetics and genome evolutionary laboratory at University Malaya. The salinity and oxygen level of the water was kept at a standard level during the experiment, and the temperature varied from 24 °C, 28 °C, 32 °C, 36 °C. Until the first 3 hours post-stress acclimatization, prawn's survival profiles were very similar for all the temperatures and the peak of mortality reached 36 °C, 12 hours

post stress. The prawns showed more erratic swimming, low food intake, pale and yellowish body, lethargic behavior but melodized lesions, especially in muscle by increasing the temperature. The RNA pool of each organ including hemolymph, muscle, and hepatopancreas at each temperature were studied for one metabolite pathway, five different direct and indirect immunological pathways and one environmental pathway through gene expression analysis. Fifteen genes that were coding this pathway were selected from *M. rosenbergii* transcriptome data from AGAGLE internal database. The samples were proceeded for gene expression rate reading by high trough put expression study. The achieved data were later analyzed by the LIVAK method, and Multiple-ANOVA test confirmed the significance of the results.

Gene expression of hemolymph under thermal shock for all under studies temperatures, 24 ℃, 28 ℃, 32 ℃ and 36 ℃ were analyzed for both time point sampling. First Time Point Sampling is sample collections six hours after thermal shock acclimatization start. Second Time Point Sampling is sample collections twelve hours after thermal shock acclimatization start. The gene's expression profiling discussion was arranged by the related tissue and time point sampling for each studied gene.

4.1 Laboratory Experiment

4.1.1 Genetic adaption

Different Gene clusters expression analysis in different tissues within six and twelve hours after acclimatization. The gene expression comparison among all the three studied organs showed that, by six hours acclimatization's, hemolymph tissue is more active in metabolite reaction in comparison with other two studied tissue. But, by increasing the thermal acclimatization's time to twelve hours, the tissues gene expression activity changes and hepatopancreas tissue, act as the highest gene expression tissue. chaperone

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pathway and apoptosis have more activity in hepatopancreas, hemolymph, and muscle, respectively. The significancy of the expression pattern is shown in (Table 4.1 to 4.11).

So, the answer to the initial hypothesis is that: Different tissue will have the different expression across the different temperature.

Hemolymph tissue shows the most affecting tissue under different thermal shock in short-term acclimatization. But, hepatopancreas shows the most affecting tissue under different thermal shock and long-term acclimatization.

The overall pattern of the all tissues gene expression shows that there is a steady increase by temperature increase, but there is a sharp gene expression decrease after long hours thermal acclimatization's which shows the level of the gene expression goes back to the control level. It confirms that animals are adapting through gene adaption process by long-term stress (Figure 4.1 to 4.10) and gene expression is data on available in supplementary section.

The overall pattern of the metabolite gene expression (Figure 4.6). Lectin pathway gene expression (Figure 4.7) in all tissue's gene expression shows that there is a steady increase by temperature increase up to 32 °C, but there is a sharp gene expression decrease after long hours thermal acclimatization's which shows the level of the gene expression goes back to the control level at 36 °C. The same pattern happened for PPO pathway (Figure 4.8). So, gene expression study confirms that animals are adapting through gene adaption process by long-term stress. It also confirms that there is a significant increase by temperature increase up to 32 °C, but there is a piercing gene expression decrease after long hours thermal acclimatization's which shows the level of the gene expression goes back to the control level at 36 °C in chaperon and apoptosis pathway (Figure 4.9-4.10).

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Table 4.1: The table shows Thermal stress influence on metabolite Pathway coding gene expression in different tissues, 6h after the treatment (a), 12h after the treatment (b) in laboratory condition.

Table(a).Thermal stress influence on metabolite pathway in different tissues, 6h after the treatment			
6h	Hemo	Muscle	Hepato
C	8.470115	8.03729	10.12069
24°C	7.10063	6.607312	7.672541
28°C	8.671132	8.016558	9.04858
32°C	10.57967	9.443917	10.54008
36°C	10.84881	8.444943	9.542155

Table(b).Thermal stress influence on metabolite pathway in different tissues, 12h after the treatment			
12h	Hemo	Muscle	Hepato
C	8.470115	8.144469	10.12069
24°C	6.086935	6.676872	6.107793
28°C	9.418013	8.379649	9.795831
32°C	12.91831	11.37408	15.2973
36°C	6.406583	7.412852	9.542155

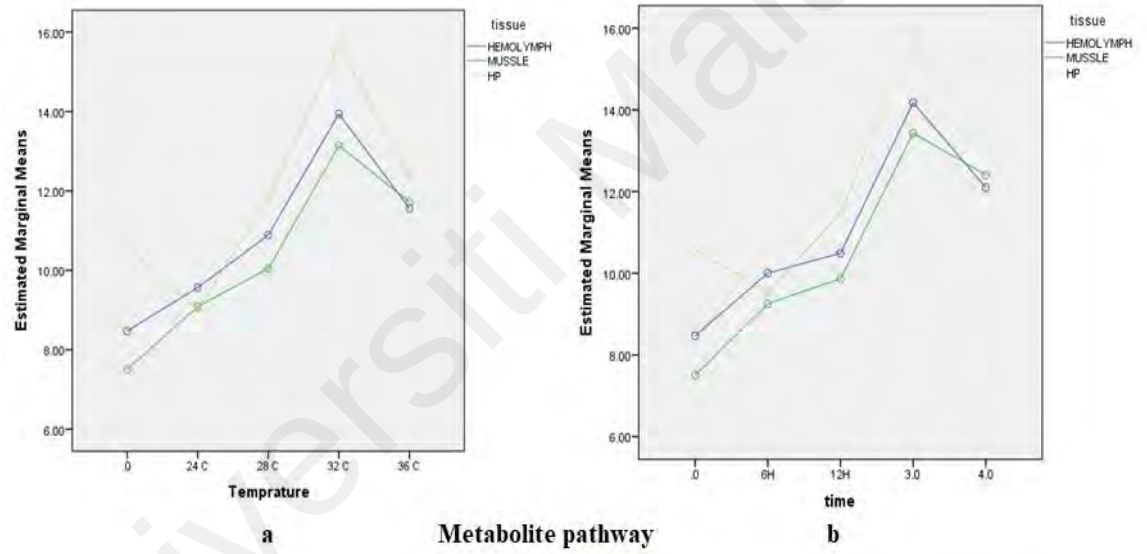

Figure 4.1: The figure shows Thermal stress influence on Metabolite pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory (a) and lectin Pathway coding gene expression in different tissues, 6h after the treatment, and 12h after the treatment in laboratory.

The gene expression comparison among all the three studied organs showed that, by six hours acclimatization's, hemolymph tissue is more active in metabolite reaction in comparison with other two studied tissue (Table 4.1). But, by increasing the thermal acclimatization's time to twelve hours, the tissues gene expression activity changes and hepatopancreas tissue, act as the highest gene expression tissue (Figure 4.1).

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Table 4.2: Descriptive Statistics of metabolite Pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory (PW1).

Dependent Variable: Metabolite pathway

Temprature	tissue	Mean	Std. Deviation	N
.0	HEMOLYMPH	8.4701	.16605	10
	MUSSLE	7.5061	.17485	10
	HP	10.5600	.14456	10
	Total	8.8454	1.30593	30
24 C	HEMOLYMPH	9.5647	3.41250	140
	MUSSLE	9.0880	2.91889	140
	HP	9.0161	3.50165	139
	Total	9.2234	3.28833	419
28 C	HEMOLYMPH	10.8913	3.35100	134
	MUSSLE	10.0477	2.95882	140
	HP	11.8285	2.64814	139
	Total	10.9208	3.07649	413
32 C	HEMOLYMPH	13.9419	2.50349	140
	MUSSLE	13.1432	3.11414	140
	HP	15.6629	3.53686	140
	Total	14.2493	3.24873	420
36 C	HEMOLYMPH	11.5606	3.92654	140
	MUSSLE	11.7239	4.26526	140
	HP	12.3581	3.93410	140
	Total	11.8808	4.05006	420
Total	HEMOLYMPH	11.4425	3.68036	564
	MUSSLE	10.9394	3.69139	570
	HP	12.1935	4.13348	568
	Total	11.5246	3.87337	1702

The gene expression comparison among all the three studied organs showed that, by six hours acclimatization's, hemolymph tissue gene expression is highly significant in metabolite reaction in comparison with other two studied tissue (Table 4.2).

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Table 4.3: Pairwise Comparisons of Metabolite Pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory.

Dependent Variable: **Metabolite Pathway**

(I) Temperature	(J) Temperature	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
.0	24 C	-.378	.634	1.000	-2.158	1.403
	28 C	-2.077*	.634	.011	-3.859	-.296
	32 C	-5.404*	.633	.000	-7.184	-3.623
	36 C	-3.035*	.633	.000	-4.816	-1.255
24 C	.0	.378	.634	1.000	-1.403	2.158
	28 C	-1.700*	.232	.000	-2.353	-1.046
	32 C	-5.026*	.231	.000	-5.677	-4.376
	36 C	-2.658*	.231	.000	-3.308	-2.007
28 C	.0	2.077*	.634	.011	.296	3.859
	24 C	1.700*	.232	.000	1.046	2.353
	32 C	-3.327*	.232	.000	-3.980	-2.674
	36 C	-.958*	.232	.000	-1.611	-.305
32 C	.0	5.404*	.633	.000	3.623	7.184
	24 C	5.026*	.231	.000	4.376	5.677
	28 C	3.327*	.232	.000	2.674	3.980
	36 C	2.368*	.231	.000	1.718	3.019
36 C	.0	3.035*	.633	.000	1.255	4.816
	24 C	2.658*	.231	.000	2.007	3.308
	28 C	.958*	.232	.000	.305	1.611
	32 C	-2.368*	.231	.000	-3.019	-1.718

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

The gene expression comparison among different tempraturs showed that, by six- and twelve-hours acclimatization's, in 28 °C gene expression is more stable in metabolite reaction in hemolynph in comparison with other studied tempraturs (Table 4.3).

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Table 4.4: Thermal stress influence the lectin pathway coding gene expression in different tissues, 6h after the treatment (c), 12h after the treatment (d) in laboratory condition

Table(c).Thermal stress influence on lectin pathway in different tissues, 6h after the treatment			
6h	Hemo	Muscle	Hepato
C	9.440538	10.72268	10.69348
24°C	7.452775	8.961151	8.915808
28°C	10.6994	10.3389	13.38675
32°C	16.36999	12.84418	19.38828
36°C	15.86374	12.088	12.49883

Table(d).Thermal stress influence on lectin pathway in different tissues, 12h after the treatment			
12h	Hemo	Muscle	Hepato
C	9.440538	10.72268	10.69348
24°C	5.418887	4.63869	7.387356
28°C	13.52376	11.03524	15.21091
32°C	22.93427	18.83421	23.41331
36°C	7.643291	6.603565	12.49883

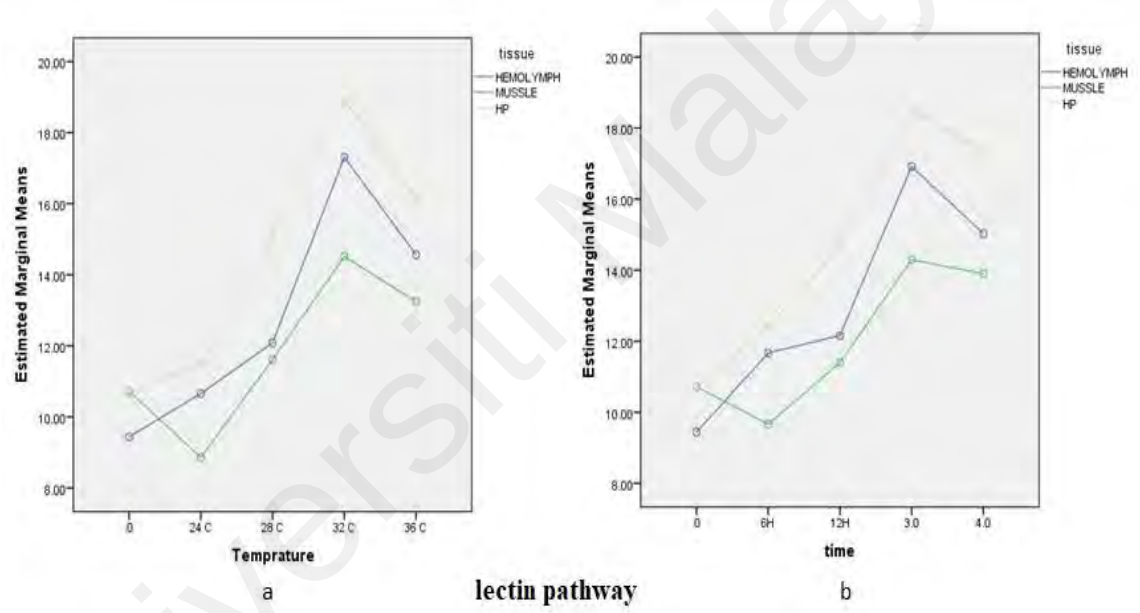

Figure 4.2: The figure shows Thermal stress influence on lectin pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory (a) and lectin Pathway coding gene expression in different tissues, 6h after the treatment, and 12h after the treatment in laboratory.

The gene expression comparison among all the three studied organs showed that, by six hours acclimatization's, hemolymph tissue is more active in Lectin pathway in comparison with other two studied tissue (Table 4.4, 4.5). But, by increasing the thermal acclimatization's time to twelve hours, the tissues gene expression activity changes and hepatopancreas tissue, act as the highest gene expression tissue (Figure 4.2).

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Table 4.5: Descriptive Statistics of lectin Pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory (PW2).

Dependent Variable: **lectin pathway**

Temperature	tissue	Mean	Std. Deviation	N
.0	HEMOLYMPH	9.4405	.10901	10
	MUSSLE	10.7227	.05940	10
	HP	10.6935	.11802	10
	Total	10.2856	.61531	30
24 C	HEMOLYMPH	10.6589	2.75273	140
	MUSSLE	8.8595	2.93903	140
	HP	11.4931	2.02664	139
	Total	10.3344	2.82108	419
28 C	HEMOLYMPH	12.0851	2.53462	134
	MUSSLE	11.6168	1.30146	140
	HP	15.1335	1.97375	139
	Total	12.9523	2.53100	413
32 C	HEMOLYMPH	17.3091	2.60759	140
	MUSSLE	14.5138	2.73523	140
	HP	18.8997	2.86858	140
	Total	16.9075	3.28053	420
36 C	HEMOLYMPH	14.5632	5.86480	140
	MUSSLE	13.2489	3.81796	140
	HP	16.1524	5.62235	140
	Total	14.6548	5.30524	420
Total	HEMOLYMPH	13.5961	4.49617	564
	MUSSLE	12.0363	3.51301	570
	HP	15.3439	4.36182	568
	Total	13.6570	4.35806	1702

The gene expression comparison among all the three studied organs showed that, by six hours acclimatization's, hemolymph tissue is more active in Lectin pathway in comparison with other two studied tissue (Table 4.5).

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Table 4.6: Thermal stress influence on the prophenoloxidase pathway coding gene expression in different tissues, 6h after the treatment (e), 12h after the treatment (f).

Table(e).Thermal stress influence on PPo pathway in different tissues, 6h after the treatment			
6h	Hemo	Muscle	Hepato
C	9.298664	11.60447	12.80299
24°C	9.015934	11.46868	11.46881
28°C	13.02228	13.72692	14.4684
32°C	20.69258	13.38854	23.80184
36°C	18.13482	11.7178	12.5286

Table(f).Thermal stress influence on lectin pathway in different tissues, 12h after the treatment			
12h	Hemo	Muscle	Hepato
C	9.298664	11.60447	12.80299
24°C	7.499195	6.003088	5.748359
28°C	16.7753	11.66856	15.77974
32°C	26.44635	19.00794	25.77988
36°C	11.04564	3.864946	12.5286

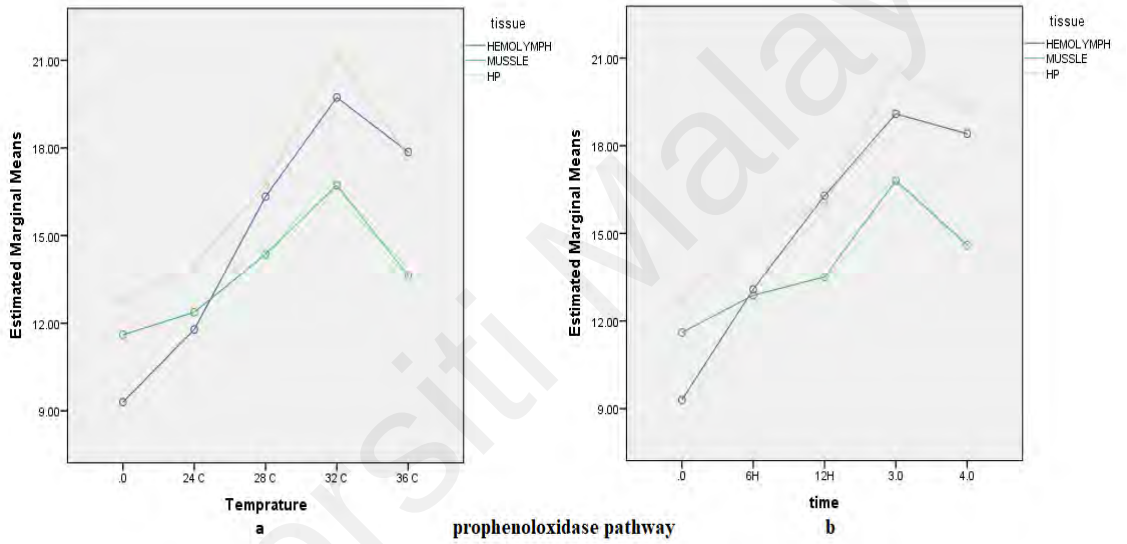

Figure 4.3: The figure shows Thermal stress influence on lectin pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory (a) and lectin Pathway coding gene expression in different tissues, 6h after the treatment, and 12h after the treatment in laboratory.

The gene expression comparison among all the three studied organs showed that, by six hours acclimatization's, hemolymph tissue is more active in Prophenolixdase pathway in comparison with other two studied tissue (Table 4.6, 4.7, 4.8). But, by increasing the thermal acclimatization's time to twelve hours, the tissues gene expression activity changes and hepatopancreas tissue, act as the highest gene expression tissue (Figure 4.3).

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Table 4.7: Thermal stress influence the PPO pathway coding gene expression in different tissues, 6h after the treatment (c), 12h after the treatment (d) in laboratory condition.

Dependent Variable: **PPO pathway**

(I) time	(J) time	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
.0	6H	-2.435*	.782	.019	-4.632	-.237
	12H	-4.012*	.782	.000	-6.210	-1.814
	3.0	-7.607*	.789	.000	-9.825	-5.388
	4.0	-6.204*	.789	.000	-8.423	-3.986
6H	.0	2.435*	.782	.019	.237	4.632
	12H	-1.578*	.269	.000	-2.334	-.821
	3.0	-5.172*	.290	.000	-5.986	-4.358
	4.0	-3.770*	.290	.000	-4.584	-2.956
12H	.0	4.012*	.782	.000	1.814	6.210
	6H	1.578*	.269	.000	.821	2.334
	3.0	-3.594*	.291	.000	-4.411	-2.778
	4.0	-2.192*	.291	.000	-3.009	-1.376
3.0	.0	7.607*	.789	.000	5.388	9.825
	6H	5.172*	.290	.000	4.358	5.986
	12H	3.594*	.291	.000	2.778	4.411
	4.0	1.402*	.310	.000	.532	2.272
4.0	.0	6.204*	.789	.000	3.986	8.423
	6H	3.770*	.290	.000	2.956	4.584
	12H	2.192*	.291	.000	1.376	3.009
	3.0	-1.402*	.310	.000	-2.272	-.532

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

The gene expression comparison among all the three studied organs showed that, by six hours acclimatization's, hemolymph tissue is more active in PPO pathway in comparison with other two studied tissue (Table 4.7).

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Table 4.8: Pairwise Comparisons of PPO Pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory (PW1).

Dependent Variable: **PPO pathway**

(I) Tempratur e	(J) Temprature	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
.0	24 C	-1.475	.747	.484	-3.573	.624
	28 C	-4.576*	.747	.000	-6.675	-2.476
	32 C	-7.989*	.746	.000	-10.087	-5.891
	36 C	-5.166*	.746	.000	-7.264	-3.068
24 C	.0	1.475	.747	.484	-.624	3.573
	28 C	-3.101*	.274	.000	-3.871	-2.331
	32 C	-6.515*	.273	.000	-7.281	-5.748
	36 C	-3.692*	.273	.000	-4.458	-2.925
28 C	.0	4.576*	.747	.000	2.476	6.675
	24 C	3.101*	.274	.000	2.331	3.871
	32 C	-3.413*	.274	.000	-4.183	-2.644
	36 C	-.591	.274	.311	-1.360	.179
32 C	.0	7.989*	.746	.000	5.891	10.087
	24 C	6.515*	.273	.000	5.748	7.281
	28 C	3.413*	.274	.000	2.644	4.183
	36 C	2.823*	.273	.000	2.057	3.589
36 C	.0	5.166*	.746	.000	3.068	7.264
	24 C	3.692*	.273	.000	2.925	4.458
	28 C	.591	.274	.311	-.179	1.360
	32 C	-2.823*	.273	.000	-3.589	-2.057

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

The gene expression comparison among different tempraturs showed that, by six- and twelve-hours acclimatization's, in 28 °C gene expression is more stable in metabolite reaction in hemolynph in comparison with other studied tempraturs (Table 4.8).

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Table 4.9: Thermal stress influence the chaperone pathway coding gene expression in different tissues, 6h after the treatment (g), and 12 h after the treatment (h) in laboratory condition.

Table(g).Thermal stress influence on Chaperone pathway in different tissues, 6h after the treatment			
6h	Hemo	Muscle	Hepato
C	9.476048	11.48105	13.46277
24°C	9.199444	11.37197	12.93831
28°C	15.81477	15.05703	16.36506
32°C	20.81888	16.55704	24.36772
36°C	12.76807	11.3653	13.10362

Table(h).Thermal stress influence on Chaperone pathway in different tissues, 12h after the treatment			
12h	Hemo	Muscle	Hepato
C	9.476048	11.48105	13.46277
24°C	7.494892	5.951996	5.136358
28°C	19.03053	16.10062	18.92013
32°C	26.56018	20.60068	25.42137
36°C	6.556685	3.815271	13.10362

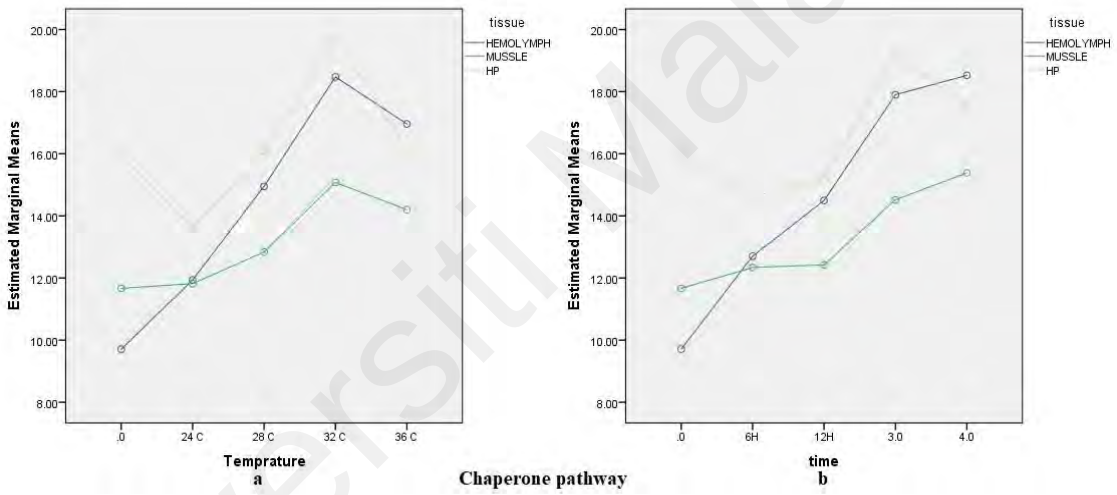

Figure 4.4: The figure shows Thermal stress influence on chaperone pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory (a) and lectin Pathway coding gene expression in different tissues, 6h after the treatment, and 12h after the treatment in laboratory.

The gene expression comparison among all the three studied organs showed that, by six hours acclimatization's, hemolymph tissue is more active in Chaperon pathway in comparison with other two studied tissue (Table 4.9, 4.10, 4.11). But, by increasing the thermal acclimatization's time to twelve hours, the tissues gene expression activity changes and hepatopancreas tissue, act as the highest gene expression tissue (Figure 4.4).

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Table 4.10: Thermal stress influence the chaperone pathway coding gene expression in different tissues, 6h after the treatment (c), 12h after the treatment (d) in laboratory condition.

Dependent Variable: **chaperone pathway**

(I) Temprature	(J) Temprature	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
.0	24 C	-.009	.711	1.000	-2.007	1.989
	28 C	-2.181*	.711	.022	-4.180	-.182
	32 C	-5.305*	.711	.000	-7.302	-3.307
	36 C	-3.322*	.711	.000	-5.320	-1.325
24 C	.0	.009	.711	1.000	-1.989	2.007
	28 C	-2.172*	.261	.000	-2.905	-1.439
	32 C	-5.295*	.260	.000	-6.025	-4.565
	36 C	-3.313*	.260	.000	-4.043	-2.583
28 C	.0	2.181*	.711	.022	.182	4.180
	24 C	2.172*	.261	.000	1.439	2.905
	32 C	-3.123*	.261	.000	-3.856	-2.391
	36 C	-1.141*	.261	.000	-1.874	-.409
32 C	.0	5.305*	.711	.000	3.307	7.302
	24 C	5.295*	.260	.000	4.565	6.025
	28 C	3.123*	.261	.000	2.391	3.856
	36 C	1.982*	.260	.000	1.253	2.712
36 C	.0	3.322*	.711	.000	1.325	5.320
	24 C	3.313*	.260	.000	2.583	4.043
	28 C	1.141*	.261	.000	.409	1.874
	32 C	-1.982*	.260	.000	-2.712	-1.253

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

The gene expression comparison among different tempraturs showed that, by six- and twelve-hours acclimatization's, in 28 °C gene expression is more stable in metabolite reaction in hemolynph in comparison with other studied tempraturs (Table 4.10).

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Table 4.11: Pairwise Comparisons of chaperone pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory (PW1).

Dependent Variable: **chaperone pathway**

(I) time	(J) time	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
.0	6H	-.776	.721	1.000	-2.803	1.250
	12H	-1.587	.721	.279	-3.614	.440
	3.0	-4.745*	.728	.000	-6.791	-2.699
	4.0	-4.718*	.728	.000	-6.764	-2.671
6H	.0	.776	.721	1.000	-1.250	2.803
	12H	-.811*	.248	.011	-1.509	-.113
	3.0	-3.969*	.267	.000	-4.720	-3.218
	4.0	-3.942*	.267	.000	-4.693	-3.190
12H	.0	1.587	.721	.279	-.440	3.614
	6H	.811*	.248	.011	.113	1.509
	3.0	-3.158*	.268	.000	-3.911	-2.405
	4.0	-3.131*	.268	.000	-3.884	-2.377
3.0	.0	4.745*	.728	.000	2.699	6.791
	6H	3.969*	.267	.000	3.218	4.720
	12H	3.158*	.268	.000	2.405	3.911
	4.0	.027	.286	1.000	-.775	.830
4.0	.0	4.718*	.728	.000	2.671	6.764
	6H	3.942*	.267	.000	3.190	4.693
	12H	3.131*	.268	.000	2.377	3.884
	3.0	-.027	.286	1.000	-.830	.775

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

The gene expression comparison among different tempraturs showed that, by six- and twelve-hours acclimatization's, in 28 °C gene expression is more stable in metabolite reaction in hemolynph in comparison with other studied tempraturs (Table 4.11).

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Table 4.12: Thermal stress influence on the apoptosis pathway coding gene expression in different tissues, 6h after the treatment (i), 12h after the treatment (j) in laboratory condition.

Table(g).Thermal stress influence on Apoptosis pathway in different tissues, 6h after the treatment			
6h	Hemo	Muscle	Hepato
C	10.54437	12.57645	14.4399
24°C	10.57851	12.59043	11.51252
28°C	16.50441	15.83118	16.17922
32°C	20.50443	17.83121	25.18631
36°C	15.12798	8.55032	8.557972

Table(g).Thermal stress influence on Apoptosis pathway in different tissues, 12h after the treatment			
6h	Hemo	Muscle	Hepato
C	10.54437	12.57645	14.4399
24°C	7.560969	6.602363	6.477722
28°C	18.63469	16.35187	18.75688
32°C	27.6347	20.35198	25.75688
36°C	6.558783	3.155012	8.557972

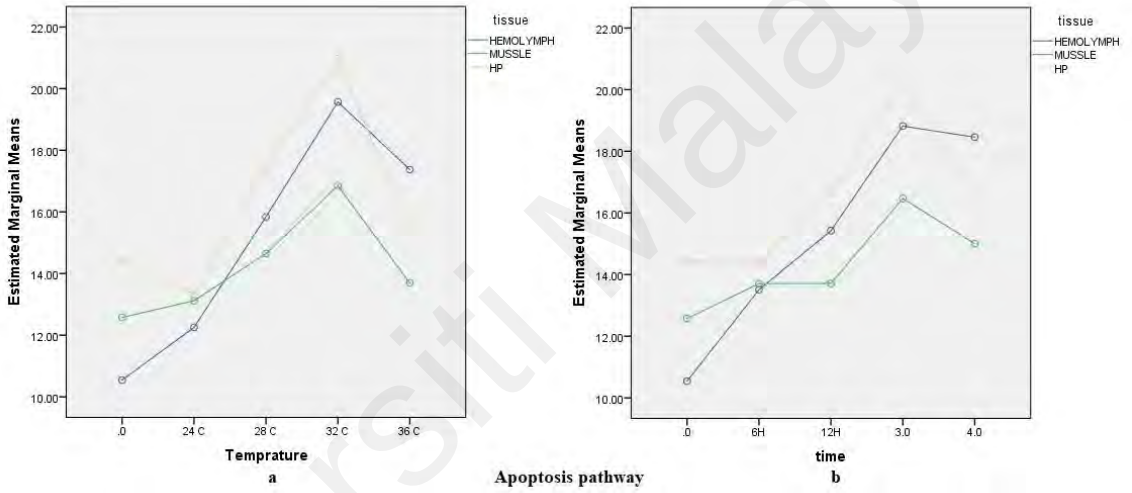

Figure 4.5: The figure shows Thermal stress influence on Apoptosis pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory (a) and lectin Pathway coding gene expression in different tissues, 6h after the treatment, and 12h after the treatment in laboratory.

The gene expression comparison among all the three studied organs showed that, by six hours acclimatization's, hemolymph tissue is more active in Apoptosis pathway in comparison with other two studied tissue (Table 4.12, 4.13, 4.14). But, by increasing the thermal acclimatization's time to twelve hours, the tissues gene expression activity changes and hepatopancreas tissue, act as the highest gene expression tissue (Figure 4.5).

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Table 4.13: Thermal stress influence the apoptosis pathway coding gene expression in different tissues, 6h after the treatment (c), 12h after the treatment (d) in laboratory condition.

Dependent Variable: **apoptosis pathway**

(I) Temprature	(J) Temprature	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
.0	24 C	-.385	.720	1.000	-2.410	1.639
	28 C	-3.455*	.721	.000	-5.480	-1.429
	32 C	-6.608*	.720	.000	-8.633	-4.584
	36 C	-2.939*	.720	.000	-4.964	-.914
24 C	.0	.385	.720	1.000	-1.639	2.410
	28 C	-3.069*	.264	.000	-3.812	-2.326
	32 C	-6.223*	.263	.000	-6.963	-5.483
	36 C	-2.554*	.263	.000	-3.293	-1.814
28 C	.0	3.455*	.721	.000	1.429	5.480
	24 C	3.069*	.264	.000	2.326	3.812
	32 C	-3.154*	.264	.000	-3.896	-2.411
	36 C	.516	.264	.512	-.227	1.258
32 C	.0	6.608*	.720	.000	4.584	8.633
	24 C	6.223*	.263	.000	5.483	6.963
	28 C	3.154*	.264	.000	2.411	3.896
	36 C	3.669*	.263	.000	2.930	4.409
36 C	.0	2.939*	.720	.000	.914	4.964
	24 C	2.554*	.263	.000	1.814	3.293
	28 C	-.516	.264	.512	-1.258	.227
	32 C	-3.669*	.263	.000	-4.409	-2.930

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

The gene expression comparison among different tempraturs showed that, by six- and twelve-hours acclimatization's, in 28 °C gene expression is more stable in metabolite reaction in hemolynph in comparison with other studied tempraturs (Table 4.13).

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Table 4.14: Pairwise Comparisons of apoptosis pathway coding gene expression in different tissues and different temperatures after the treatment in laboratory.

Dependent Variable: **apoptosis pathway**

(I) time	(J) time	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b] Lower Bound	Upper Bound
.0	6H	-1.350	.772	.804	-3.519	.819
	12H	-2.744*	.772	.004	-4.914	-.574
	3.0	-5.984*	.779	.000	-8.174	-3.793
	4.0	-4.170*	.779	.000	-6.360	-1.980
6H	.0	1.350	.772	.804	-.819	3.519
	12H	-1.394*	.266	.000	-2.141	-.647
	3.0	-4.633*	.286	.000	-5.437	-3.830
	4.0	-2.820*	.286	.000	-3.624	-2.016
12H	.0	2.744*	.772	.004	.574	4.914
	6H	1.394*	.266	.000	.647	2.141
	3.0	-3.240*	.287	.000	-4.046	-2.433
	4.0	-1.426*	.287	.000	-2.232	-.620
3.0	.0	5.984*	.779	.000	3.793	8.174
	6H	4.633*	.286	.000	3.830	5.437
	12H	3.240*	.287	.000	2.433	4.046
	4.0	1.813*	.306	.000	.954	2.672
4.0	.0	4.170*	.779	.000	1.980	6.360
	6H	2.820*	.286	.000	2.016	3.624
	12H	1.426*	.287	.000	.620	2.232
	3.0	-1.813*	.306	.000	-2.672	-.954

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

The gene expression comparison among different tempraturs showed that, by six- and twelve-hours acclimatization's, in 28 °C gene expression is more stable in metabolite reaction in hemolynph in comparison with other studied tempraturs (Table 4.14).

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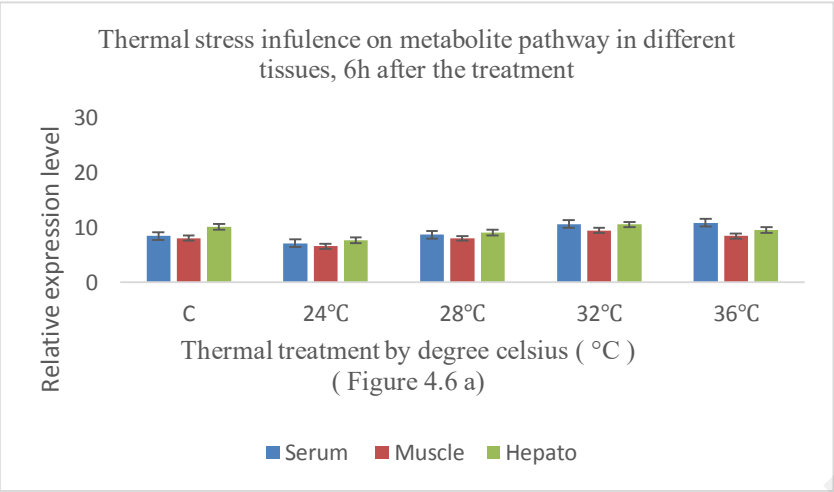

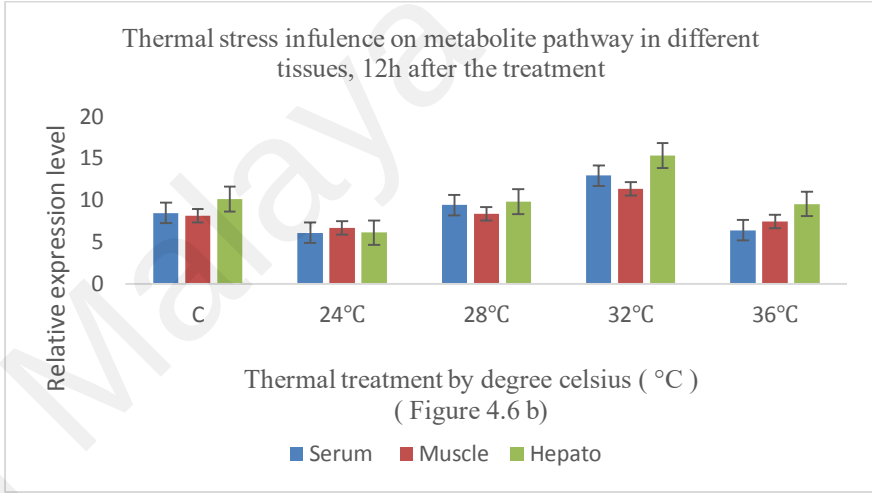

Figure 4.6: The picture shows Thermal stress influence on metabolite pathway in different tissues, 6h after the treatment (a), 12h after the treatment (b). A different color is used for organ differentiation. Blue, represents hemolymph, orange represents muscle and grey represent hepatopancreas.

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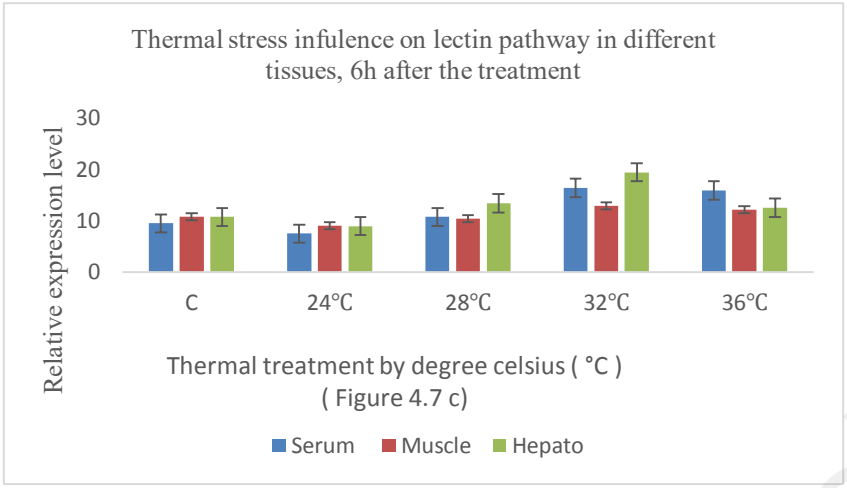

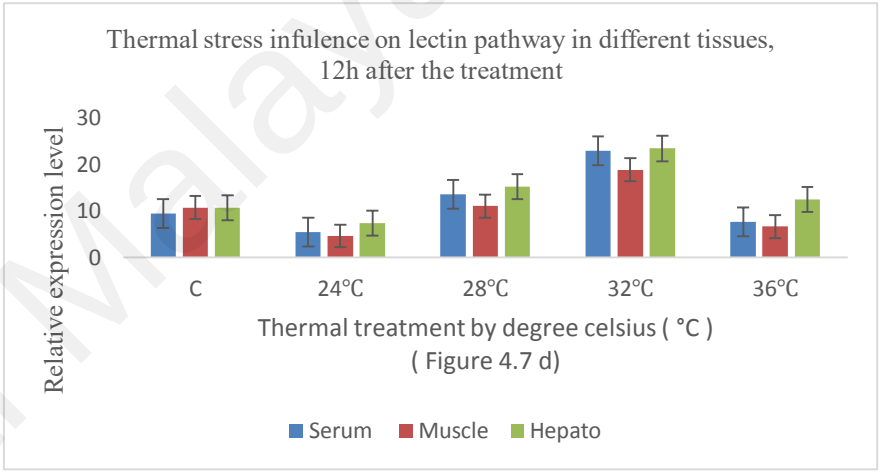

Figure 4.7: Continued. Thermal stress influences the lectin pathway in different tissues, 6h after the treatment (c), 12h after the treatment (d). A different color is used for organ differentiation. Blue, represents hemolymph, orange represents muscle and grey represent hepatopancreas.

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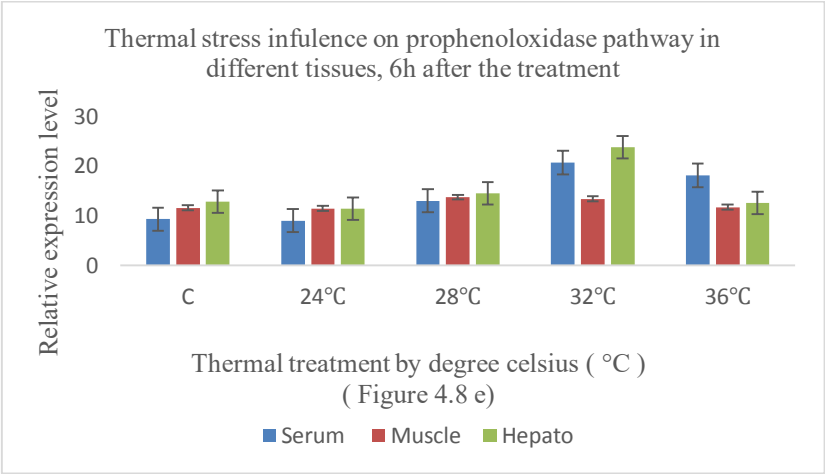

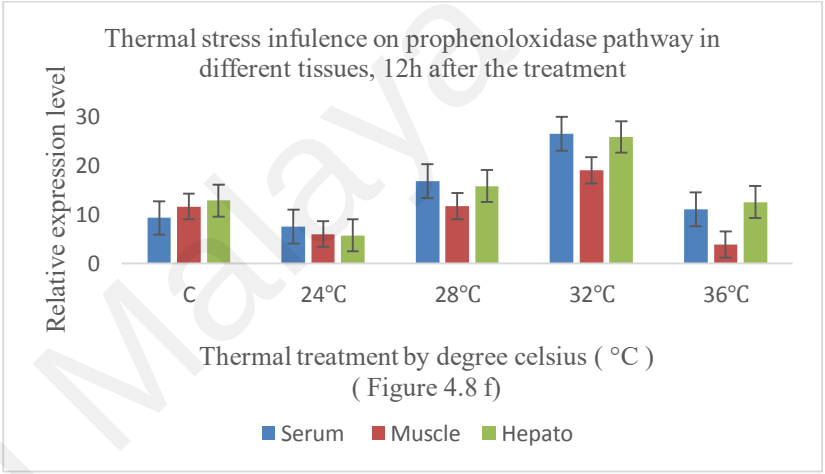

Figure 4.8: Continued. Thermal stress influences the lectin pathway in different tissues, 6h after the treatment (e), 12h after the treatment (f). A different color is used for organ differentiation. Blue, represents hemolymph, orange represents muscle and grey represent hepatopancreas.

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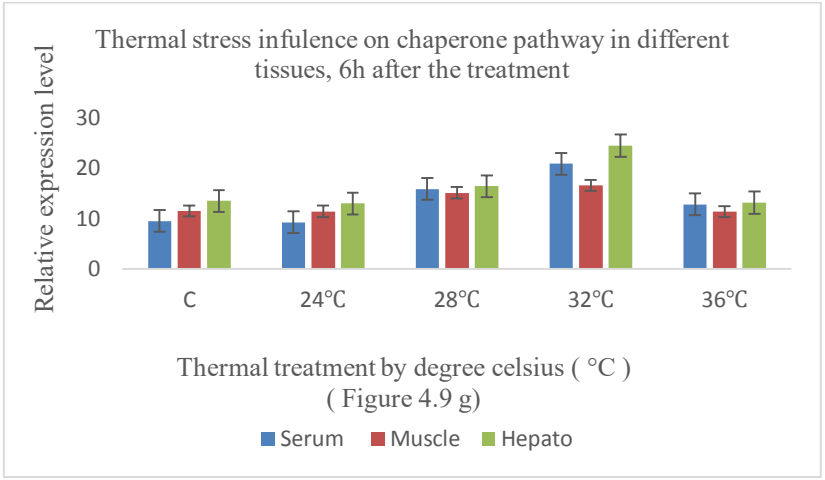

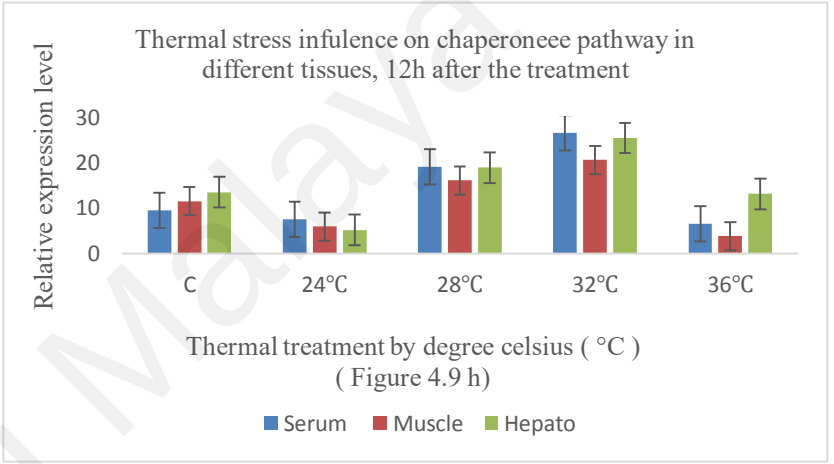

Figure 4.9: Continued. Thermal stress influences the lectin pathway in different tissues, 6h after the treatment (g), 12h after the treatment (h). A different color is used for organ differentiation. Blue, represents hemolymph, orange represents muscle and grey represent hepatopancreas.

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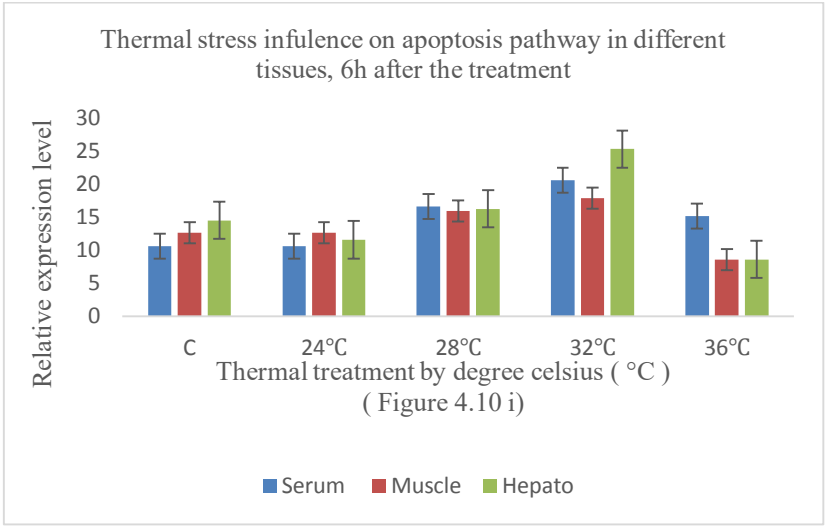

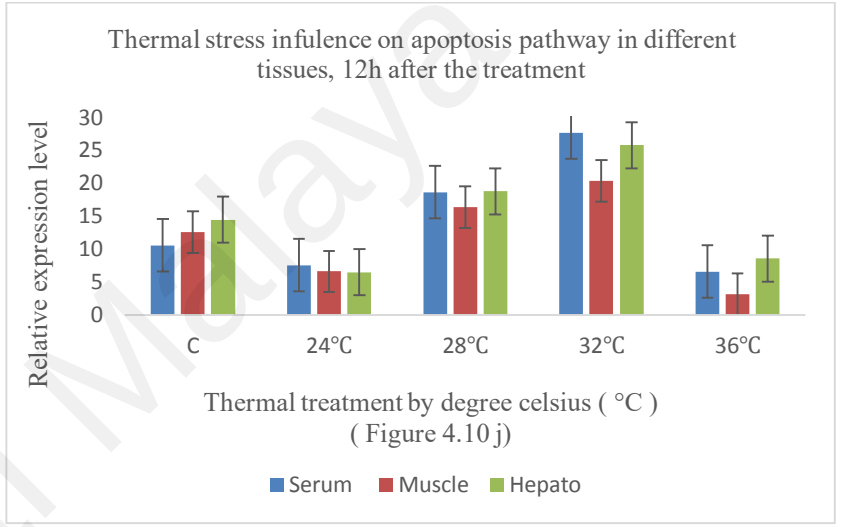

Figure 4.10: Continued. Thermal stress influence on the apoptosis pathway in different tissues, 6h after the treatment (i), 12h after the treatment (j). A different color is used for organ differentiation. Blue, represents hemolymph, orange represents muscle and grey represent hepatopancreas.

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The second concern in this study was to obtain insights into different gene cluster in adverse environmental circumstances. To achieve this purpose, we screened different gene clusters in three different studied tissues under three different environmental circumstances. Hemolymph, muscle, and hepatopancreas tissues were studied through metabolite, innate immune-related and environmental controller genes. So, the second hypothesis is:

H_0: Different gene cluster will have the same expression across the different tissue.

H_a: Different gene cluster will have the same expression across the tissue.

This study confirmed that, in the hemolymph tissue, the gene expression comparison among all the studied pathways coding gene showed that all of them have higher expression level in 32 °C, 36 °C and expression rate dropped at 28 °C, 24 °C, respectively, in both six- and twelve-hours post-stress thermal acclimatization. It is also illustrated that Chaperone and prophenoloxidase pathways have higher expression levels in comparison with metabolite, lectin, chaperone, apoptosis and Ppo genes, in both six- and twelve-hours post-stress acclimatization in hemolymph (Table 4.15 to 4.17).

Table 4.15: The Table shows Thermal stress influence a different pathway in hemolymph, 6h after the treatment (a), 12h after the treatment (b) in laboratory condition.

6h	Lectin	Metabolite	PPO	Chapron	Apoptosis
C	9.440538	8.470115	9.298664	9.476048	10.54437
24°C	7.452775	7.10063	9.015934	9.199444	10.57851
28°C	10.6994	8.671132	13.02228	15.81477	16.50441
32°C	16.36999	10.57967	20.69258	20.81888	20.50443
36°C	15.86374	10.84881	18.13482	12.76807	15.12798

6h	Lectin	Metabolite	PPO	Chapron	Apoptosis
C	9.440538	8.470115	9.298664	9.476048	10.54437
24°C	5.418887	6.086935	7.499195	7.494892	7.560969
28°C	13.52376	9.418013	16.7753	19.03053	18.63469
32°C	22.93427	12.91831	26.44635	26.56018	27.6347
36°C	7.643291	6.406583	11.04564	6.556685	6.558783

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Table 4.16: The Table shows thermal stress influence a different pathway in muscle, 6h after the treatment (c), and 12 h after the treatment (d) in laboratory condition.

6h	Lectin	Metabolite	PPO	Chapron	Apoptosis
C	10.72268	8.03729	11.60447	11.48105	12.57645
24°C	8.961151	6.607312	11.46868	11.37197	12.59043
28°C	10.3389	8.016558	13.72692	15.05703	15.83118
32°C	12.84418	9.443917	13.38854	16.55704	17.83121
36°C	12.088	8.444943	11.7178	11.3653	8.55032

12h	Lectin	Metabolite	PPO	Chapron	Apoptosis
C	10.72268	8.144469	11.60447	11.48105	12.57645
24°C	4.63869	6.676872	6.003088	5.951996	6.602363
28°C	11.03524	8.379649	11.66856	16.10062	16.35187
32°C	18.83421	11.37408	19.00794	20.60068	20.35198
36°C	6.603565	7.412852	3.864946	3.815271	3.155012

Table 4.17: The Table shows thermal stress influence a different pathway in hepatopancreas, 6h after the treatment (e), and 12 h after the treatment (f) in laboratory condition

6h	Lectin	Metabolite	PPO	Chapron	Apoptosis
C	10.69348	10.12069	12.80299	13.46277	14.4399
24°C	8.915808	7.672541	11.46881	12.93831	11.51252
28°C	13.38675	9.04858	14.4684	16.36506	16.17922
32°C	19.38828	10.54008	23.80184	24.36772	25.18631
36°C	12.49883	9.542155	12.5286	13.10362	8.557972

12h	Lectin	Metabolite	PPO	Chapron	Apoptosis
C	10.69348	10.12069	12.80299	13.46277	14.4399
24°C	7.387356	6.107793	5.748359	5.136358	6.477722
28°C	15.21091	9.795831	15.77974	18.92013	18.75688
32°C	23.41331	15.2973	25.77988	25.42137	25.75688
36°C	12.49883	9.542155	12.5286	13.10362	8.557972

The gene expression comparison among all the five studied pathways coding gene groups showed that they have higher expression level at 32 °C, 28 °C and expression rate dropped at 36 °C, 24 °C, respectively, in both six- and twelve-hours post-stress in hemolymph. It is also illustrated that Chaperone and prophenoloxidase pathways and PRRs genes have higher expression levels in comparison with metabolite, lectin in both six- and twelve-hours post-stress in hemolymph. The comparison between two time point

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sampling shows that all the genes have higher expression at 32 °C, 28 °C and expression rate dropped at 24 °C, 36 °C in twelve hours post stress but 32 °C, 28 °C and expression rate dropped at 36 °C, 24 °C in six hours post-stress in muscle.

The gene expression comparison among all the three studied organs showed that by six hours acclimatization's, metabolite and immune-related pathways Ppo, prophenoloxidase, lectin have more activity and gene expression increasing in hepatopancreas, hemolymph, and muscle, respectively (Figure 4.11 to 4.13) and suplemenry data on gene expression is available in appendix IV.

The overall pattern of the all tissues gene expression in hemolymph shows that there is a steady increase by temperature increase up to 32 °C, but there is a sharp gene expression decrease after long hours thermal acclimatization's which shows the level of the gene expression goes back to the control level at 36 °C. It confirms that animals are adapting through gene adaption process by long-term stress (Figure 4.11).

The overall pattern of the all tissues gene expression in muscle shows that there is a steady increase by temperature increase up to 32 °C, but there is a sharp gene expression decrease after long hours thermal acclimatization's which shows the level of the gene expression goes back to the control level at 36 °C. It confirms that animals are adapting through gene adaption process by long-term stress (Figure 4.12).

The overall pattern of the all tissues gene expression in heptopanceras shows that there is a steady increase by temperature increase up to 32 °C, but there is a sharp gene expression decrease after long hours thermal acclimatization's which shows the level of the gene expression goes back to the control level at 36 °C. It confirms that animals are adapting through gene adaption process by long-term stress (Figure 4.13).

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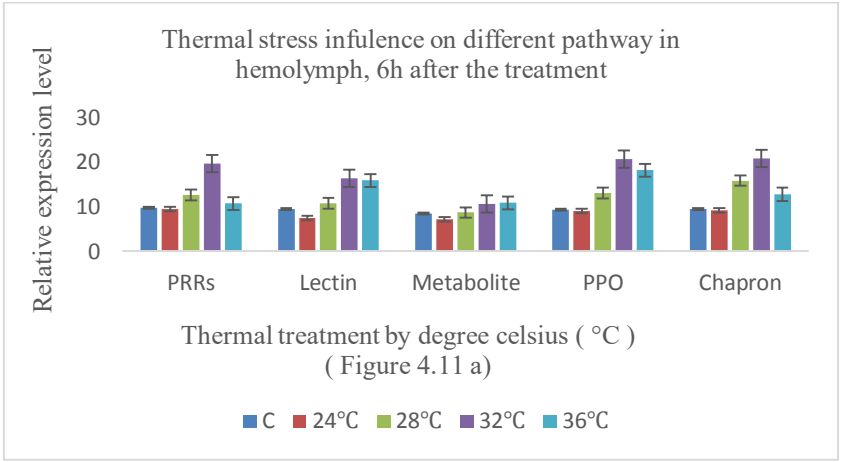

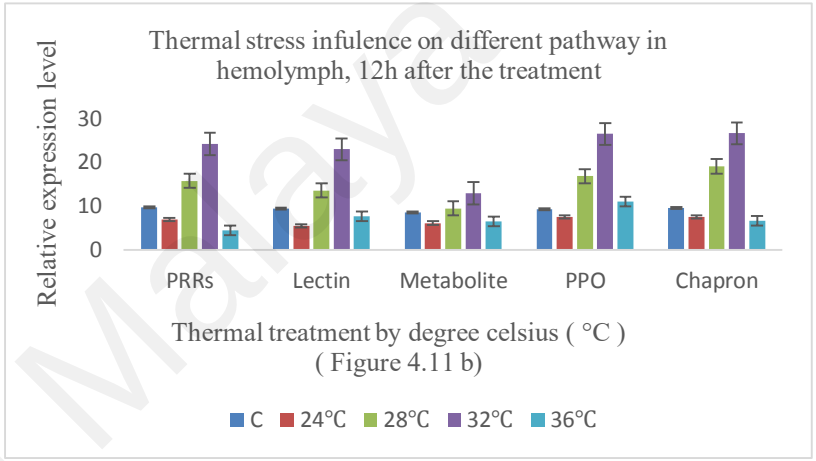

Figure 4.11: Thermal stress influence a different pathway in hepatopancreas, 6h after the treatment (a), and 12 h after the treatment (b). Different color has been used for organ differentiation. Blue, represents Control, orange represents 24 °C and grey represent 228 °C, yellow represent 32 °C and dark blue represent 36 °C.

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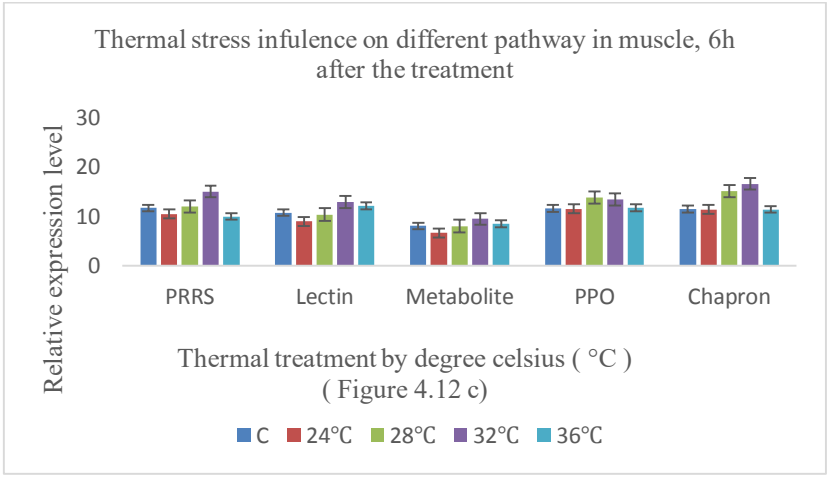

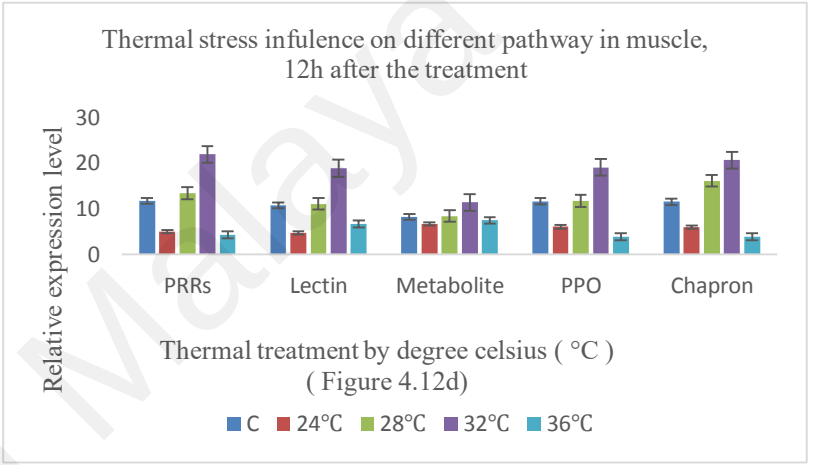

Figure 4.12 Continued. Thermal stress influences a different pathway in hepatopancreas, 6h after the treatment (c), and 12 h after the treatment (d). Different color has been used for organ differentiation. Blue, represents Control, orange represents 24 °C and grey represent 28 °C, yellow represent 32 °C and dark blue represent 36 °C.

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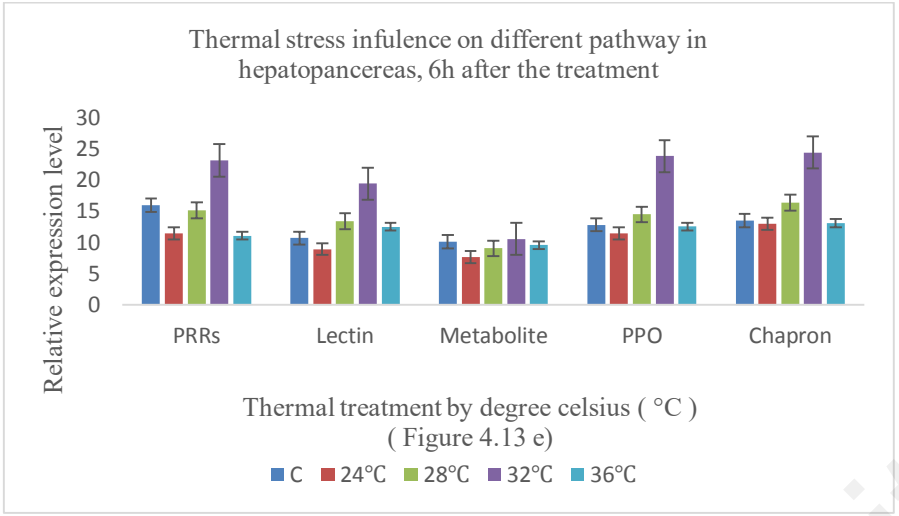

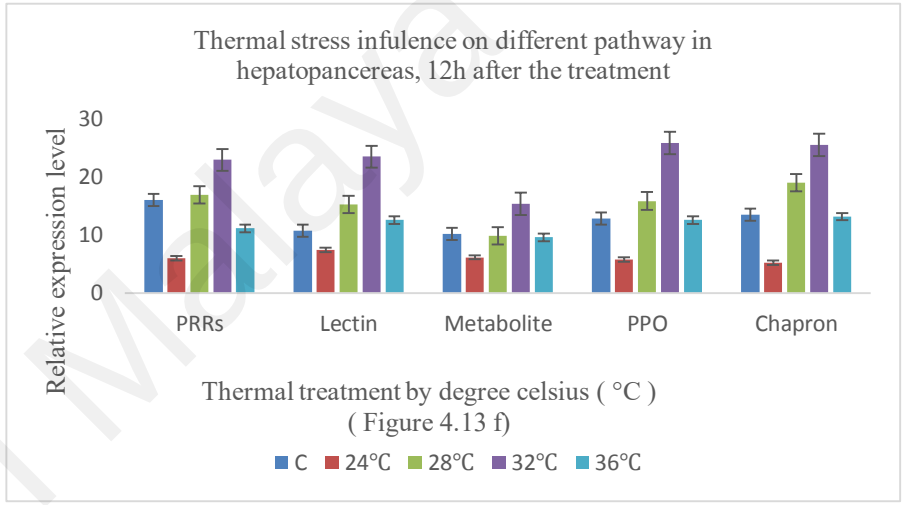

Figure 4.13: Continued. Thermal stress influence a different pathway in hepatopancreas, 6h after the treatment (e), and 12 h after the treatment (f). Different color has been used for organ differentiation. Blue, represents Control, orange represents 24 °C and grey represent 28 °C, yellow represent 32 °C and dark blue represent 36 °C.

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The gene expression comparison among all the five studied pathways coding gene groups showed that they have higher expression levels at 32 °C, 28 °C and expression rate dropped at 36 °C, 24 °C, respectively, in both six- and twelve-hours post-stress in hemolymph. It is also illustrated that Chaperone and prophenoloxidase pathways and Ppo genes have higher expression levels in comparison with metabolite, lectin in both six- and twelve-hours post-stress in hemolymph. The comparison between two time point sampling shows that all the genes have higher expression at 32 °C, 28 °C and expression rate dropped at 24 °C, 36 °C in twelve hours post stress but 32 °C, 28 °C and expression rate dropped at 36 °C, 24 °C in six hours post-stress in hepatopancreas.

4.1.2 Non-Genetic adaptation:

Morphological parameters

Morphological parameters are considered as non-genetic adaptions factors which are temporary symptoms and are not passed to the next generation.

H_0: There is the presence of non-genetic adaption to thermal tolerance

H_a: There is non- presence of non-genetic adaption to thermal tolerance

H_0: GMP will adapt physiologically in line to temperature changes.

H_a: GMP will not-adapt physiologically in line to temperature changes.

To achieve more insights into this objective, morphological parameters were studied in laboratory condition. The morphological parameters such as weight, body color, hemocyte color, abdomen and uropods, hepatopancreas somatic index (HSI) has been studied before and after the experiment.

The physiological parameters such as total hemocyte count, hemocyte Cell viability assays, phenoloxidase activity, protein quantification and histo-architecture has been studied before and after the experiment.

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4.1.2.1 Weight

The comparison between the weight of the control and heat-treated groups showed that the metabolism rate increased. As a result, the weight of the exposed animal groups in comparison with the control group was significantly reduced. The average weight of the control group was 26.22± 0.55, and the average weights of the treated group were 25.36±0.99, 25.68±0.99, 25.63±1.3, 25.63±1.42 respectively for 24 °C, 28 °C, 32 °C, 36 °C.

4.1.2.2 Body color

The body color of normal prawn was usually greenish to brownish grey (Figure 4.14). The heat-treated prawn showed discoloration such as whitish, orange dot-like pattern or dusty grey.

Figure 4.14: Representative images of the body color variation and rostral (broken) and black dots and flakes between control (A) and treated prawn, 12 hours under 32 °C (B).

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4.1.2.3 Hemocyte color

The hemocyte color of normal prawn was usually blue or greenish blue (Figure 4.15). The heat-treated prawn showed dark coloration by increasing time and temperature and discoloration in 12h after the test at 36 °C.

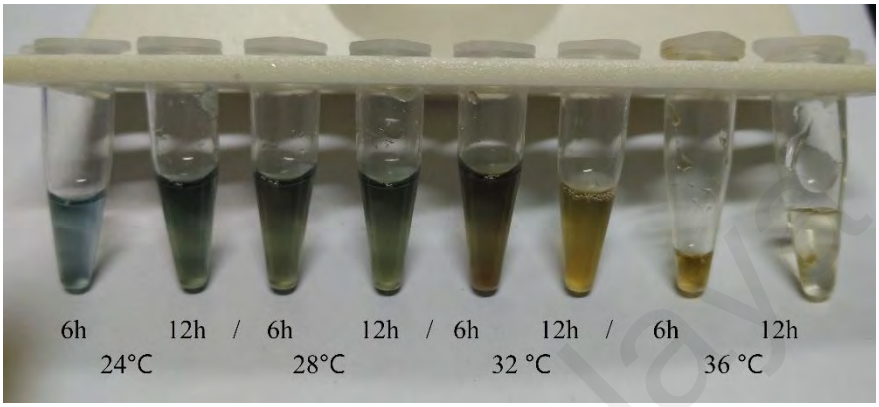

Figure 4.15: Representative Image of the hemocyte color variation by temperature changes and time points.

4.1.2.4 Abdomen and Uropods

Compared to the normal animal, the treated prawn exhibited black spots and black formation around their 2nd, 3rd somites. Also, deformed uropods and telson were found in treated prawns at high temperature.

4.1.2.5 Hepatopancreas Somatic Index (HSI)

The mean HIS value of control group was 2.4±0.06. The mean body weight and HIS values for prawns in control groups increased significantly between 24 °Cand 28°Cdegrees and decreased significantly at 32°Cand 36°Cduring the experimental period (Figure 4.16).

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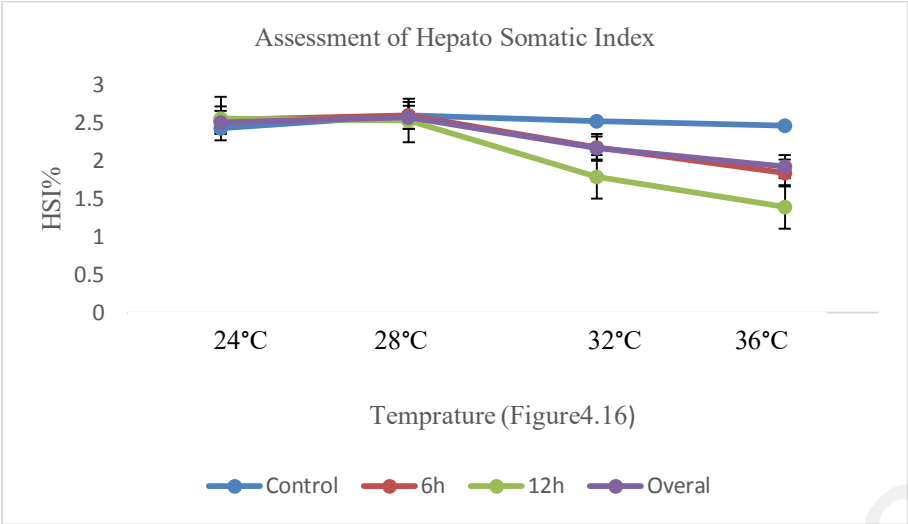

Figure 4.16: The chart shows the assessment of hepato somatic index. While blue color is representing the control samples, orange color represents 6h after the thermal shock, grey color represents 12h after the thermal shock, and yellow color represents the overall behavior of the HIS index during thermal shock.

4.1.2.6 Total hemocyte count

Statistical analysis showed that there was a significant difference ($P<0.05$) for total hemocyte cell count between the control group and heat-treated group. The analyzed data showed that the number of the hemocyte cells at 24 ℃ is in the same range between the two-time points. But there is a significant increase in the hemocyte concentration in twelve hours at 28℃ while there is a significant decrease at twelve-time points at 36℃. It confirms that the number of the hemocyte is significantly decreased by increasing the temperature up to 28 °C and it has a significant sharp decrease by increasing the temperature at 32 °C and 36 °C, respectively (Figure 4.17).

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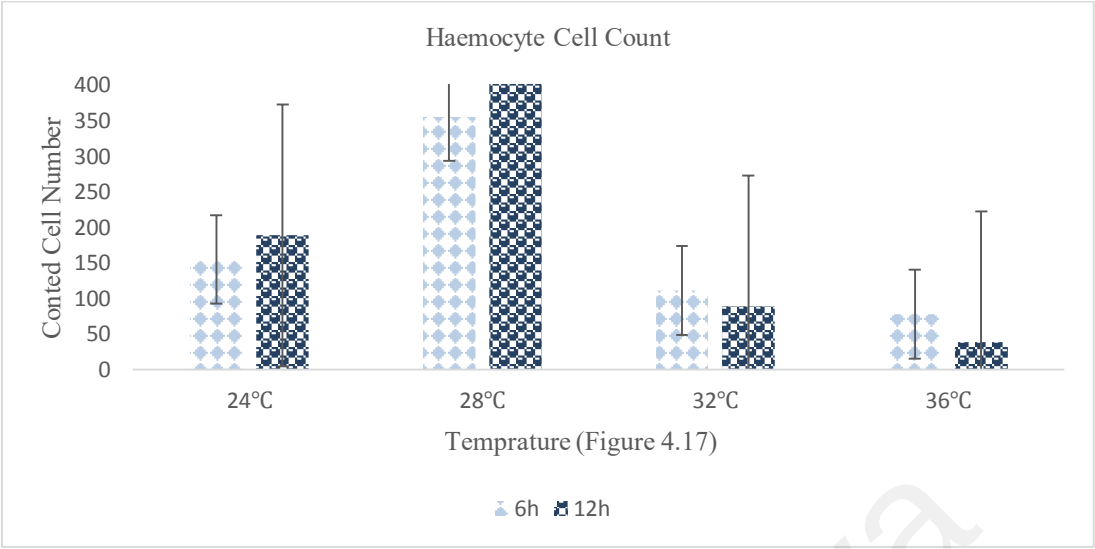

Figure 4.17: The chart shows the haemocyte cell count at different temperatures and time points. Light blue is representing 6h after the thermal shock, and dark blue is representing 12 after the thermal shock.

4.1.2.7 Hemocyte Cell viability assays

Based on the MTT assays, increasing temperature has a negative effect on hemocyte cells viability. High temperature decreased cell viability in hemocyte (13–18% decrease, depending on the temperature) ($p<0.05$) (Figure 4.18).

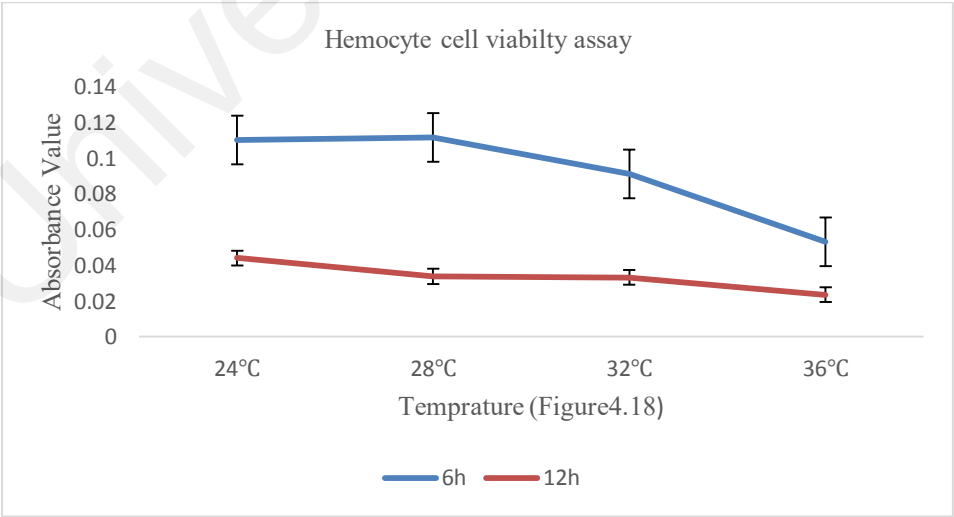

Figure 4.18: The chart shows the Hemocyte cell viability assay at different temperatures and time points. The blue color is representing 6h after the thermal shock and orange are representing 12 after the thermal shock.

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4.2.1.8 Phenoloxidase activity

The assessment of the changes in enzymatic oxidation of the substrate L-DOPA revealed a linear decrease of PO activity by increasing the temperature of the plasma. This linear decrease in PO activity was recognized for the enzymatic conversion of o-diphenols into o-quinones by phenoloxidase enzymes in met and oxy forms (Figure 4.19).

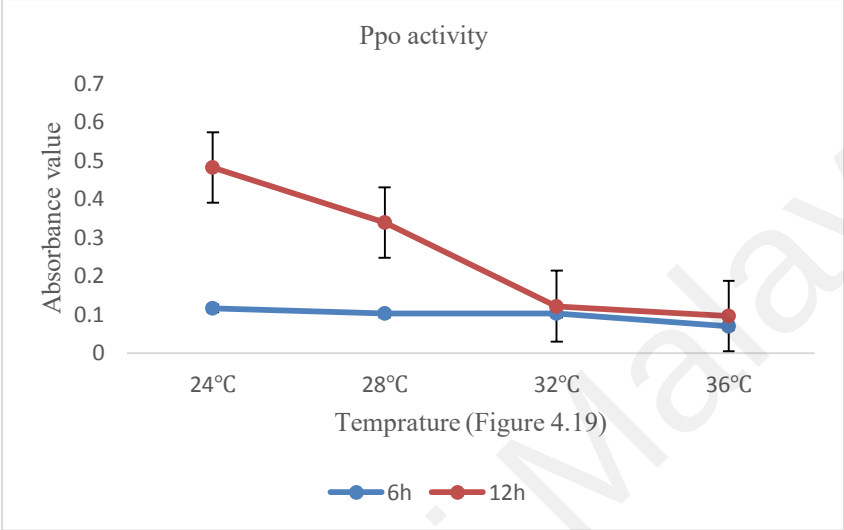

Figure 4.19: The chart shows the PPO activity at different temperatures and time points. The blue color is representing 6h after the thermal shock and orange are representing 12 after the thermal shock.

4.1.2.9 Protein quantification

The assessment of the Bradford assay protein quantification revealed a linear decrease of PO activity by increasing the temperature for hemocyte, muscle, and hepatopancreas. This linear decrease in total protein content demonstrates a high decrease in the enzymatic activities either metabolical or immunological (Figure 4.20).

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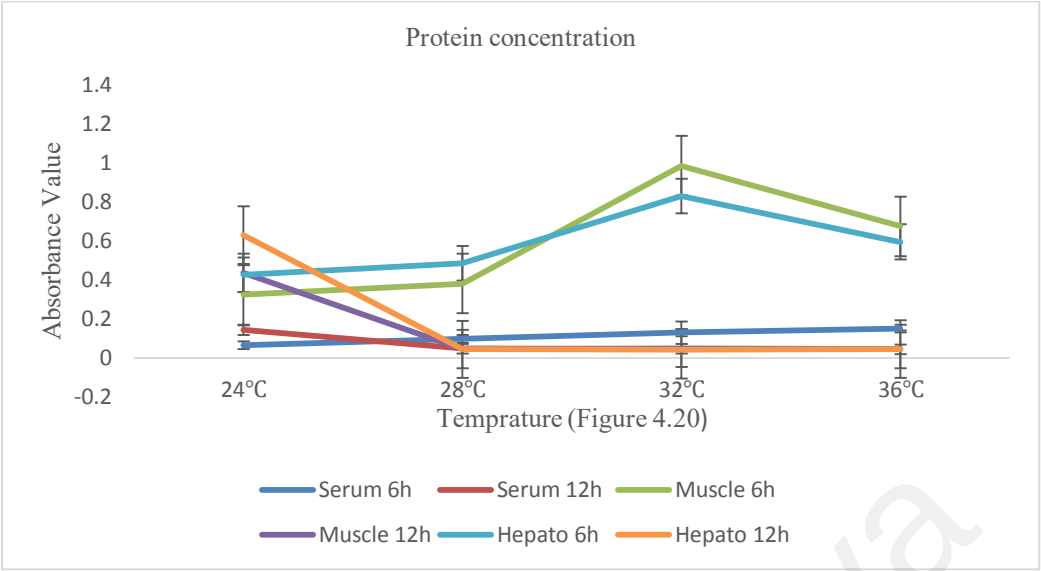

Figure 4.20: The chart shows the protein concentration at different temperatures and time points. The blue color is representing 6h after the thermal shock and orange are representing 12 after the thermal shock.

The chart shows the Protein concentration at different temperatures and time points. Light blue color is representing hemolymph protein concentration 6h after the thermal shock, and orange color is representing hemolymph protein concentration 12h after the thermal shock. Grey color is representing muscle protein concentration 6h after the thermal shock, and the yellow color is representing muscle protein concentration 12h after the thermal shock. Dark blue color is representing hepatopancreas protein concentration 6h after the thermal shock, and the green color is representing hepatopancreas protein concentration 12h after the thermal shock.

4.1.2.10 Histo-architecture of the prawns exposed to the thermal shock

4.1.2.10.1 Hepatopancreas

The histoarchitecture of prawn's hepatopancreas resulted in changes in the number of B-cells (Blasenzellen cells), R-cells (Restzellen cells), E-cells (Embryonic cells), F-cells (Fibrillenzellen cells), the interstitial-sinus was filled with abnormal infiltrated hemocytes (AIH), and the epithelial-tubule showed ruptured basal laminae (RBL)

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(Figure 4.21). The histoarchitecture of hepatopancreas group exhibited a number of E-Cell, IS, HI in the interstitial sinus of the hepatopancreas tubules, coagulation (CO), the formation of the abnormal lumen (ALU) and necrotic Hepatopancreatic tubule (NT) were observed by increasing the temperature (Figure 4.21). Similarly, the histopathological changes such as hemocyte infiltration (HI) in the interstitial sinus of the hepatopancreas tubules, the formation of the abnormal lumen, Blasenzellen cell, thickened basal laminae (TBL), Tissue debris (TD) were observed at 36 °C treated animal groups (Figure 4.21). The histoarchitecture of hepatopancreas in the groups showed tissue debris, hemocyte infiltration, coagulation, necrotic hepatopancreas tubule (NT), ruptured basal laminae (RBL) and necrotic cells of hepatopancreas (NCH) by increasing temperature (Figure 4.21).

4.1.2.10.2 Muscle

The histoarchitecture of muscle in groups showed abnormalities in muscle tissues and necrotic muscles infiltrated by hemocytes (Figure 4.22). The groups exhibited structurally altered muscle fibers, necrotic striated muscles, and shrinkage of muscular fibers (Figure 4.22). In groups, necrosis and lesions in striated muscle cells and increased eosinophilia of the cytoplasm and pyknotic conditions were seen small arrows in the (Figure 4.22). In heat-treated groups, necrotic striated muscle and muscle fibers, complete degenerated muscle fibers with fragmentation and flocculation, damaged myofilaments, the formation of tissue dippers in the muscle of treated *M. rosenbergii* (Figure 4.22) were observed.

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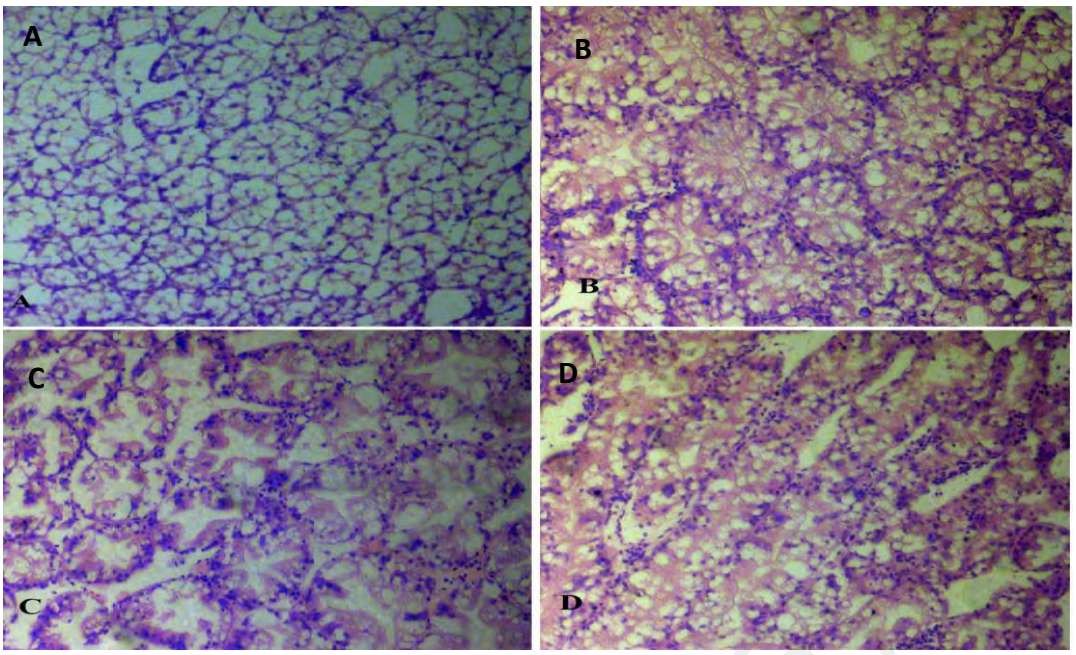

Figure 4.21: (A) Histological cross sections of the hepato of 24 ℃ the formation of the abnormal lumen, Blasenzellen cell, thickened basal laminae (TBL), Tissue debris (TD) (10× H&E). (B) Histological cross sections of the hepatopancreas of 28 ℃ exposed group showing healthy and normal hepato tissues (10× H&E stain). (C and D) Histological cross sections of the hepato of 32 ℃ and 36 ℃ treated prawns showing Tubule coagulation (10× H&E).

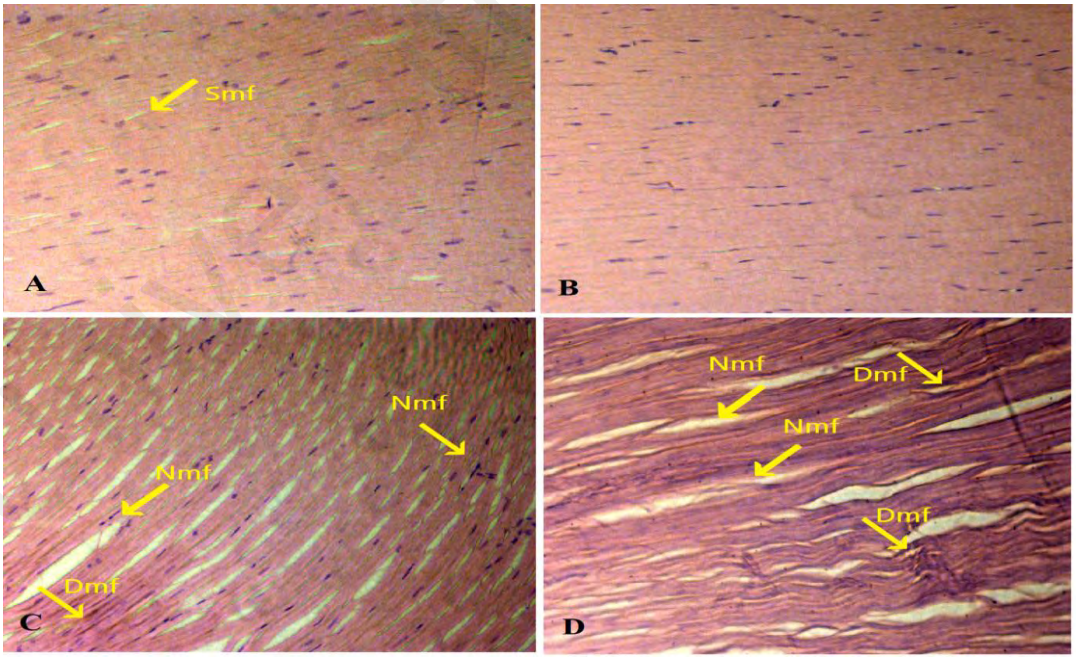

Figure 4.22: (A) Histological cross sections of the muscle of 24 ℃ exposed group Shrinkage of muscular fiber (SMF) (10× H&E). (B) Histological cross sections of the muscle of 28 ℃ exposed group showing healthy and normal muscle tissues, striated muscle fibers clearly (10× H&E stain). (C and D) Histological cross sections of the muscle of 32 ℃ and 36 ℃ exposed group showing necrotic musculature (NMF) and degenerated muscle fibers (DMF) (10× H&E).

4.1.2.10.3 Histological gradations

In control condition for prawns, normal histological structures were noted. In treated groups, the histopathological severity increases as temperature increases. At 36 °C, the extended severe histological changes observed when compared to control and other groups. Based on the percentage of the severity of hepatopancreas and muscles histology, five gradations were formulated to have holistic information on the temperature impact on *M. rosenbergii* (Tables 4-18, 4-19). The gradations were −: None (0%), + mild (<10%), ++: moderate (10–50%), +++: severe (50–70%), ++++: extended severe (>70%).

Table 4.18: The table shows the Effect of thermal shock in Muscle histology *M. rosenbergii* −−: None (0%), +: mild (<10%), ++: moderate (10–50%), +++: severe (50–70%). Data were represented as a mean of 125 slides (25 slides/prawn) in each group.

Effect of thermal shock in Muscle histology *M. rosenbergii*

Cells	Temperature				
	Control	24°C	28°C	32°C	36°C
Necrotic muscle fibers	_	-	-	++	+++
Necrotic striated muscle	_	-	-	++	++
Degeneration	_	_	-	++	++
Mean	_	_	-	++	++++

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Table 4.19: The table shows the Effect of thermal shock in Hepatopancreas histology *M. rosenbergii*. —: None (0%), +: mild (<10%), ++: moderate (10–50%), +++: severe (50–70%). Data were represented as a mean of 125 slides (25 slides/prawn) in each group.

Effect of thermal shock in Hepatopancreas histology *M. rosenbergii.*

Cells	Temperatures				
	Control	24°C	28°C	32°C	36°C
Necrotic tubule	_	-	-	++	+++
R cell	_	-	-	++	++
B cell	_	_	-	++	+++
E cell	_	_	_	++	+++
F cell	_	_	_	++	+++
Thickened Basal Laminae	_	_	_	++	+++
Infiltrated hemocytes	_	_	_	++	+++
Tissue debris	_	_	_	++	+++
Tubule coagulation	_	_	_	++	+++
Mean	_	_	-	++	++++

4.1.3 Correlation studies and gene network

To get more insights into the studied gene correlations and studied gene network analysis based on the gene expression reading in different time, temperatures and environmental circumstance, the gene expression data is analysed by JMP. SAS for correlation study and Gene network prediction program. Since hepatopancreas shows more significance gene expression in comparison with the hemolymph and muscle, its correlation study and gene network analysis are presented in result chapter. Hemolymph and muscle analysis are demonstrated in appendixII.

The multivariate gene expression shows that in control condition while the animal is not under environmental stress, the gene expression pattern shows less metabolite activity, so animal is in tun state and the genes shows negative correlations. By increasing

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the temperature animal move to the active stage in 28 °C which is reported previously as best temperature for *M. rosenbergii.*in this stage PPO, chaperone and Hsp 70 showed positive correlation while the rest of the genes are in negative correlation or tun state. In 32 °C, the overall gene expression patter shows fluctuations in relation between the gene's expression pattern. In early stage of 36 °C, all the studied gene shows positive correlation which shows high metabolite activity in cells. In morphological observation in this stage we observed that the animals in laboratory condition showed weight loose, decrease in hemocyte cell count, Ppo activity and hemocyte color and cell viability. The correlation study confirms that all the studied gene can be used as biomarkers in 32 °C. This researches data interpretation confirms that Hsp70, Ppo1 and PpoIII can be consider as potential biomarkers for study *M. rosenbergii* genomic adaption under thermal stress (Figure 4.23 to 4.25) and suplemenry data on gene expression is available in apendixII.

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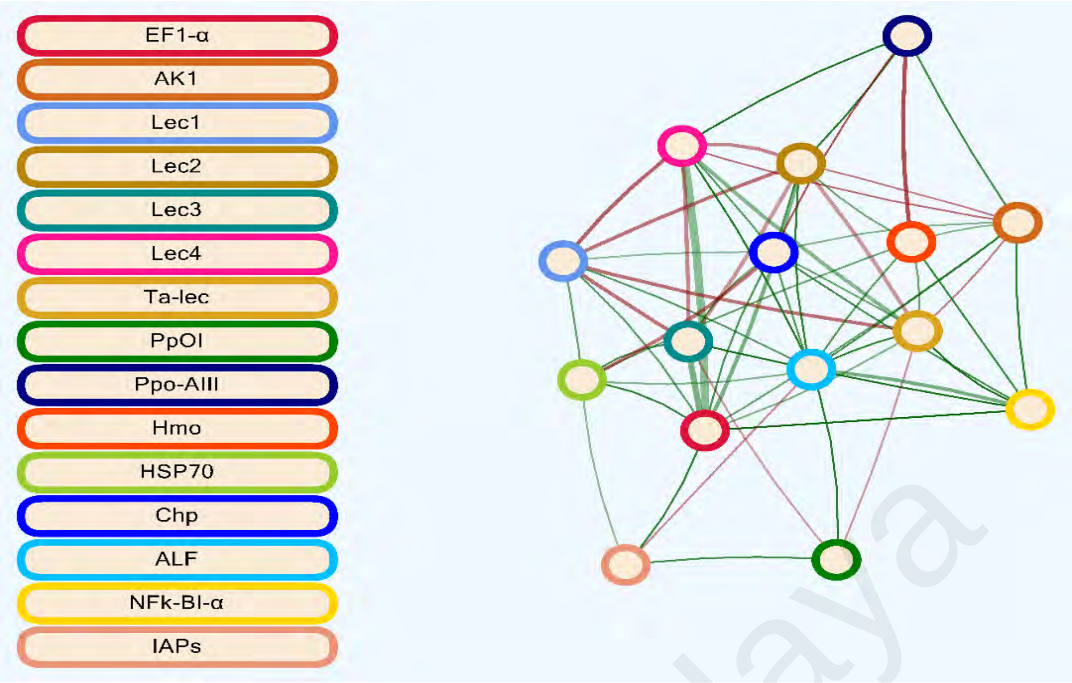

Figure 4.23: Hepatopancreas gene network prediction in laboratory control condition.

Figure 4.23, 4.24, 4.25 are showing Gene network prediction in hepatopancreas in laboratory condition. The table on the left of each picture is resembling the under studied genes which are represented by a specific color in this gene network demonstration. The pictures are showing the Thermal stress influence a different genes correlation in networking format in Hepatopancreas in laboratory control condition. The pictures are showing the Thermal stress influence a genes cluster and gene network prediction in Hepatopancreas, control (a) 6h after the treatment 24 °C (b), 12h after the treatment 24 °C (c). Thermal stress influences a different pathway in Hepatopancreas, 6h after the treatment 28 °C (d), 12h after the treatment 28 °C (e). 6h after the treatment 32 °C (f), 12h after the treatment 32 °C (g). 6h after the treatment 36 °C (h), 12h after the treatment 36 °C (i). Different color demonstrates the different genes. The refrence color is demonstrated in the left side of the picture and each buble represent the same color gene.

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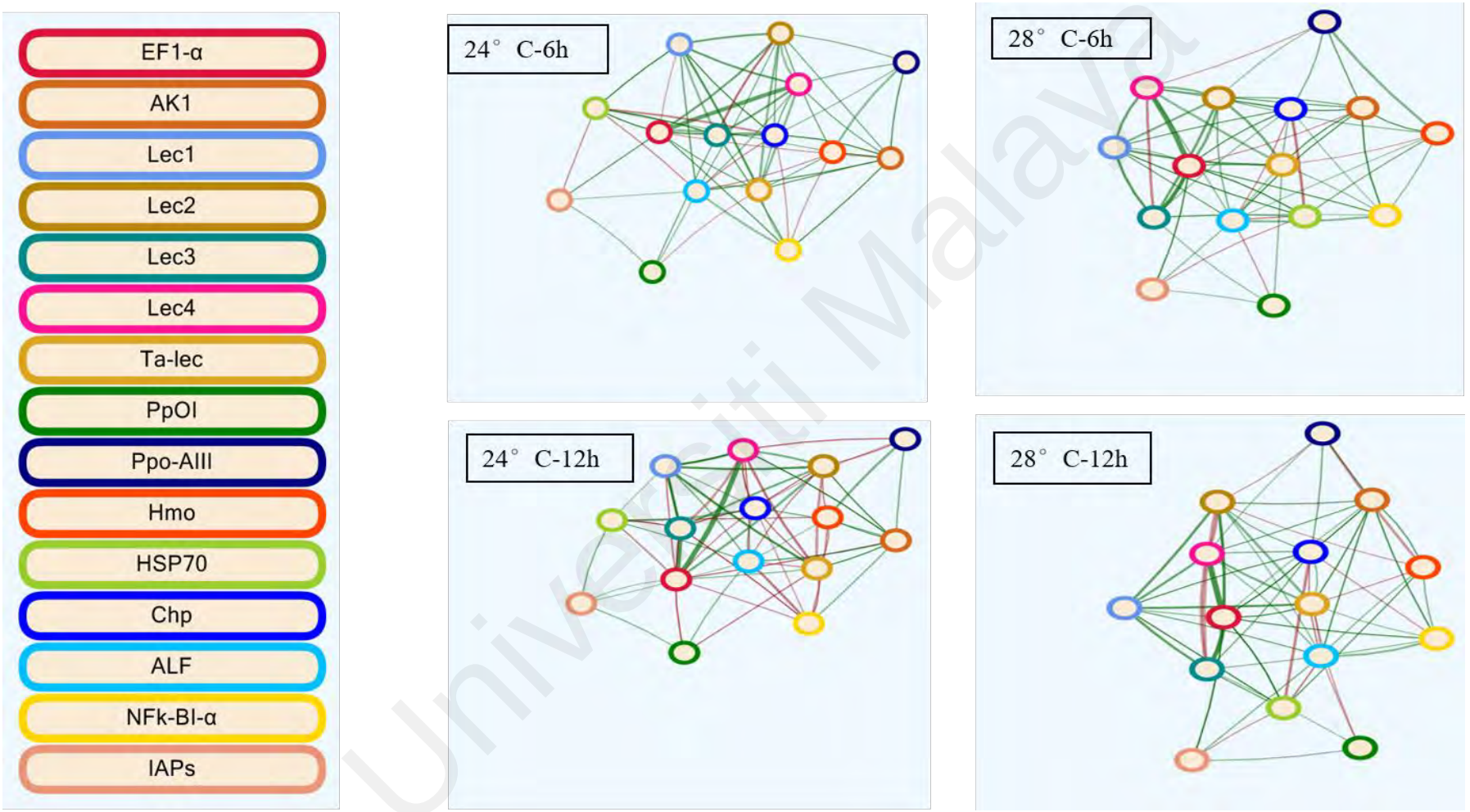

Figure 4.24: Hepatopancreas gene network prediction in laboratory control condition.

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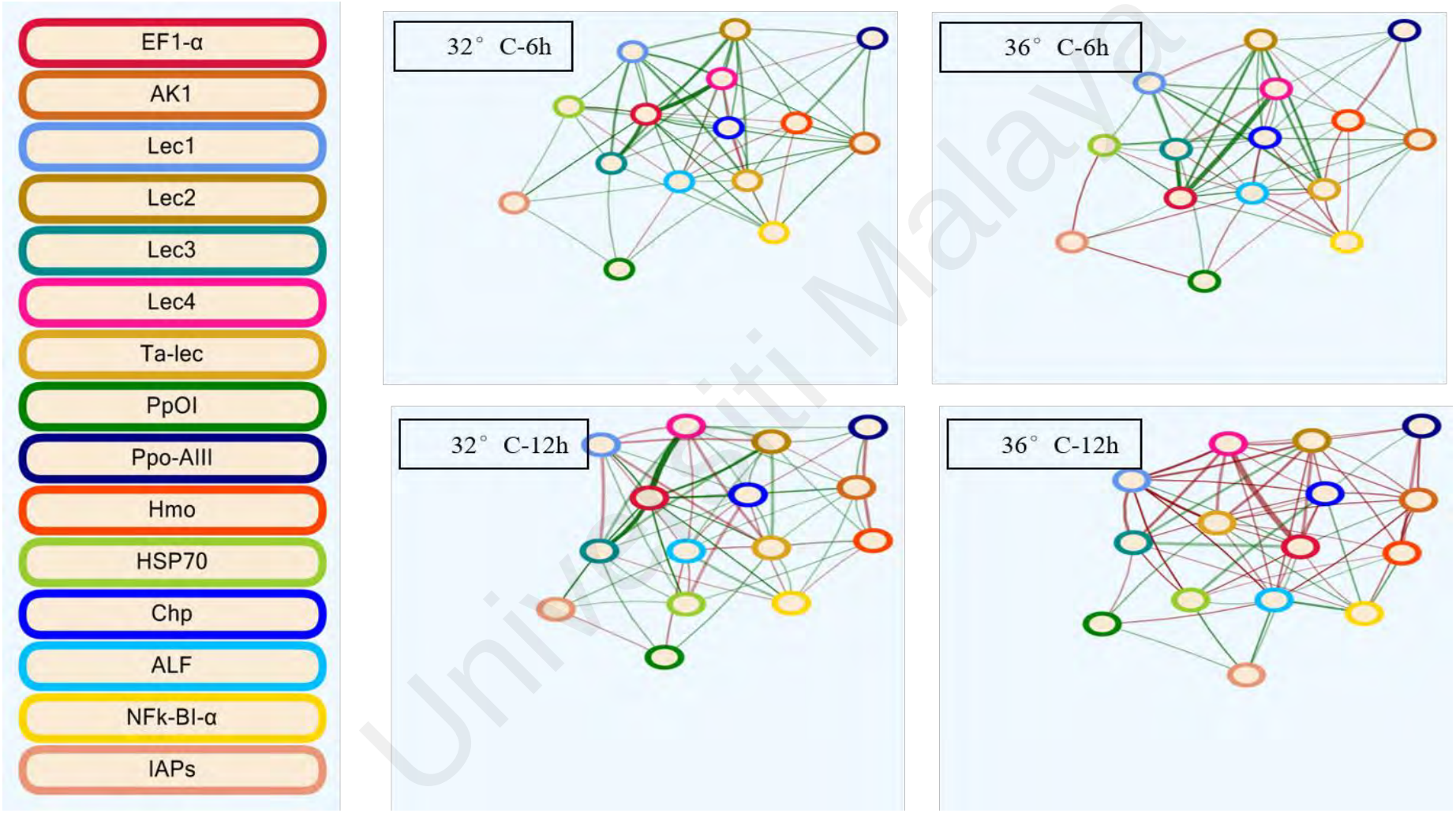

Figure 4.25: Hepatopancreas gene network prediction in laboratory condition.

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4.2 Farms experiment

Environmental circumstances adaption in well-managed farm and bad-managed farm. Analysis of the sample collection from a population is the initial source of the result for genetics and genomic assays. The analytical factors are consist of molecular markers from the molecular level at DNA, RNA or protein. In such this study a large number of samples with high throughput reading data is needed for accurate statistical analysis achievement.

All the organisms have a complex of interactive genes which are interacting in molecular level while they are interacting with environmental factors, simultaneously. These simultaneous interactions create a complex of gene-environment interactions which later on create the adaption of the individuals under advert circumstances.

Phenotype expression is an environmental-dependent factor. The environment is concerned with the surrounding circumstances which can affect an organism. Genotype is an environmental-interactor factor where the genes roles as main factors. Genes are expressed through gene regulatory system which has the flexibility to adapt to the environment. Gene expression might be modified, silenced or boosted by the effect of internal and external factors to the cell to regulatory mechanism respond. This consequence reaction will create a range of the phenotypes which is called norm of the population. Genotype, environment interactions (GEI) can be studied by investigation upon phenotypic traits. Achieving the principle behind GEI would be possible through high-throughput techniques which can have enough statical depth. So, the studied traits can be analyzed in terms of genotypic and environmental effects. The genomic methods analysis has a great impact in GEI analysis by using (genome × environment × space ×time) as main factors which describe gene expression at different environmental circumstances in different space and different time scale. Understanding GEI can be simplified by running an experiment under a controlled condition which considered

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standard index and comparing it with the natural environment. So, in this study, we looked at gene expression behavior in three different environments. The first environment is a laboratory scale, the second environment is a well-management farm, and the third environment is a bad-management farm.

To get insights to GEI in the trait complicated behavior, two methods are common in statistical analysis. One is selective genotyping analysis, and another one is bulked sergeant sample analysis. In this study, we used bulked sample analysis (BSA) as a sampling method to find out the best representative gene by selecting a limited number of the *M. rosenbergii* male individuals in an entire pooling area as bulks samples. To get deeper in concept, we describe two important elements in BSA samples. They represent the individuals collected from selected populations in this study. We looked at the markers that represent all types of biomarkers at the RNA level in different tissues and different pathways under the environmental variation. Our main concern is the effect of thermal conditions on these markers.

4.2.1 Environmental circumstances adaption in Well-managed farm

Besides genomic studies, phenotypic study and biological index studies have been done to get more evaluation insights into animal adaption and genomics improvement. So, with respect to all these concerns, the first objective of this study is:

H_0: Different gene cluster will have the same expression across the different tissue.

H_a: Different gene cluster will have the same expression across the tissue.

The second objective is defined as below:

H_0: Different tissue will have the same gene expression across the different temperature in the well-managed farm.

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H_a: Different tissue will have the different gene expression across the different temperature well-managed farm.

To assess GMP's immune genes thermal tolerance, animals were collected from two different farms with different management types. Farm A was considered a well-managed farm with up to the standard husbandry. Feeding rate, water quality, salinity, oxygen level and water temperature were measured at the different times of the day starting from sunrise to sunset for six days. A number of sixty male prawns were collected randomly from different parts of the pond. The animals were immediately dissected in the field and stored in liquid nitrogen. The prawns showed more erratic swimming, low food intake, pale and yellowish body, lethargic behavior in the afternoon by increasing temperature. The RNA pool of each organ including serum, muscle, and hepatopancreas at each temperature was studied for five different direct and indirect immunological pathways, through gene expression analysis. Fifteen genes that were coding this pathway were selected from GMP transcription data. The samples were proceeded for gene expression rate reading by high trough put expression study. The samples later on proceeded for immune histochemistry studies in animal genetics and genome evolutionary laboratory in University Malaya. The achieved data were later analyzed by the livek method and Multiple-ANOVA test which confirmed the significance of the results (Table 4.20 to 4.22).

In farm A, the water parameters were measured by the oximeter machine. Water salinity, water oxygen level, water transparency, pH, water alkalinity, NH_3-N, NO_3-N were in the standard range. The pond was equipped with enough water circulator which supplied enough oxygen for animal growth. The water temperature in natural habitat in Malaysia started from 24 °C from early morning, it reached to 28 °C by 11am and 32 °C by 3 pm (32 °C a), and the temperature was at the same level, 32 °C (32 °C b) by 7 pm

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(Figur 4.27 to 4.30).

H_0: Different Gene clusters expression analysis in different tissues in natural habitat under an environmental condition in well-managed farm.

H_a: Different tissue will have the same gene expression across the different temperature in the well-managed farm.

Table 4.20: The Table shows Thermal stress influence a different pathway in hemolymph in well-managed farm.

	Lectin	Metabolite	PPO	Chapron	Apoptosis
24°C	9.482015	6.669545	11.43436	11.88143	11.55503
28°C	9.510038	7.547649	16.72028	13.93414	16.50707
32a°C	15.93357	12.0203	19.81003	17.48006	18.51605
32b°C	21.61959	12.81269	26.4367	21.38182	25.55738

Table 4.21: The Table shows thermal stress influence a different pathway in muscle in well-managed farm.

	Lectin	Metabolite	PPO	Chapron	Apoptosis
24°C	6.921549	7.56419	11.37437	11.6647	11.31646
28°C	11.39469	7.148815	14.20646	11.60642	13.26441
32a°C	11.53191	9.911456	16.30375	14.84725	15.34225
32b°C	15.22632	11.46437	19.34886	17.13333	16.29093

Table 4.22: The Table shows thermal stress influence a different pathway in hepatopancreas in well-managed farm.

	Lectin	Metabolite	PPO	Chaperone	Apoptosis
24°C	10.37442	6.014866	14.97758	13.50819	14.26612
28°C	15.16237	9.1551	17.57759	14.98164	17.51602
32a°C	17.3361	12.52742	22.08338	21.45003	18.53796
32b°C	21.80175	13.9776	26.73629	27.05229	20.55642

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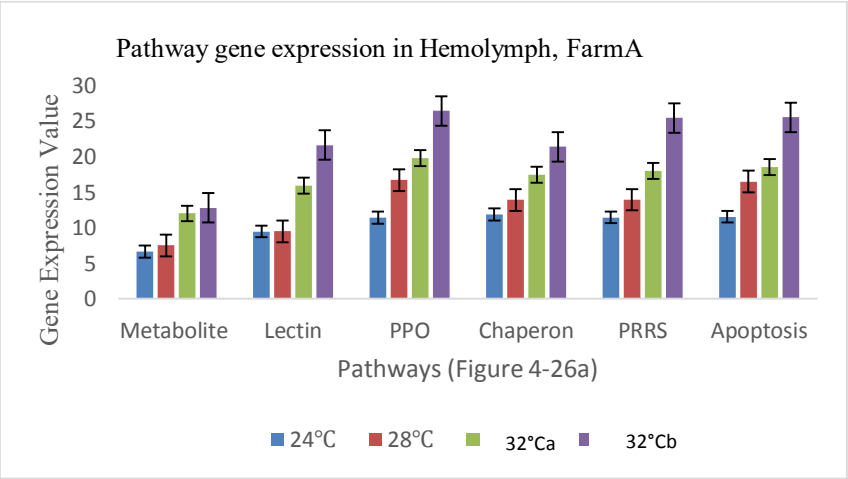

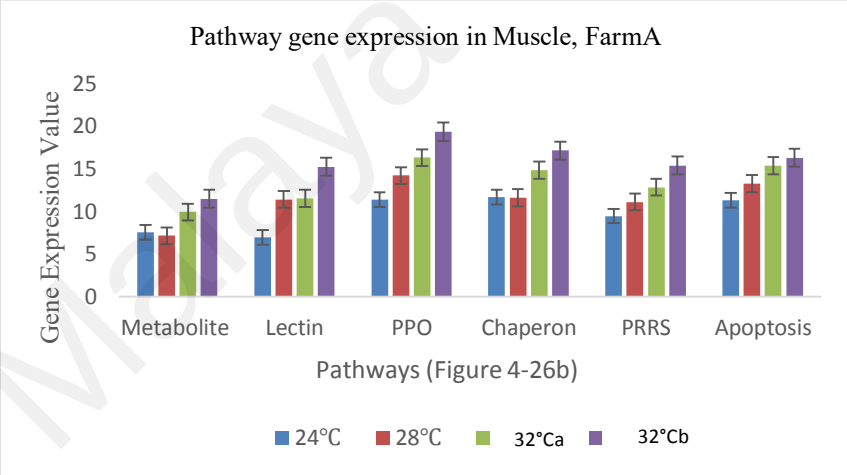

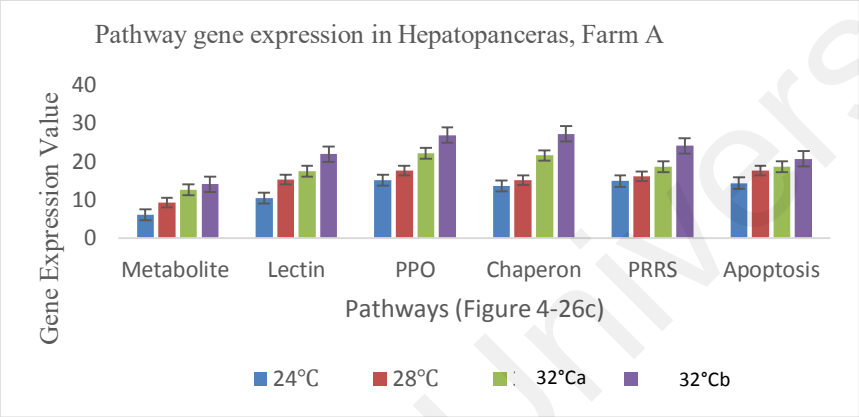

Figure 4.26: The picture shows Thermal stress influence on pathway coding gene in different tissues in different temperatures in the well-managed farm. Hemolymph (a), Muscle (b), Hepatopancreas (c).

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This gene expression analysis has been done according to bulk sampling in the natural environment. The data is demonstrating the norm of the collected samples from the farm at a different temperature.

The gene expression comparison among all the three studied organs showed that, by increasing the temperature, metabolite pathway coding gene, lectin pathway coding gene (Figure 4.27), prophenoloxidase (Figure 4.28). pathway coding gene and chaperone pathway coding genes have a higher expression level in hepatopancreas, hemolymph, and muscle, respectively (Figure 4.29).

The gene expression comparison among all the three studied organs showed that, by increasing the temperature, apoptosis pathway coding gene, Ppo pathway coding gene have a higher expression level in hemolymph, hepatopancreas, and muscle, respectively (Table 4.23 to 4.28) and suplemenry data on gene expression is available in apendix IV.

Table 4.23: The table shows Thermal stress influence on metabolite Pathway coding gene expression in different tissues in well-managed farm.

Thermal stress influence on metabolite pathway in different tissues, in well-managed farm.			
6h	Hemo	Muscle	Hepato
24°C	6.669545	7.56419	6.014866
28°C	7.547649	7.148815	9.1551
32a°C	12.0203	9.911456	12.52742
32b°C	12.81269	11.46437	13.9776

Table 4.24: Thermal stress influence the lectin pathway coding gene expression in different tissues well-managed farm.

Thermal stress influence on lectin pathway in different tissues, in well-managed farm.			
	Hemo	Muscle	Hepato
24°C	9.482015	6.921549	10.37442
28°C	9.510038	11.39469	15.16237
32a°C	15.93357	11.53191	17.3361
32b°C	21.61959	15.22632	21.80175

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Table 4.25: Thermal stress influence on the prophenoloxidase pathway coding gene expression in different tissues well-managed farm.

Thermal stress influence on PPO pathway in different tissues, in well-managed farm.			
6h	Hemo	Muscle	Hepato
24°C	11.43436	11.37437	14.97758
28°C	16.72028	14.20646	17.57759
32a°C	19.81003	16.30375	22.08338
32b°C	26.4367	19.34886	26.73629

Table 4.26: Thermal stress influence the chaperone pathway coding gene expression in different tissues well-managed farm.

Thermal stress influence on Chaperone pathway in different tissues, in well-managed farm.			
6h	Hemo	Muscle	Hepato
24°C	11.88143	11.6647	13.50819
28°C	13.93414	11.60642	14.98164
32a°C	17.48006	14.84725	21.45003
32b°C	21.38182	17.13333	27.05229

Table 4.27: Thermal stress influence on the a PRR pathway coding gene expression in different tissues in well-managed farm.

Thermal stress influence on PRR pathway in different tissues, in well-managed farm.			
6h	Hemo	Muscle	Hepato
24°C	11.45914	9.448886	14.7848
28°C	13.95654	11.10858	16.05082
32°C	17.99696	12.83981	18.48347
36°C	25.46039	15.38829	23.94429

Table 4.28: Thermal stress influence on the apoptosis pathway coding gene expression in different tissues in well-managed farm.

Thermal stress influence on Apoptosis pathway in different tissues, in well-managed farm.			
	Hemo	Muscle	Hepato
24°C	11.55503	11.31646	14.26612
28°C	16.50707	13.26441	17.51602
32a°C	18.51605	15.34225	18.53796
32b°C	25.55738	16.29093	20.55642

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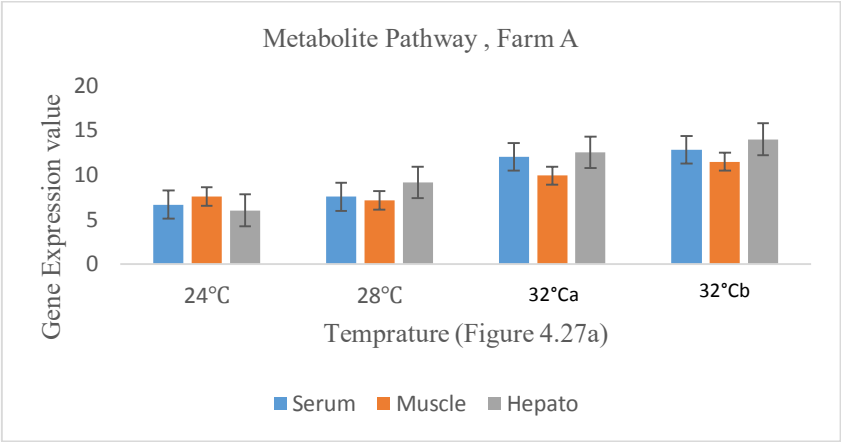

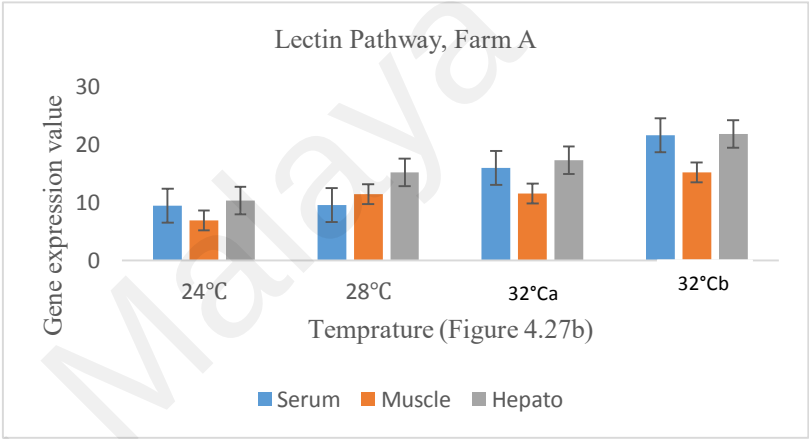

Figure 4.27: The picture shows Thermal stress influence on metabolite pathway in different tissues in the bad-managed farm (a), .Thermal stress influences the lectin pathway in different tissues, in the well-managed farm (b). Thermal stress influence on the apoptosis pathway in different tissues, in the well-managed farm (e). A different color is used for organ differentiation. Blue, represents hemolymph, orange represents muscle and grey represent hepatopancreas.

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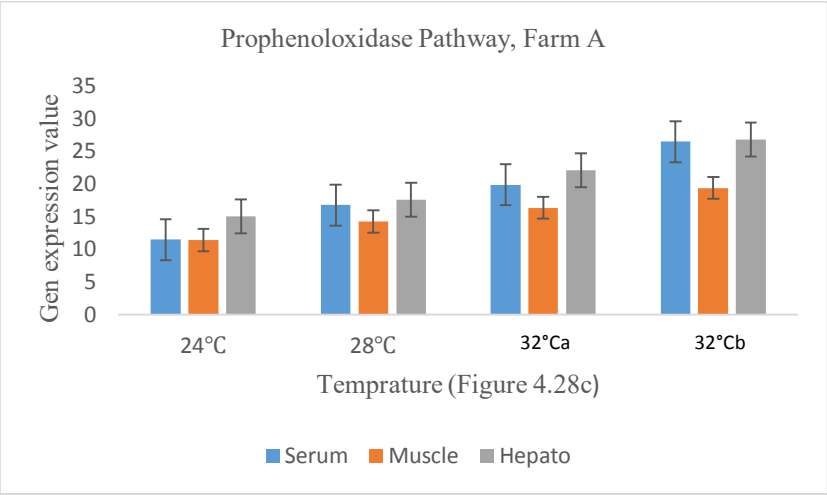

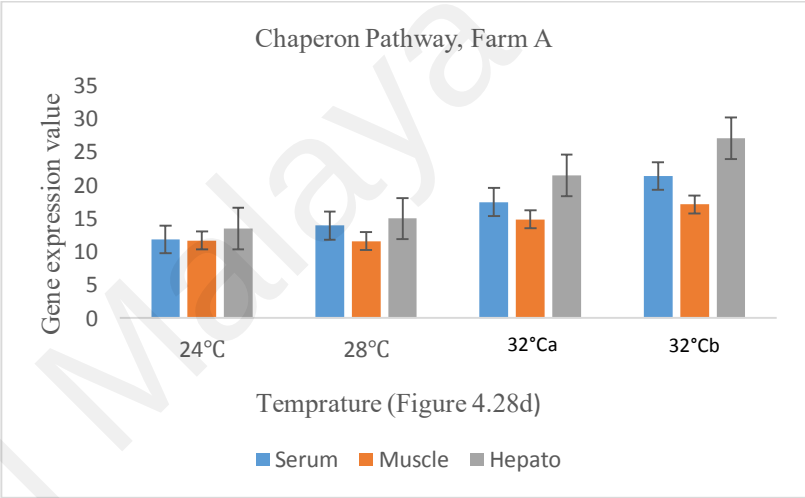

Figure 4.28: Continued. Thermal stress Influence on prophenoloxidase pathway in different tissues in the well-managed farm (c). Thermal stress influences the chaperone pathway in different tissues in the well-managed farm (d). Thermal stress influence on the apoptosis pathway in different tissues, in the well-managed farm (e). A different color is used for organ differentiation. Blue, represents hemolymph, orange represents muscle and grey represent hepatopancreas.

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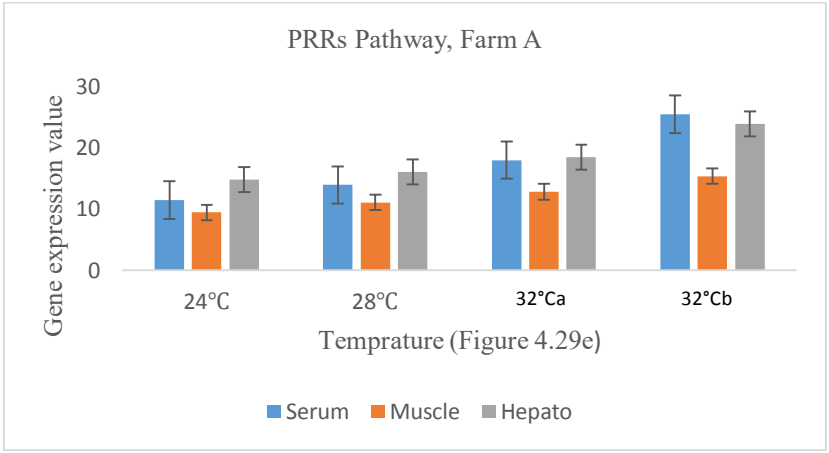

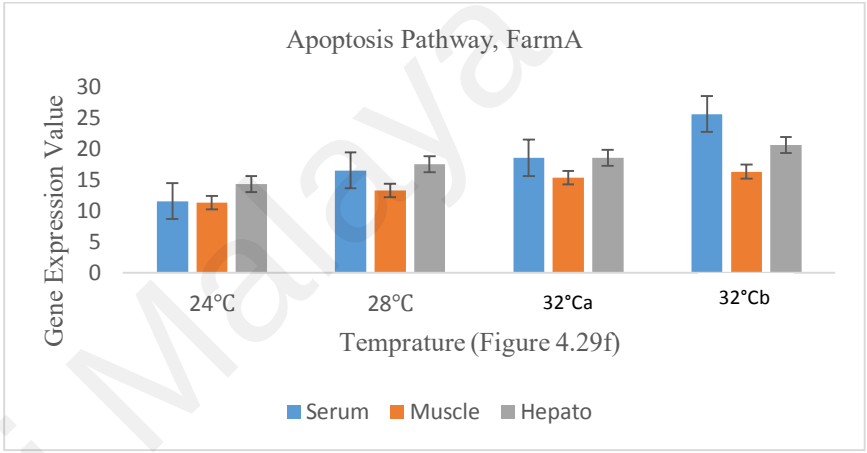

Figure 4.29: Continued. Thermal stress influences the chaperone pathway in different tissues in the well-managed farm (d). Thermal stress influence on the apoptosis pathway in different tissues, in the well-managed farm (e). A different color is used for organ differentiation. Blue, represents hemolymph, orange represents muscle and grey represent hepatopancreas.

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4.2.2 Morphological observations

4.2.2.1 Weight

The weight of the control and treated groups were reported at the end of the experiment. Due to the natural increase of the temperature, the metabolical rate increased, as a result, the weight of the exposed animal groups in comparison with laboratory control, was significantly reduced. The average weight of the control group was 25.22± 0.98, and the average weight of the treated group were 25.83±0.99, 25.83±0.99, 25.80±1.3, 25.84±1.42 respectively for 24 °C, 28 °C, 32 °C a, 32 °C b.

4.2.2.2 Body color

The body color of normal prawn was usually greenish to brownish grey. The heat-treated prawn showed discoloration such as whitish, orange dot-like pattern or dusty grey.

4.2.2.3 Hemocyte color

The hemocyte color of normal prawn was usually blue or greenish blue. The heat-treated prawn showed dark coloration by increasing time and temperature and discoloration 12h after the test at 32 °C b.

4.2.2.4 Abdomen and Uropods

Compared to the normal animal, the treated prawn exhibited black spots and black formation around their 2nd, 3rd somites. Also, deformed uropods and telson were found in treated prawns at high temperatures.

4.2.2.5 Hepatopancreas Somatic Index (HSI)

The mean HIS value of control group was 2.4±0.06. The mean body weight and HIS values for prawns in control groups increased significantly between 24 °C and 28 °C degrees and decreased significantly at 32 °C a and 32 °C b during the experimental period (Figure 4.30).

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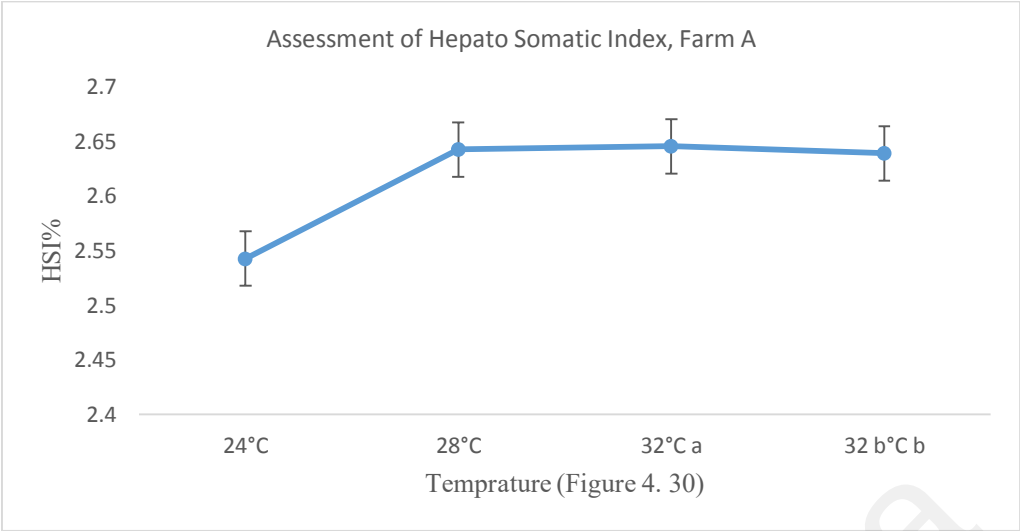

Figure 4.30: The graph shows the assessment of Hepato Somatic Index. The graph is representing the overall behavior of the HIS index during thermal shock in natural environments in the well-managed farm.

4.2.2.6 Total hemocyte count

Statistical analysis showed that there was a significant difference ($P<0.05$) for total hemocyte cell count between the control group and heat-treated group. The analyzed data showed that the number of the hemocyte cells at 24 ℃ is in the same range between the two-time points. But there is a significant increase in the hemocyte concentration at twelve hours at 28 ℃ while there is a significant decrease at twelve-time points at 32 ℃ b. It confirms that the number of the hemocyte is significantly decreased by increasing the temperature, (Figure 4.31).

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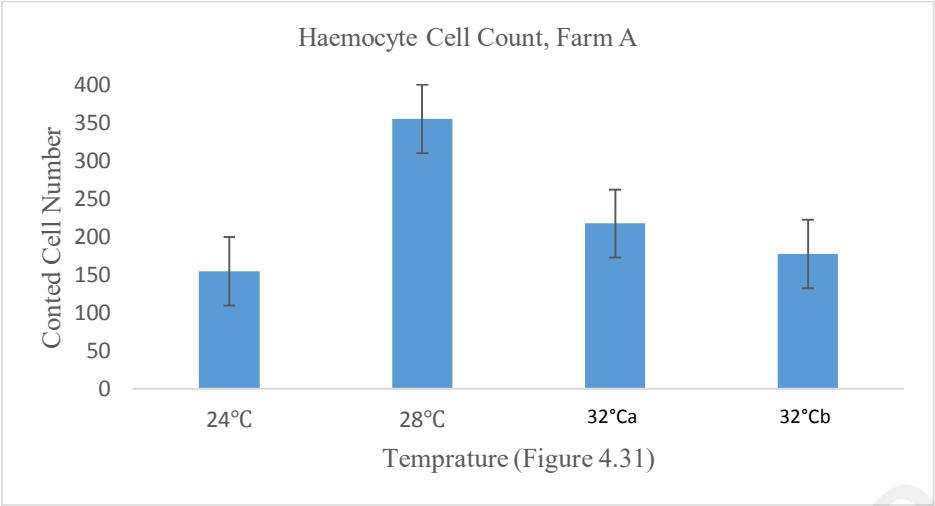

Figure 4.31: The chart shows the haemocyte cell count at different temperatures and time points. Light blue is representing the hemolymph behavior during thermal shock in natural environments in the well-managed farm.

4.2.2.7 Hemocyte Cell viability assays

Based on the MTT assays, increasing temperature has a negative effect on hemocyte cells viability. High temperature decreased cell viability in hemocytes (13–18% decrease, depending on the test) ($p<0.05$) (Figure 4.32).

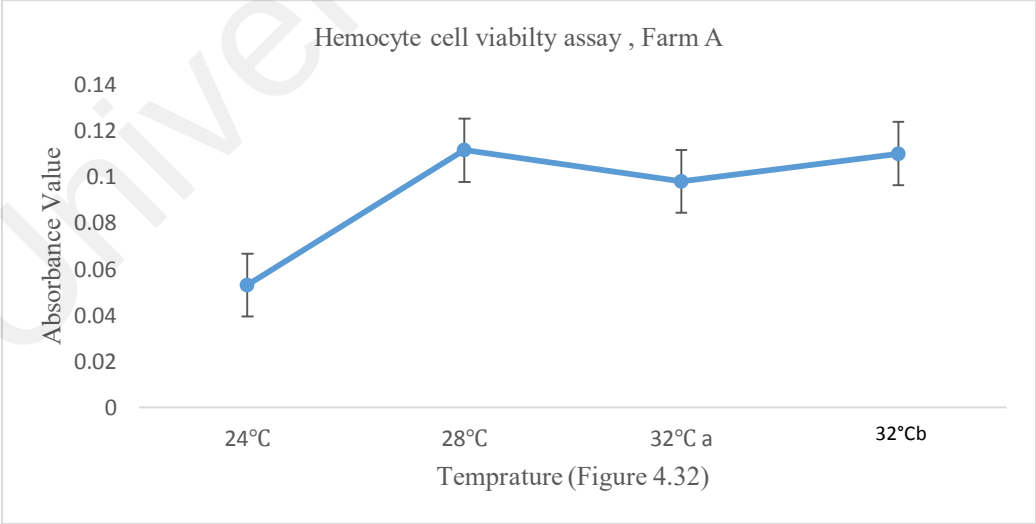

Figure 4.32: The chart shows the hemocyte cell viability assay at different temperatures. It shows the hemolymph behavior during thermal shock in natural environments in the well-managed farm.

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4.2.2.8 Phenoloxidase activity

The assessment of the changes in enzymatic oxidation of the substrate L-DOPA revealed a linear decrease of PO activity by increasing the temperature of the plasma. This linear decrease in PO activity was recognized for the enzymatic conversion of o-diphenols into o-quinones by phenoloxidase enzymes in met and oxy forms (Figure 4.33).

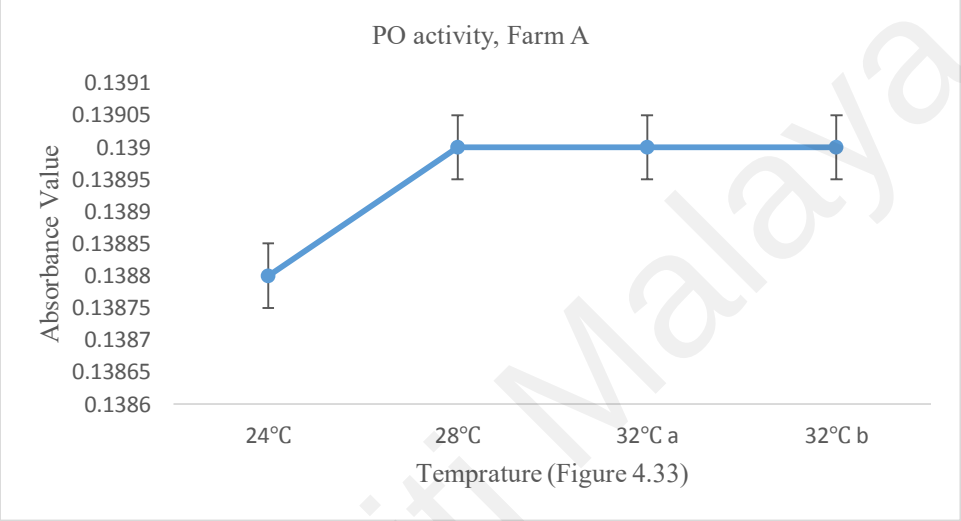

Figure 4.33: The chart shows the PPO activity at different temperatures. It shows the hemolymph behavior during thermal shock in natural environments in the well-managed farm.

4.2.2.9 Protein quantification

The assessment of the Bradford assay protein quantification revealed a linear decrease of PO activity by increasing the temperature for hemocyte, muscle, and hepatopancreas. This linear decrease in total protein content demonstrates a high decrease in the enzymatic activities either metabolical or immunological (Figure 4.34).

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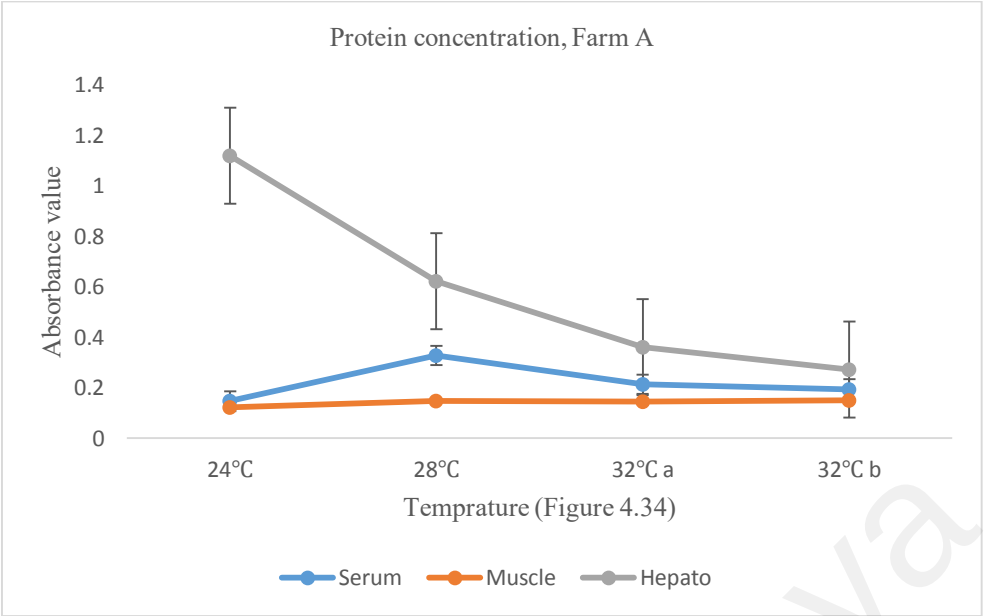

Figure 4.34: The chart shows the Protein concentration at different temperatures. Light blue color is representing hemolymph protein concentration, and orange color is representing muscle protein concentration during the thermal shock. Grey color is representing hepatopancreas protein concentration during the thermal shock in natural environments in the well-managed farm.

4.2.2.10 Histo-architecture of the prawns in different temperature in farm A

4.2.2.10.1 Hepatopancreas

The histoarchitecture of prawn's hepatopancreas resulted the number of B-cells (Blasenzellen cells), R-cells (Restzellen cells), E-cells (Embryonic cells), F-cells (Fibrillenzellen cells), the interstitial sinus was filled with abnormal infiltrated hemocytes (AIH), and the epithelial tubule showing ruptured basal laminae (RBL). The histoarchitecture of hepatopancreas group exhibited a number of E-Cells, IS, HI in the interstitial sinus of the hepatopancreas tubules, coagulation (CO), the formation of the abnormal lumen (ALU) and necrotic Hepatopancreatic tubule (NT) were observed by increasing temperature. Similarly, the histopathological changes such as hemocyte infiltration (HI) in the interstitial sinus of the hepatopancreas tubules, the formation of the abnormal lumen, Blasenzellen cell, thickened basal laminae (TBL), Tissue debris (TD) were observed. The histoarchitecture of hepatopancreas in the groups showed

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tissue debris, hemocyte infiltration, Coagulation, necrotic hepatopancreas tubule (NT), ruptured basal laminae (RBL) and necrotic cells of hepatopancreas (NCH) (Figure 4.35).

4.2.2.10.2 Muscle

The histoarchitecture of muscle in groups showed abnormalities in muscle tissues and necrotic muscles infiltrated by hemocytes. Some animals exhibited structurally altered muscle fibers, necrotic striated muscles, and shrinkage of muscular fibers. In farm A, necrosis and lesions in striated muscle cells and increased eosinophilia of the cytoplasm and pyknotic conditions were seen (small arrows in the figure). In farm A, necrotic striated muscle and muscle fibers, complete degenerated muscle fibers with fragmentation and flocculation, damaged myofilaments, the formation of tissue dippers in the muscle of some of the *M. rosenbergii* were observed (Figure 4.36).

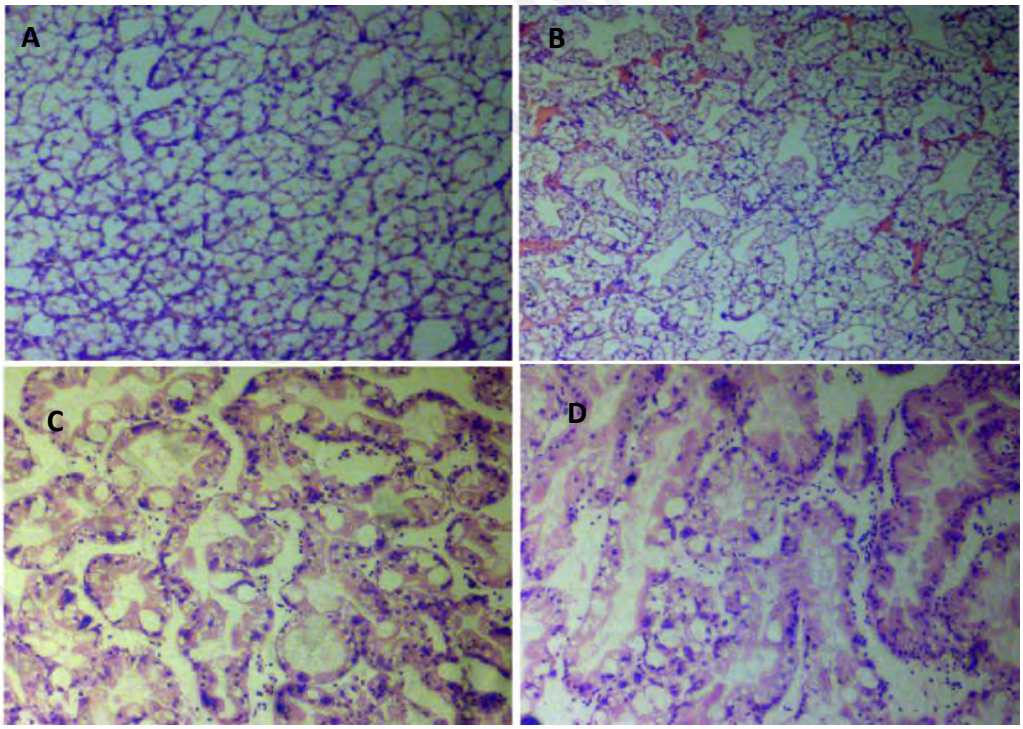

Figure 4.35: (A) Histological cross sections of the hepato of 24 ℃ the formation of the abnormal lumen, Blasenzellen cell, thickened basal laminae (TBL), Tissue debris (TD) (10× H&E). (B) Histological cross sections of the hepatopancreas of 28 ℃ exposed group showing healthy and normal hepato tissues (10× H&E stain). (C and D) Histological cross sections of the hepato of 32 ℃ and 36 ℃ treated prawns showing Tubule coagulation (10× H&E).

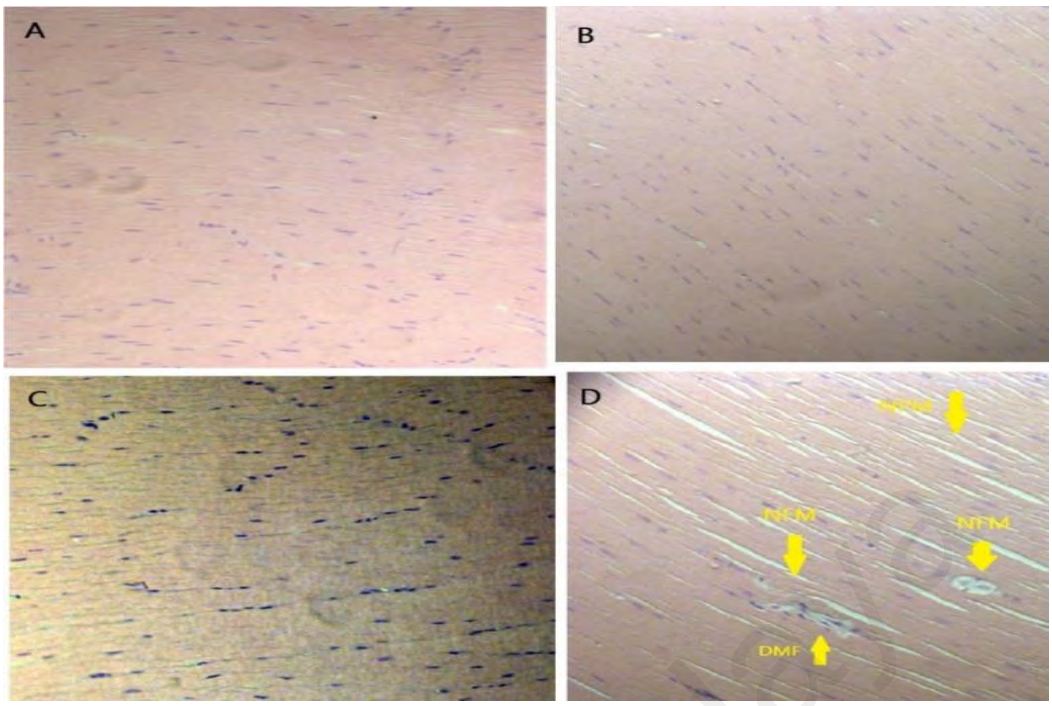

Figure 4.36: (A) Histological cross sections of the muscle of 24 ℃ exposed group Shrinkage of muscular fiber (SMF) (10× H&E). (B) Histological cross sections of the muscle of 28 ℃ exposed group showing healthy and normal muscle tissues, striated muscle fibers clearly (10× H&E stain). (C and D) Histological cross sections of the muscle of 32 ℃ and 36 ℃ exposed group showing necrotic musculature (NMF) and degenerated muscle fibers (DMF) (10× H&E).

4.2.2.10.3 Histological gradations

In control condition for prawns, normal histological structures were noted. In treated groups, the histopathological severity increases as temperature increases. At 36°C, the extended severe histological changes observed when compared to control and=other groups. Based on the percentage of the severity of hepatopancreas and muscles histology, five gradations were formulated to have holistic information on the temperature impact on *M. rosenbergii* (Tables 4.29, 4.30). The gradations were −: None (0%), + mild (<10%), ++: moderate (10–50%), +++: severe (50–70%), ++++: extended severe (>70%) (Table 4.29- 4.30).

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Table 4.29: The table shows the effect of thermal shock in hepatopancreas histology *M. rosenbergii*. −−: None (0%), +: mild (<10%), ++: moderate (10–50%), +++: severe (50–70%). Data were represented as a mean of 125 slides (25 slides/prawn) in each group.

Effect of thermal shock in Muscle histology *M. rosenbergii*					
Cells	Temperatures				
	Control	24°C	28°C	32°C a	32°C b
Necrotic muscle fiber	_	-	-	+	++
Necrotic striated muscle	_	-	-	+	+
Degeneration	_	_	-	+	++
Mean	_	_	-	+	++

Table 4.30: The table shows the Effect of thermal shock in Hepatopancreas histology *M. rosenbergii*. −−: None (0%), +: mild (<10%), ++: moderate (10–50%), +++: severe (50–70%). Data were represented as a mean of 125 slides (25 slides/prawn) in each group.

Effect of thermal shock in Hepatopancreas histology *M. rosenbergii*					
Cells	Temperatures				
	Control	24°C	28°C	32°C a	32°C b
Necrotic tubule	_	-	-	+	++
R cell	_	-	-	+	+
B cell	_	_	-	+	++
E cell	_	_	_	+	++
F cell	_	_	_	+	++
Thickened Basal Laminae	_	_	_	+	++
Infiltrated hemocytes	_	_	_	+	++
Tissue debris	_	_	_	+	++
Tubule coagulation	_	_	_	+	++
Mean	_	_	-	+	+++

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4.2.2.11 Water Sample analysis

The water samples sent for analytical Alkalinity, NH_3-N (mg/L), NO3-N (mg/L) tests and the result is as shown in (Table 4.31).

Table 4.31: Water quality of the Well-managed farm.

Parameters	Treatments	
	Standard Level	**Well-managed farm**
Water temperature (ºC)	28±1	29.58±0.121
Transparency (cm)	20±1	21.06 ± 0.166[c]
DO (mg/l)	**7**±1	6.70 ± 0.139[a]
pH	7±1	8.51 ± 0.084[a]
Alkalinity	140±1	145.59 ± 8.48[a]
NH_3-N (mg/L)	0-0.05±0.1	0.08 ± 0.005
NO_3-N (mg/L)	1.08 ± 0.01	1.08 ± 0.017

4.2.3 Environmental circumstances adaption in poor-managed farm

H_0: Different tissue will have the same gene expression across the different temprature in non-managed farm.

Ha: Different tissue will have the different gene expression across the different temprature in non-managed farm.

H_0: Different tissue will behave the same gene expression across the same temprature between Laboratory and non -managed farm.

Ha: Different tissue will not behave the same gene expression across the same temprature between Laboratory and non -managed farm.

To assess GMP's immune genes thermal tolerance, animals were collected from two different farms with different management types. Farm B was considered a poorly managed farm with non-standard husbandry. Feeding rate, water quality, salinity, oxygen level and water temperature were measured at different times of the day starting from

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sunrise to sunset for six days. A number of sixty male prawns were collected randomly from different parts of the pond. The animals were immediately dissected in the field and stored in liquid nitrogen. The prawns showed more erratic swimming, low food intake, pale and yellowish body, lethargic behavior in the afternoon by increasing temperature. The RNA pool of each organ including serum, muscle, and hepatopancreas at each temperature was studied for five different direct and indirect immunological pathways, through gene expression analysis. Fifteen genes that were coding this pathway were selected from GMP transcription data. The samples were proceeded for gene expression rate reading by high trough put expression study. The samples later on proceeded for immunohistochemistry studies in animal genetics and genome evolutionary laboratory in University Malaya. The achieved data were later analyzed by the livek method, and Multiple-ANOVA test confirmed the significance of the results.

4.2.3.1 Different Gene clusters expression analysis in different tissues in natural habitat under an environmental condition in bad-managed farm

The gene expression comparison among all the three studied organs showed that, by increasing the temperature, metabolite pathway coding gene has a higher expression level in hepatopancreas, hemolymph, and muscle, respectively.

The gene expression comparison among all the three studied organs showed that, by increasing the temperature, lectin pathway coding gene has a higher expression level in hepatopancreas, hemolymph, and muscle, respectively.

The gene expression comparison among all the three studied organs showed that, by increasing the temperature, prophenoloxidase pathway coding gene has a higher expression level in hepatopancreas, hemolymph, and muscle, respectively (Figure 4.37).

The gene expression comparison among all the three studied organs showed that, by increasing the temperature, chaperone pathway coding gene has a higher expression level

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in hepatopancreas, hemolymph, and muscle, respectively (Figure 4.38). The gene expression comparison among all the three studied organs showed that, by increasing the temperature, PRRs pathway coding gene has a higher expression level in hepatopancreas, hemolymph, and muscle, respectively (Figure 4.39).

The gene expression comparison among all the three studied organs showed that, by increasing the temperature, apoptosis pathway coding gene has a higher expression level in hepatopancreas, hemolymph, and muscle, respectively (Figure 4.37-39) and suplemenry data on gene expression is available in appendix IV.

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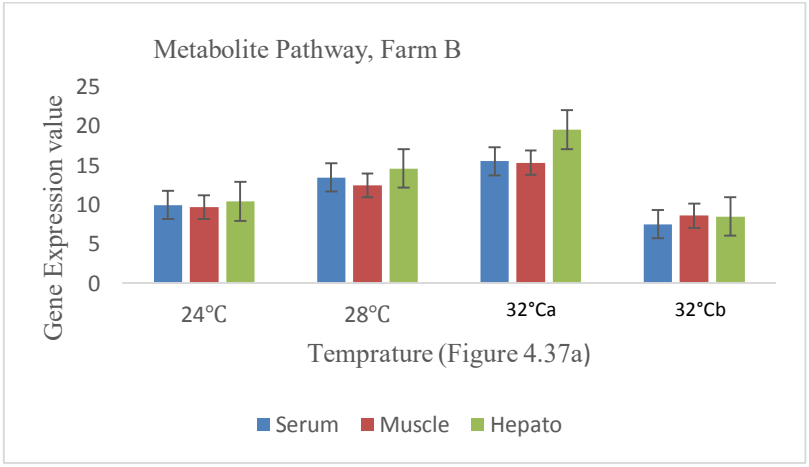

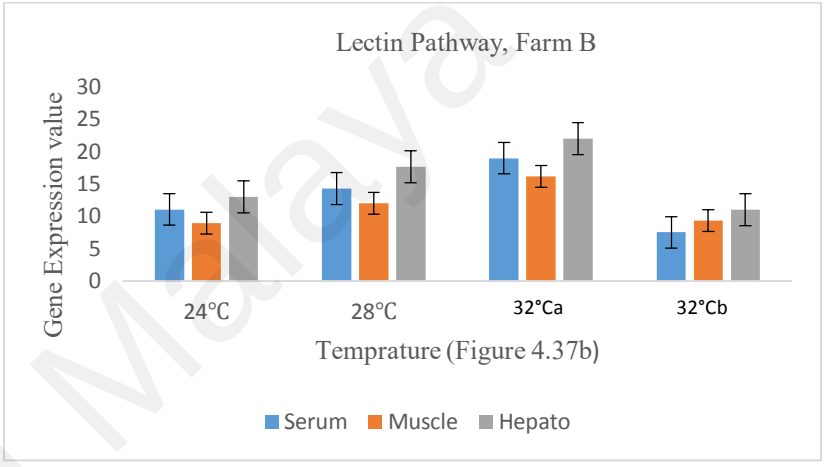

Figure 4.37: The picture shows Thermal stress influence on metabolite pathway in different tissues in the poor-managed farm (a). Thermal stress influences the lectin pathway in different tissues, in bad-managed farm (b).

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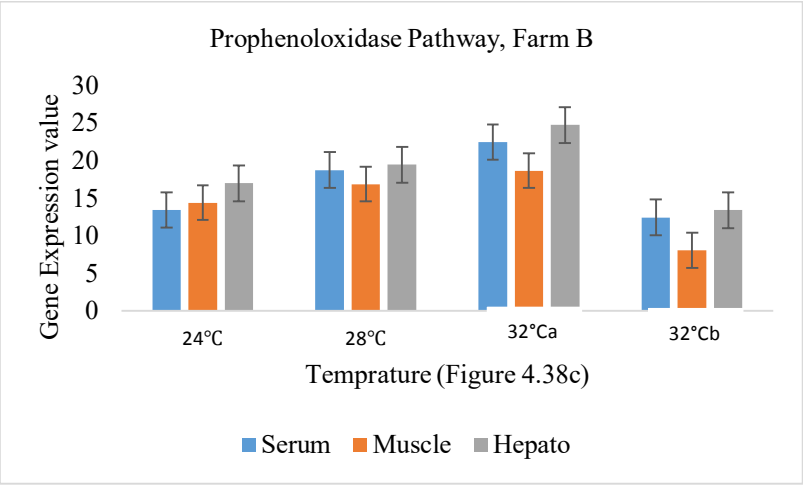

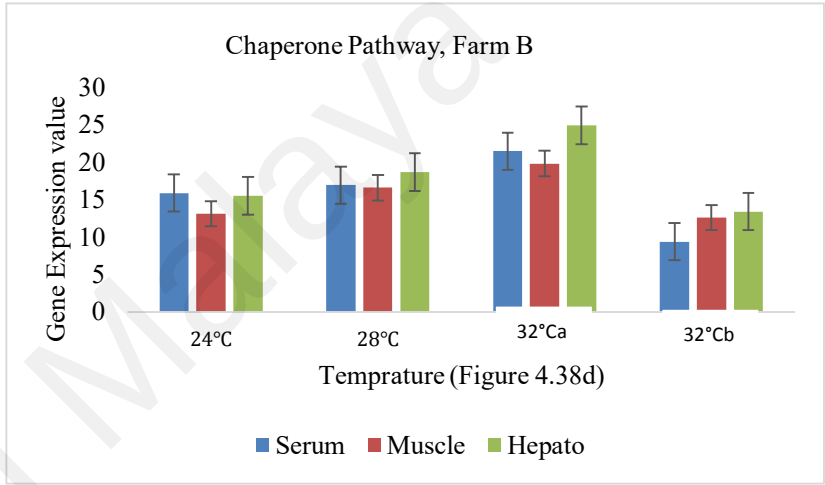

Figure 4.38: Continued. Thermal stress influence on prophenoloxidase pathway in different tissues in the poor-managed farm (c). Thermal stress influences the chaperone pathway in different tissues in bad-managed farm (d). Thermal stress influence on the apoptosis pathway in different tissues, in the bad-managed farm (e). A different color is used for organ differentiation. Blue, represents hemolymph, orange represents muscle and grey represent hepatopancreas.

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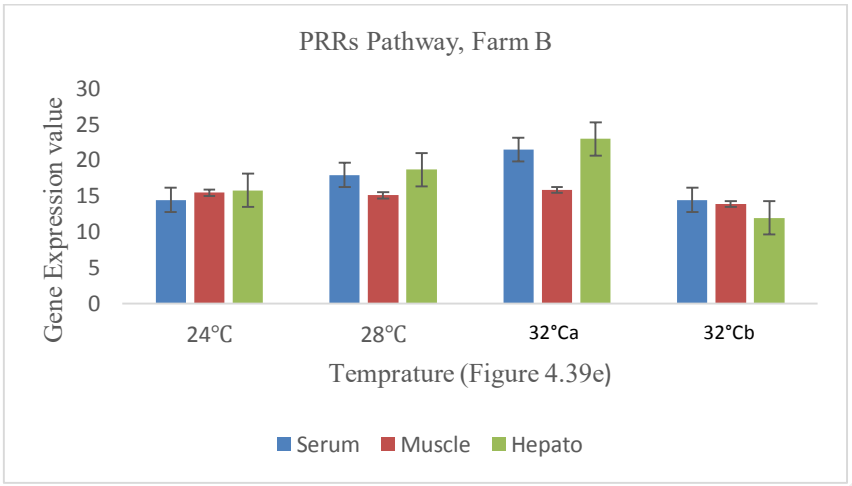

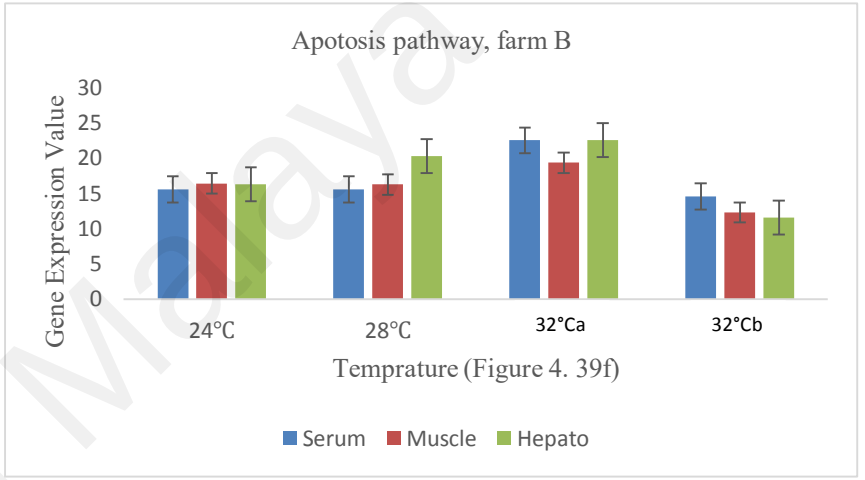

Figure 4.39: Thermal stress influence on the apoptosis pathway in different tissues, in the poor-managed farm (e). A different color is used for organ differentiation. Blue, represents hemolymph, orange represents muscle and grey represent hepatopancreas.

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4.2.4 Morphological observations

4.2.4.1 Weight

The weight of the control and treated groups were reported at the end of the experiment. Due to the heat stress, the metabolical rate increased, as a result, the weight of the exposed animal groups in comparison with control group, was significantly reduced. The average weight of the control group was 25.22± 0.55, and the average weights of the treated group were 25.73±0.99, 25.77±0.99, 25.73±1.3, 25.53±1.42 respectively for 24 °C, 28 °C, 32 °C, 36 °C.

4.2.4.2 Body color

The body color of normal prawn was usually greenish to brownish grey. The heat-treated prawn showed discoloration such as whitish, orange dot-like pattern or dusty grey.

4.2.4.3 Hemocyte color

The hemocyte color of normal prawn was usually blue or greenish blue. The heat-treated prawn showed dark coloration by increasing time and temperature and discoloration 12h after the test at 36 °C.

4.2.4.4 Abdomen and Uropods

Compared to the normal animal, the treated prawn exhibited black spots and black formation around their 2nd, 3rd somites. Also, deformed uropods and telson were found in treated prawns at high temperatures.

4.2.4.5 Hepatopancreas Somatic Index (HSI)

The mean HIS value of control group was 2.4±0.06. The mean body weight and HIS values for prawns in control groups increased significantly between 24 °C and 28 °C degrees and decreased significantly at 32 °C and 36 °C during the experimental period (Figure 4.40).

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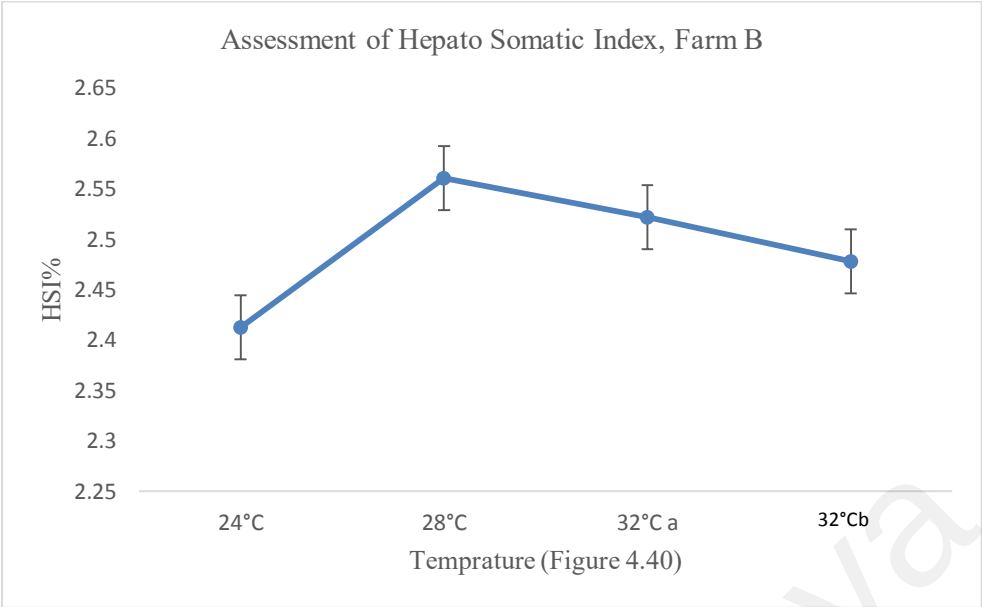

Figure 4.40: The graph shows the assessment of hepato somatic index. The graph is representing the overall behavior of the HIS index during thermal shock in natural environments in the well-managed farm.

4.2.4.6 Total hemocyte count

Statistical analysis showed that there was a significant difference ($P<0.05$) for Total Hemocyte Cell count between the control group and heat-treated group. The analyzed data showed that the number of the hemocyte cells at 24 ℃ is in the same range between the two-time points. But there is a significant increase in the hemocyte concentration at twelve hours at 28 ℃ while there is a significant decrease at twelve-time points at 36 ℃. It confirms that the number of the hemocyte is significantly decreased by increasing the temperature (Figure 4.41).

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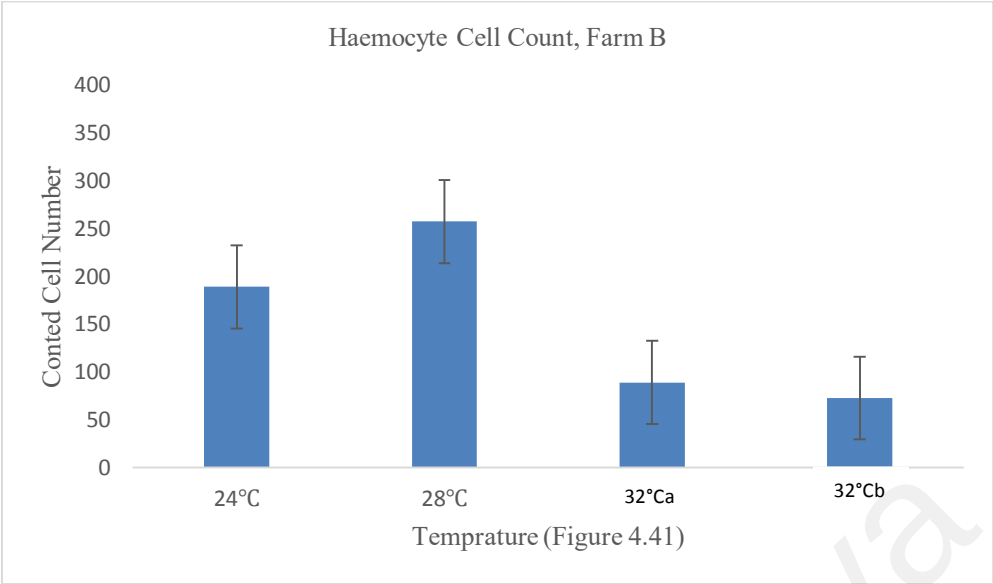

Figure 4.41: The chart shows the Haemocyte cell count at different temperatures and time points. Light blue is representing the hemolymph behavior during thermal shock in natural environments in the well-managed farm.

4.2.4.7 Hemocyte Cell viability assays

Based on the MTT assays, increasing temperature has a negative effect on hemocyte cells viability. High temperature decreased cell viability in hemocytes (13–18% decrease, depending on the test) ($p<0.05$) (Figure 4.42).

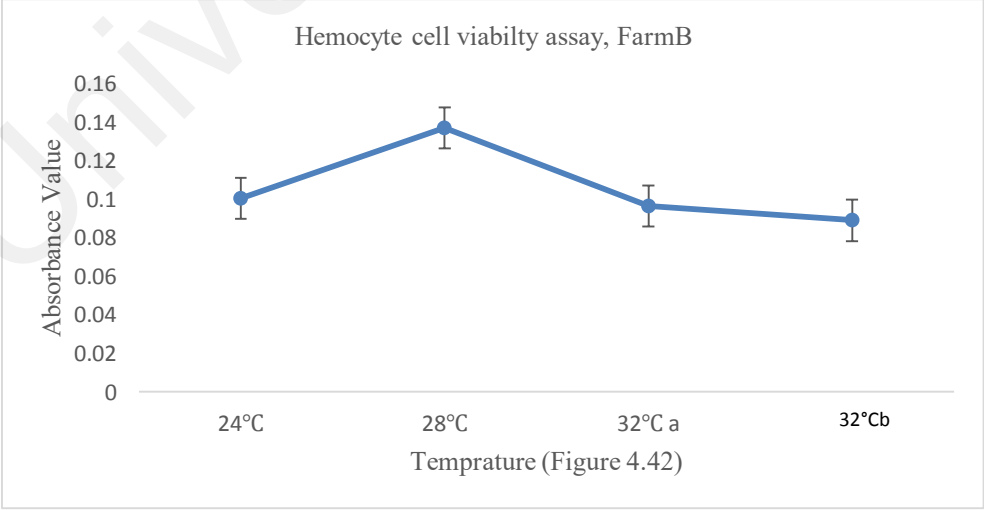

Figure 4.42: The chart shows the Hemocyte cell viability assay at different temperatures. It shows the hemolymph behavior during thermal shock in natural environments in the well-managed farm.

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4.2.4.8 Phenoloxidase activity

The assessment of the changes in enzymatic oxidation of the substrate L-DOPA revealed a linear decrease of PO activity by increasing the temperature for plasma. This linear decrease in PO activity was recognized to the enzymatic conversion of o-diphenols into o-quinones by phenoloxidase enzymes in met and oxy forms (Figure 4.43).

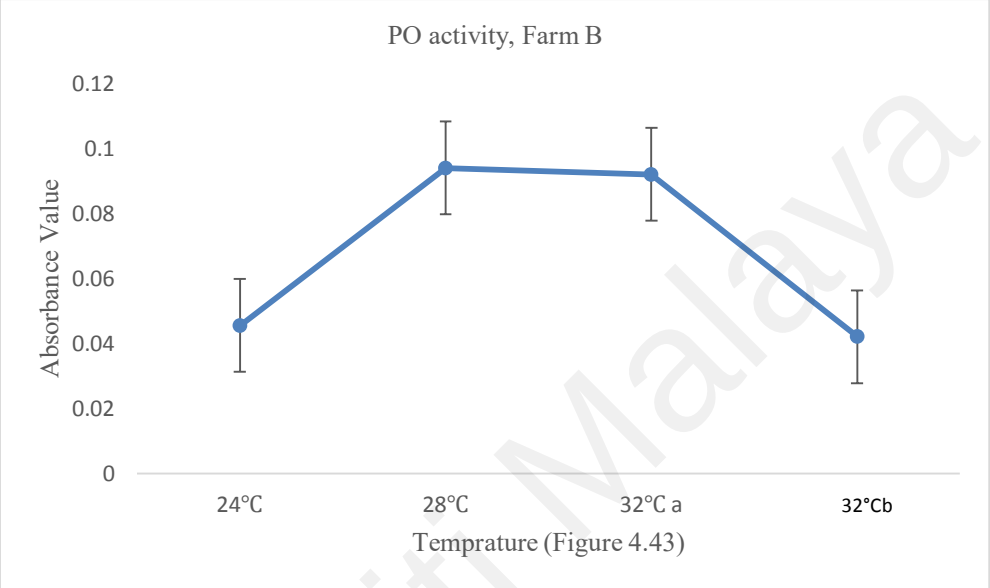

Figure 4.43: The chart shows the PPO activity at different temperatures. It shows the hemolymph behavior during thermal shock in natural environments in the well-managed farm.

4.2.4.9 Protein quantification

The assessment of the Bradford assay protein quantification revealed a linear decrease of PO activity by increasing the temperature for hemocyte, muscle, and hepatopancreas. This linear decrease in total protein content demonstrates a high decrease in the enzymatic activities either metabolical or immunological (Figure 4.44).

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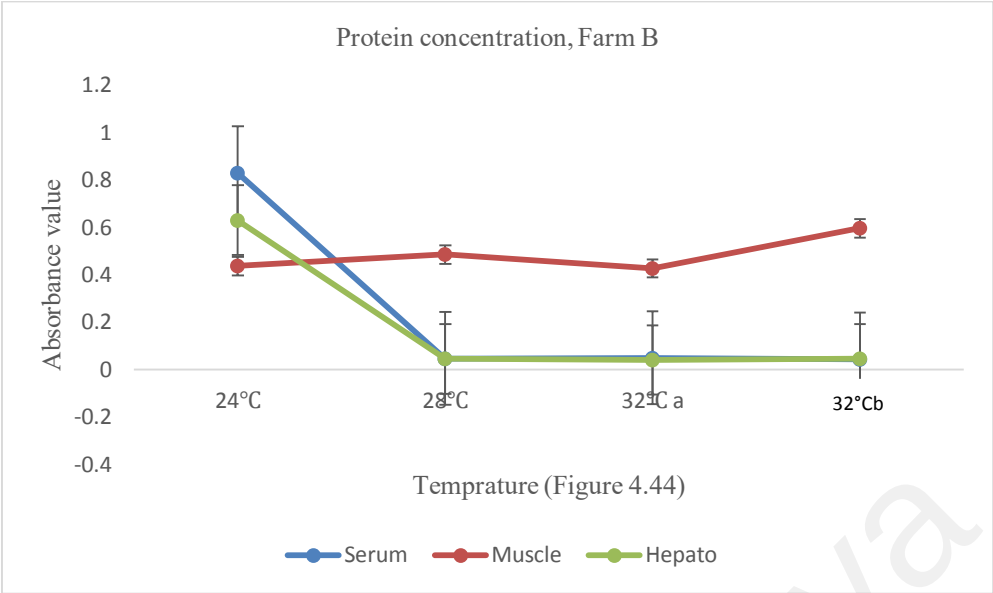

Figure 4.44: The chart shows the Protein concentration at different temperatures. Light blue color is representing hemolymph protein concentration, and orange color is representing muscle protein concentration during the thermal shock. Grey color is representing hepatopancreas protein concentration during the thermal shock in natural environments in the well-managed farm.

4.2.4.10 Histo-architecture of the prawns in different temperature in Farm B

4.2.4.10.1 Hepatopancreas

The histoarchitecture of prawn's hepatopancreas resulted in the number of B-cells (Blasenzellen cells), R-cells (Restzellen cells), E-cells (Embryonic cells), F-cells (Fibrillenzellen cells), the interstitial sinus was filled with abnormal in filtrated hemocytes (AIH), and the epithelial tubule is showing ruptured basal laminae (RBL) (Figure 4.45). The histoarchitecture of hepatopancreas group exhibited a number of E-Cell, IS, HI in the interstitial sinus of the hepatopancreases tubules, coagulation (CO), the formation of the abnormal lumen (ALU) and necrotic Hepatopancreatic tubule (NT) were observed by increasing temperature (Figure 4.45). Similarly, the histopathological changes such as hemocyte infiltration (HI) in the interstitial sinus of the hepatopancreas tubules, the formation of the abnormal lumen, Blasenzellen cell, thickened basal laminae (TBL), Tissue debris (TD) were observed in

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heat-treated groups (Figure 4.45). The histoarchitecture of hepatopancreas in the Thermal treated groups showed tissue debris, hemocyte infiltration, Coagulation, necrotic hepatopancreas tubule (NT), ruptured basal laminae (RBL) and necrotic cells of hepatopancreas (NCH).

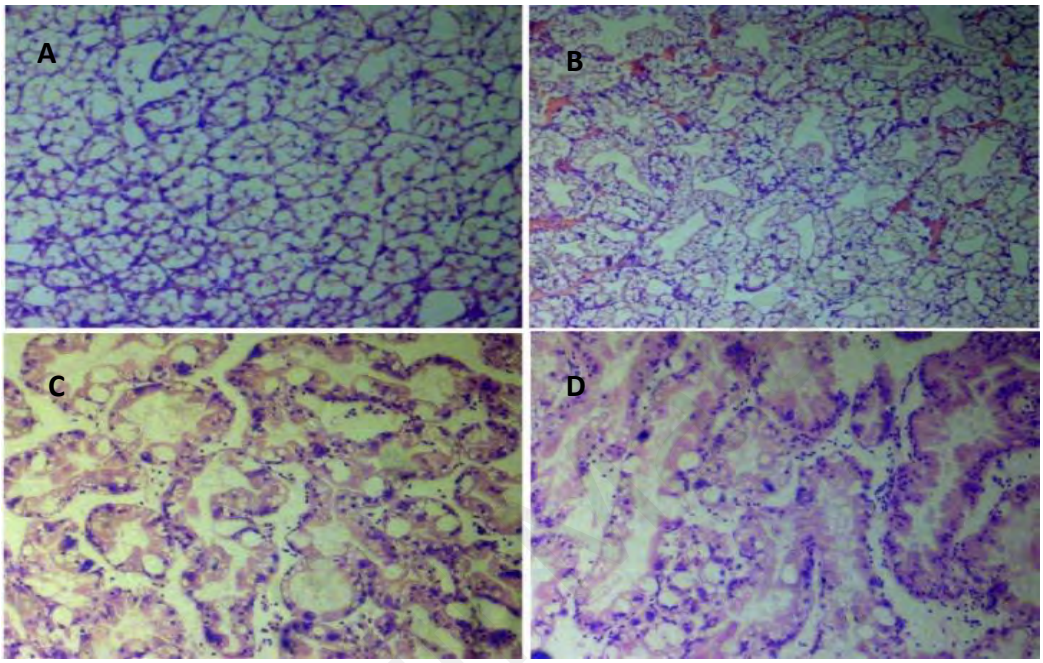

Figure 4.45: (A) Histological cross sections of the hepato of 24 ℃ the formation of the abnormal lumen, Blasenzellen cell, thickened basal laminae (TBL), Tissue debris (TD) (10× H&E). (B) Histological cross sections of the hepatopancreas of 28 ℃ exposed group showing healthy and normal hepato tissues (10× H&E stain). (C and D) Histological cross sections of the hepato of 32 ℃ and 36 ℃ treated prawns showing Tubule coagulation (10× H&E).

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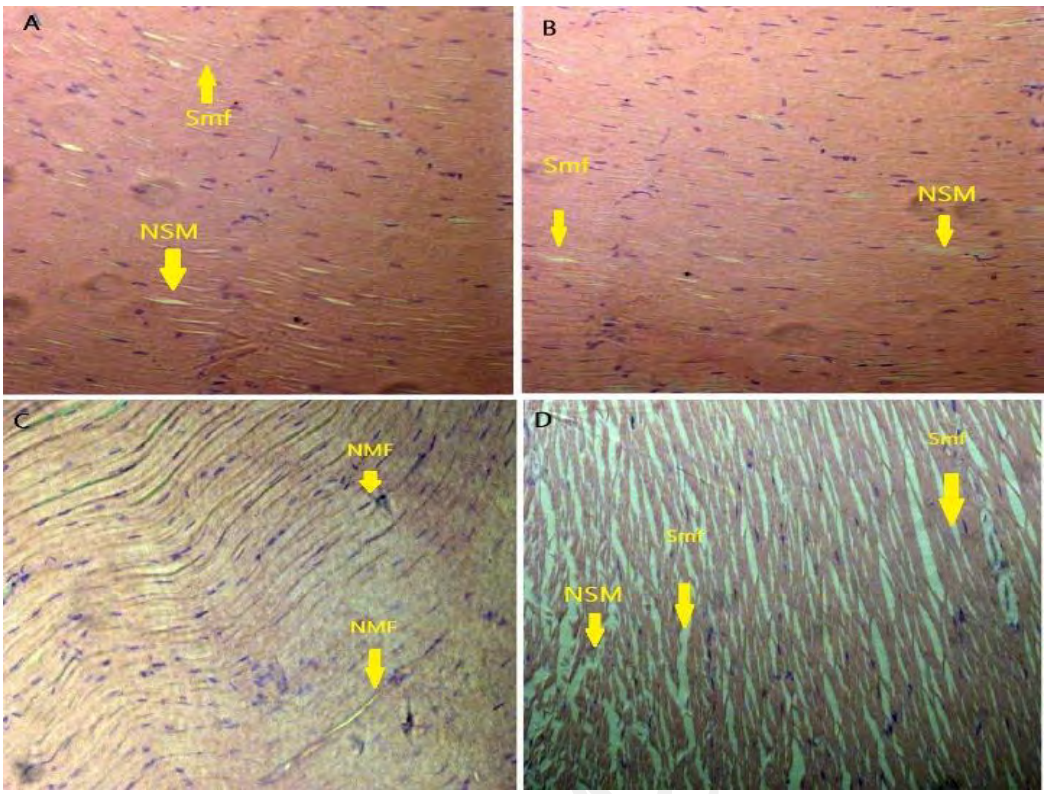

Figure 4.46: (A) Histological cross sections of the muscle of 24 ℃ exposed group Shrinkage of muscular fiber (SMF) (10× H&E). (B) Histological cross sections of the muscle of 28 ℃ exposed group showing healthy and normal muscle tissues, striated muscle fibers clearly (10× H&E stain). (C and D) Histological cross sections of the muscle of 32 ℃ and 36 ℃ exposed group showing necrotic musculature (NMF) and degenerated muscle fibers (DMF) (10× H&E).

4.2.4.10.2 Muscle

The histoarchitecture of muscle in heat treated prawns showed abnormalities in muscle tissues and necrotic muscles infiltrated by hemocytes (Figure 4.46). The majority of animals structurally altered muscle fibers, necrotic striated muscles, and shrinkage of muscular fibers (Figure 4.46). In groups, necrosis and lesions in striated muscle cells and increased eosinophilia of the cytoplasm and pyknotic conditions were seen small arrows in the (Figure 4.46). In farm B, necrotic striated muscle and muscle fibers, complete degenerated muscle fibers with fragmentation and flocculation, damaged myofilaments, the formation of tissue dippers in the muscle of the majority of *M. rosenbergii* were observed.

4.2.4.10.3 Histological gradations

In control prawns, normal histological structures were noted. In treated groups, the histopathological severity increases as temperature increases. At 36°C, the extended severe histological changes observed when compared to control and other groups. Based on the percentage of the severity of hepatopancreas and muscles histology, five radations were formulated to have holistic information on the temperature impact on *M. rosenbergii* (Tables 2 and 3). The gradations were −: None (0%), + mild (<10%), ++: moderate (10–50%), +++: severe (50–70%), ++++: extended severe (>70%) (Table 4.32, 4.33).

Table 4.32: Effect of thermal shock in Muscle histology *M. rosenbergii.*

	Control	24°C	28°C	32°C a	32°C b
Necrotic muscle fiber	_	+	+	++	+++
Necrotic striated muscle	_	+	+	++	++
Degeneration	_	+	+	++	++
Mean	_	+	+	++	++++

−−: None (0%), +: mild (<10%), ++: moderate (10–50%), +++: severe (50–70%). Data were represented as a mean of 125 slides (25 slides/prawn) in each group.

Table 4.33: Effect of thermal shock in Hepatopancreas histology *M. rosenbergii.*

	Control	24°C	28°C	32°C a	32°C b
Necrotic tubule	_	+	+	++	+++
R cell	_	+	+	++	++
B cell	_	+	+	++	+++
E cell	_	+	+	++	+++
F cell	_	+	+	++	+++
Thickened Basal Lamina	_	+	+	++	+++
Infiltrated hemocyte	_	+	+	++	+++
Tissue debris	_	+	+	++	+++
Tubule coagulation	_	+	+	++	+++
Mean	_	+	+	++	++++

−−: None (0%), +: mild (<10%), ++: moderate (10–50%), +++: severe (50–70%). Data were represented as a mean of 125 slides (25 slides/prawn) in each group.

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4.2.5 Water Sample analysis

The water samples sent for analytical Alkalinity, NH_3-N (mg/L), NO_3-N (mg/L) tests and the result is as below (Table 4.34).

Table 4.34: Water Sample analysis of poor managed farm**.**

Parameters	Treatments	
	Standard Level	poor managed farm
Water temperature (ºC)	8±1	30.40±0.033
Transparency (cm)	20±1	32.13 ± 1.179[a]
DO (mg/l)	7±1	5.99±0.536[ab]
pH	7±1	7.58± 0.384[b]
Alkalinity	140±1	124.65±4.92 [b]
NH_3-N (mg/L)	0-0.05±0.1	0.13 ± 0.042
NO_3-N (mg/L)	1.08 ± 0.01	1.22 ± 0.029

4.2.6 Correlation studies and gene network

To get more insights into the studied gene correlations and studied gene network analysis based on the gene expression reading in different time, temperatures and environmental circumstance, the gene expression data is analysed by JMP. SAS for correlation study and Gene network prediction program. Since hepatopancreas shows more significance gene expression in comparison with the hemolymph and muscle, its correlation study and gene network analysis are presented in result chapter. Hemolymph and muscle analysis are demonstrated in and appendixII.

The multivariate gene expression shows that in control condition while the animal is not under environmental stress, the gene expression pattern shows less metabolite activity, so animal is in tun state and the genes shows negative correlations. By increasing the temperature animal move to the active stage in 28°C which is reported previously as best temperature for *M. rosenbergii.*in this stage PPO, chaperone and Hsp 70 showed positive correlation while the rest of the genes are in negative correlation or tun state. In 32°C, the overall gene expression patter shows fluctuations in relation between the gene's expression pattern. In early stage of 36°C, all the studied gene shows positive correlation

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which shows high metabolite activity in cells. In morphological observation in this stage we observed that the animals in laboratory condition showed weight loose, decrease in hemocyte cell count, Ppo activity and hemocyte color and cell viability. The correlation study confirms that all the studied gene can be used as biomarkers in 32 °C. This researches data interpretation confirms that Hsp70, Ppo1 and PpoIII can be consider as potential biomarkers for study *M. rosenbergii* genomic adaption under thermal stress (Figure 4.37, 38) and suplemenry data on gene expression is available in apendix III.

4.2.7 Gene network prediction

Gene network prediction program predict that, incresing the temprature will decrease the consistency of the genes relation. It predict that the corelation between lectin 4 and other genes might be increased by increasing the temprature. This increased corelation is shown by ticker line in the network prediction (Figure 4.47). Gene network analysis are demonstrated in appendix I.

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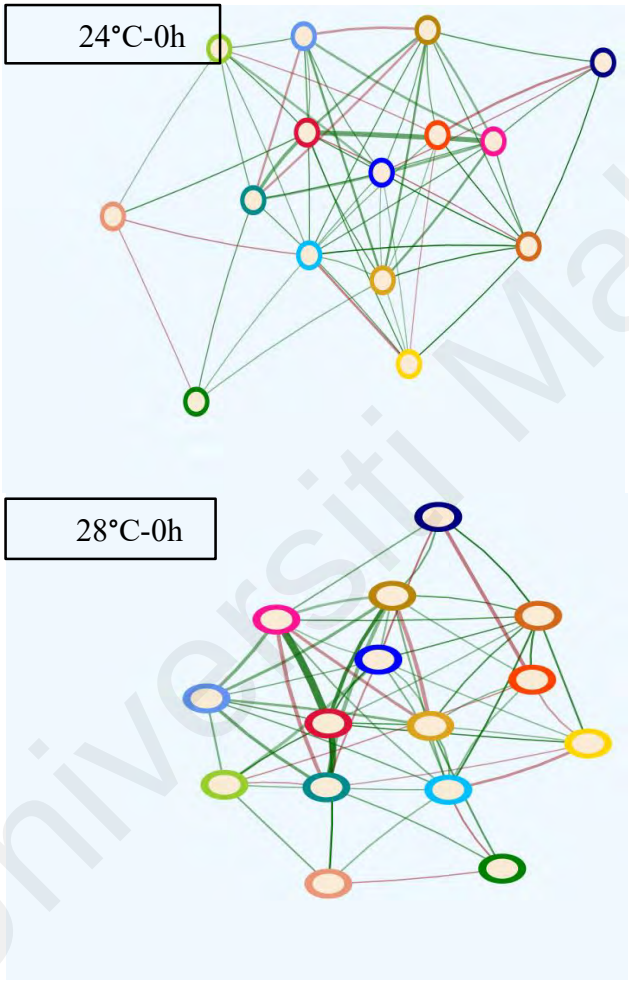

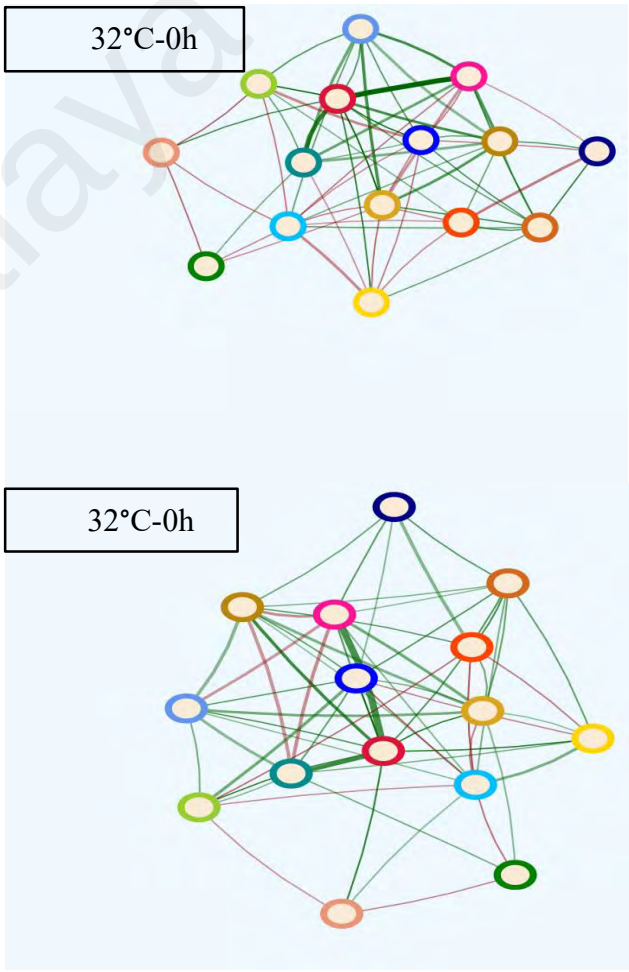

Figure 4.47: Farm 1 - Hepatopancreas

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CHAPTER 5: DISCUSSION

The Macro-environment consists of the total environmental parameters that a species is physically exposed to. This open environment has a significant impact on the species survival chance. In the current fluctuating climate circumstances, a species survival is dependent on its adaptive nature to the environmental changes. These adaptive characteristics can be either physiological or genetical adaptation that are avouching the species survival simultaneously. Among all environmental parameters, temperature is the most important environmental factor which has a direct effect on the physiological response of crustaceans. This critical environmental factor has been notified in the early steps of the scientific researches. Many reports have been released that shows the temperature has a direct impact on kinetic characteristics of enzymes (P. Hochachka & G. Somero, 2002), metabolite pathways and energy utilization (Levinton, 1983), oxygen compensation (Mangum, 1963) and also osmotic regulations (Rodriguez, 1981), negative effect on larval development in Echinaster species (Watts et al., 1982), direct effect on Palaemonetes longirostris larvae inhibition (Antonopoulou & Emson, 1989) , and juvenile crabs hide beneath the habitat before gaining tolerance to the environmental circumstances (Knne, 1970). Few years after these reports, laboratory experiments with concerns on temperature had been reported. Survival and larvae development of *Menippe mercenaria* was faster in low temperature in comparison with higher tested temperature in comparison with *Menippe. adina*. And *M.adina* showed greater tolerance to low water temperature in comparison with *M. mercenaria* (Susan & Bert, 1993). An observatory study has been reported that no fertilized female was observed in summer between mid-Julys till August in Mississippi river. This report supported the idea that annual differences in temperature and high temperature has great influence in animal mating, spawning, molting and survival rate in a crustacean. This study confirmed that the adaptation of marine species is a kind of divergence to the environmental circumstance's

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tolerance. It was also mentioned that this adaptation pattern is not only between different species but also whitin a population and among individuals. And it promotes the distribution of a specie´s pattern according to its physiological differences in allopatry (Brown & Bert, 1993).

It has also been reported that water temperature is an important physical factor which effects on the fish growth rate (Brett & Groves, 1979) (Corey et al., 1983). The fish and subsequently prawns growth factors rate will increase by increasing water temperature up to the animals tolerance level (Jobling, 1993). Low temperature shows metabolite rate decreasing in fish which cause reduction in its production (Jhingran & Pullin, 1985). Later on innate immune system of the crustacean as an important survival factor, attracted researchers and some studies on the effect of the environmental factors such as water temperature, salinity, pH on the innate immune system has been conducted *M. rosenbergii* as a sample model of aquatic animals (Winton & Jiann, 2000). It was also reported that temperature has direct effect on food and oxygen consumption and growth rate in *M. rosenbergii* (Niu et al., 2003). It was reported that the optimal temperature range for freshwater prawn, *M. rosenbergii*, culture is between 20- 28° C, depending on the latitude and environmental circumstances (Uddin et al., 2010). There are many reports available which are performing the importance of the water temperature on aquatic animal's growth rate, survival and production in different parts of the world. These reports are emphasizing that aquaculture has important impact in providing nutritional burdens in the populated world. The increasing population is subsequently increasing the food demand and nutrition's. This increasing demand will cause increase in production of aquatic animals such as fishes, prawns, mollusks, lobsters, etc., and even aquatic plants such as different types of the weeds. Among all, prawns have a great potential culture for large cultures as low-cost animal but high nutrition animal for human consumption. Of these, giant freshwater prawn, *Macrobrachium rosenbergii*, has specific characteristics

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such as high nutritional value and large size that make it desirable for human consumption. So, the high market demand and export values also low culture cost are reasons of a great increase in this aquatic animal s production (FAO, 2011a) FAO reported that the production of *M.rosenbergii* was initially 66628 tone in 1997 and increased to 220254 tone in 2012 (FAO, 2011b). Even though *M. rosenbergii* is a popular food resource for Malaysians and there is good demand in market, the production of this animal is still low in Malaysia (413.28 tone) which cause the increase in the market price (25 RM /kg) (Yusoff, 2015). Malaysia is a South Asian tropical country with high humidity and an average of 27 °C temperature (NOAA, 2012). In order to culture the prawns in Malaysia's fluctuating environment, this animal need to adapt rapidly to the environmental circumstances. Aquacultures animals have limited tolerance range to water quality and temperature. Critical temperatures for crustacean and invertebrate species have been observed in different species by study different parameters such as innate immune system, growth rate, fertilization, ventilation rate, accumulation of heavy metals, heart rate etc. (Banu & Christianus, 2016). Their best possible survival rate and growth factor are observed at their optimal environment circumstances, and each water parameters can directly affect on their survival rate. As an aquatic animal, *M. rosenbergii* survival and growth depend on the water environment circumstances. So, it's important for Malaysian farmers to know about *M. rosenbergii* optimum condition for better growth and survival rate (Pauly & Kinne, 2010). Having a good insight into *M. rosenbergii* tolerance limits and optimal survival and growth condition will help farmers to increase the culture yield which will encourage them to increase their farming and develop their broodstock. Therefore, this study was conducted to investigate the effect of water temperature on the innate immune system susceptibility to the water temperature and adaption range of this animal.

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5.1 Genomic discussion

Providing a molecular response range resulting from environmental changes is important to develop preventional strategies, which avoid mortalities related to a climatic parameter in the natural environment-and in-aquaculture farms. Here, through the essential pathway's gene expression analysis, we tried to identify resistance-related gene expression signatures in prawn succumbing water temperature changes. To understand the mechanisms that cause resistance in prawns, some studies have been directed to identify gene regulation by environmental changes. Particular attention has been paid to temperature because of its catastrophic impact on prawn production worldwide (C. J. Conway & Martin, 2000; T. J. Conway et al., 1994a; T. J. Conway et al., 1994b; Hornbach et al., 2016; Hughes et al., 2003; Pachauri et al., 2014; Sagarin et al., 1999; Stillman, 2003). Even though it's generally argued that breaking temperature limits can cause high level of mortality in a limited time, still, fluctuations in differential gene expression that may control the mortality event have been rarely investigated. This study is expected to be a novel report regarding of gene analysis pathways of prawns under heat shock. We provide the conditions for a heat shock challenge which is up to the temperature that kills prawns within few minutes after the heat shock.

This study, therefore, explored and studied the immune-gene range in prawns succumbing to heat shock through high-throughput gene expression profiling of experimented prawns. Revealed immunological gene responses, which were previously identified as transcriptional signatures for *M. rosenbergii*, were defined as striking functional categories potentially involved in gradual death caused by environmental changes. The study has been conducted in three different locations consisting of a laboratory condition, a well-managed farm, and a poorly-managed farm.

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In this study, three different tissues of *M. rosenbergii* including hemolymph, hepatopancreas and muscle were genetically and physiologically screened to get more insight in to their physiological importance in animal survival and adaptation to the advert environmental circumstances. This is the first report to study the *M. rosenbergii* hemolymph, hepatopancreas and muscle gene expression under heat shock treatment.

Hemolymph is an important organ in a crustacean's survival because it controls the open circulatory system. Nutrients, hormones and oxygen are distributed in the hemolymph. Thus, hemolymph has a critical role in the animal´s survival. Since hemolymph is a circulatory fluid, it fills the interior (the hemocoel) of the animal's body. It will, therefore, show initial effects against environmental changes in terms of gene expression and its cell numbers. Also, Hemolymph contains hemocyanin which is a copper-based protein, and its color turns blue by oxygenation. The dark blue color is the natural color of the healthy animal's hemolymph, and any environmental changes will make changes in its natural color (Fredrick & Ravichandran, 2012). Previous studies have been conducted to study the hemolymph role in pathogenic infective conditions (Agundis et al., 2000; Fredrick & Ravichandran, 2012; Madeira, 2014; Myung & Choi, 2010; Nellaiappan & Sugumaran, 1996; Pereyra et al., 2004). In this study, Gene expression profiling was studied as genetic adaptation, and hemocyte cell number, hemocyte protein content and hemocyte color changes were examined as non-genetic adaptation at different temperatures and time intervals.

Hepatopancreas is another important organ in prawn. It's the source of nutrient absorbance and storage. It synthesizes the food digestive enzymes. Also, it is the source of lipid and energy storage. This hepatopancreatic stored energy will later be used in a starving situation, molting or reproduction and during growth. In this study, we observed the hepatopancreatic gene expression profile as genetic-adaptation and hepatopancreatic

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somatic index and hepatopancreas weight and hepatopancreas Histo-architecture under different thermal conditions and environments (W. Wang et al., 2014) as non-genetic adaptive parameters or physiological function (H. Jiang et al., 2009; W. Wang et al., 2014; ZhaoZhiYing et al., 2009).

Muscle is another important organ of the prawn body because it's the largest organ of the body and has the main functional role in the animal´s movement. It is also the source of accumulated nutrient's which will be use in food consumption. In this study, we looked at its gene expression as genetic-adaptation, histopathology and protein content during thermal treatment (Sriket, 2014) as non-genetic adaptation. The denatured muscle fiber and damaged myofilaments in high temperature were put in the laboratory and the poorly-managed farm while most of the animals from the well-managed farm confirmed normal muscle histo structural pattern (Cheng & Chen, 1998; Sriket, 2014). The same muscle histo structural pattern had been reported for *M. rosenbergii* under pathogenic infections (Cheng & Chen, 1998).

Gene expression data analysis of the metabolite coding genes, arginine kinase, showed that the gene expression of both metabolite coding genes, arginine kinase and small heat shock 70, in 24 °C are significantly up regulated in *M. rosenbergii's* hepatopancreas (Arockiaraj et al., 2011; Junprung et al., 2017). The revealed data illustrate that six hours after starting the heat shock experiment, the expression of both studied metabolite controlling genes were significantly up-regulated from 24 °C degree up to 32 °C. Their expression follows by a rapid fall up to 36 °C hemolymph, hepatopancreas and muscle, respectively. Also, in twelve hours after the heat shock experiment the gene expression rate increases rapidly from 24 °C up to 28 °C. It shows a slight fall up to 32 °C but a sharp fall up to 36 °C, which is dormant with the control expression level in *M. rosenbergii* hepatopancreas, hemolymph and muscle, respectively.

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In this study, pathway studies showed that antiviral-related genes were up-regulated upon heat-shock challenge. Most of the antiviral-related genes that were analyzed mediated the RNAi machinery. This process had been proven to perform antiviral mechanism in different organisms, including crustaceans (Bartlett et al., 2002) (Bartlett et al., 2002; Labreuche & Warr, 2013) Prophenoloxidase genes which were to be up-regulated by heat shock, and were involved in double-stranded RNA mechanism in recognition and degradation by increasing temperature (Labreuche et al., 2013; (Aliyari & Ding, 2009). This gene up-regulation was also observed in *Fenneropenaeus chinensis* shrimp in response to WSSV infection (Li et al., 2013). In this study, other antiviral coding genes were found to be up-regulated by the heat shock up to 28 °C and started to decrease the regulation from 32 °C up to 36 °C. Our findings; therefore, revealed that heat shock may activate RNAi mechanism. It might activate other antiviral defense pathways. However, it was previously stated that the activation of the antiviral host defense appeared to be not sufficient for protecting shrimp during environmental changes (Condamine et al., 2013) so, other groups of genes need to be activated for environmental defenses.

A preliminary observation showed that the prawn´s mortality by high temperature was preceded by a decrease in AMPs regulation. In another study, under non-lethal microbial challenge (heat-killed viral pathogenes WSSV), AMPs at mRNA level revealed an intense drop during the initial hours post-injection in hemocytes (reviewed in (Rosa & Barracco, 2010; Somboonwiwat et al., 2008). However, AMPs expression again returned to initial expression levels at 24–72 h after non-lethal injection, because of the hematopoiesis activation (Bachère et al., 2004). The decrease in AMPs mRNA levels might be related to the deficiency of the prawn's cellular and humoral reactions in the immune responses. It also might be because of the hemocyte reproduction for maturation by hematopoietic tissues. These conditions may cause the prawn´s death. By comparing the total studied coding genes in heat shock challenge with their differential expression

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in viral infection studies, our preliminary observation showed a dramatic increase for the modulated genes upon a heat shock challenge. This finding may indicate that the early steps of mortality by heat shock has a stronger effect on the suppression of the studied genes.

Anti-lipopolysaccharide factor gene shows a moderate up-regulation at 24 °C and 28 °C and a sharp up-regulation at 32 °C and a rapid fall at 36 °C at six hours after the heat shock experiment in hemolymph, hepatopancreas and muscle, respectively. It illustrates a steep increase expression at 24 °C and 28 °C follows by a steady increase at 32 °C and a sharp fall at 36 °C, twelve hours after the heat shock experiment in *M. rosenbergii* hepatopancreas, hemolymph and muscle, respectively.

NF-kappa B inhibitor alpha shows a moderate up-regulation at 24 °C and 28 °C and a sharp up-regulation at 32 °C and a rapid fall at 36 °C at six hours after the heat shock experiment in hemolymph, hepatopancreas and muscle, respectively. It illustrates a steep increase expression at 24 °C and 28 °C and a steady increase at 32 °C, and a sharp fall at 36 °C, twelve hours after the heat shock experiment in *M. rosenbergii* hepatopancreas, hemolymph and muscle, respectively. The insufficient gene expression of the prawn´s immune-related transcripts may have repressed the prawn's physiological systems and will cause early mortality (within 12 hours after challenge). Results also showed that genes involved in the proPO activating system, such as the prophenoloxidase 1 were strongly down-regulated by increasing temperature. The activity of proPO system against high environmental changes have been well established (Amparyup et al., 2013; Cerenius & Söderhäll, 2004). Previous studies on viral infections showed down-regulation of proPO genes during WSSV challenge as a pathogen which might be affected by the environmental stability (Luna-González et al., 2012). On the other hand, the small heat shock 70 gene were reported to be expresssed in Farfantepenaeus paulensis in response

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to *F. solani* non-lethal infection (Perazzolo et al., 2011) and also in *F. chinensis* infected under WSSV (Shihao Li et al., 2013). According to our findings, the initial activation and control of the proPO can be compromised in virus-infected cells are effective in this experiment. Therefore, high-level heat challenge could obstruct the proPO activation or the proPO system activators and reduce the host immune defense.

Prophenoloxidase 1 and prophenoloxidase activating enzyme gene expression, illustrates a steady increase at 24 °C, 28 °C and 32 °C and plateau expression at 36 °C, six hours after the heat shock experiment in hemolymph, hepatopancreas and muscle, respectively. It shows a different pattern twelve hours after the test by significant increase at 24 °C, 28 °C and 32 °C hours and a sharp fall at 36°C in *M. rosenbergii* hepatopancreas, hemolymph and muscle, respectively.

Hemocyanin shows a moderate up-regulation at 24 °C and 28 °C and a sharp up-regulation at 32°C and a marked down-regulation at 36 °C, 6 hours after the heat shock experiment in hemolymph, hepatopancreas and muscle, respectively. It also shows a steady increase at 24 °C, 28 °C and 32 °C and a sharp fall at 36 °C twelve hours' time point after the heat shock experiment in *M. rosenbergii* hepatopancreas, hemolymph and muscle, respectively.

Lectin family exists in circulating fluids, either as cell surface receptors or soluble proteins (Marques & Barracco, 2000). Different types of lectins have been diagnosed in a crustacean. Lectin1, lectin2, lectin3, l3ctin4 and C-type lectins are the most recognized lectins in crustaceans. C-type lectins are the largest group of immune function that is mostly found in a crustacean´s hepatopancreas (Zhang & Dong, 2007). Many lectins have been purified and characterized from a crustacean´s hemolymph (Vázquez et al., 2009). In crustaceans, lectins have been reported to contribute to innate immune responses, including prophenoloxidase activation, encapsulation1.nodule formation of hemocyte,

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opsonin formation, antibacterial activity, and antifungal activity and may also be contributing to injury healing (Vazquez et al., 2013). It is reported that lectin serves a decisive role for both physiological and pathological processes with exclusive interactions between intricate carbohydrates that include glycoproteins, glycolipids, polysaccharides or proteoglycans (Vázquez-Mendoza et al., 2013).

Gene expression study of C-type lectin family genes, including Lectin1, Lectin2, Lectin3, Lectin4 and Tachylectin genes shows a moderate up regulation at 24 °C, 28 °C, six hours after the heat shock experiment. The gene expression study confirms steady up-regulation in gene expression from up to 32 °C and rapid decrease in gene expression at 36 °C, six hours after the heat shock experiment in hemolymph, hepatopancreas and muscle, respectively. This group of genes shows significant up-regulation twelve hours after the test in in 24 °C, 28 °C up to 32 °C and a sharp down regulation in 36 °C, twelve hours after the heat shock experiment in *M. rosenbergii* hepatopancreas, hemolymph and muscle, respectively.

In response to this architecture, a family of proteins that were termed as pattern recognition proteins (PRPs) will be exerted by the immune system to initiate downstream mechanisms. Heat shock proteins are considered as chaperone molecules that can protect other macromolecules from degradation. Even though some of the proteins from this family have other functions. In some organisms such as fish and bacteria the expression patterns of the heat shock protein genes have been linked to adaptation to thermal environments. These examples are proof that, there are many instances of environmental impacts on gene expression. However, it is important to keep in mind that there is a very complex interaction between genes and the environment which describes individual phenotype and characteristics.

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Chaperonin and a small heat shock 70 protein gene show a steady expression rate at 24 °C, 28 °C and 32 °C and a sharp fall at 36 °C in six hours' time point in *M. rosenbergii´s* hemolymph, hepatopancreas and muscle, respectively. This gene illustrates a steady increase expression at 24 °C, 28 °C and 32 °C and a sharp down-regulation in 36 °C, twelve hours after the heat shock experiment in *M. rosenbergii* hepatopancreas, hemolymph and muscle, respectively.

Apoptosis is genetically programed cell death. This pathway will eliminate harmful cells (such as a viral cell) and damaged cells. This pathway is very important in some of the normal processes such as the development of the embryo, metamorphosis and more importantly in the immune defense (Wen et al., 2012). Apoptosis pathway plays an important key role in a crustacean´s immune defense against viral attacks. Many viruses have different strategies to inhibit apoptosis in host cells during viral infections. The virus will try to delay the host cell´s capability till enough vital offspring viruses has produced (Wang et al., 2008). Even though the viruses always try to inhibit apoptosis, there is an exception in some viruses that they try to use apoptosis pathway to spread their progeny virus to other cells. The most important molecules which mediate the apoptosis pathways are caspase (Elmore, 2007). Caspase overexpression was diagnosed in *M, japonicas* under WSSV infection (Jiann & Mo, 2016).

Inhibitor of Apoptosis protein shows a moderate up-regulation at 24 °C and 28 °C and a sharp up regulation at 32 °C and a rapid fall at 36 °C at six hours after the test in hemolymph, hepatopancreas and muscle, respectively. It illustrates a steep increase expression at 24 °C and 28 °C and a steady increase at 32 °C and a sharp fall at 36 °C, twelve hours after the heat shock experiment in *M. rosenbergii* hepatopancreas, hemolymph and muscle, respectively.

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Genes from different functional pathways and categories, including metabolism and antiviral related molecules, were demonstrated as constitutively expressed genes in circulating hemocytes over twelve hours of the heat shock, representing that the expression of these molecules are not highly affected by environmental changes. For instance, apoptosis inhibitor gene in highly up-regulated in pathogenic attacks but here it is down-regulated by environmental changes. This work studies RT-qPCR analyses at an individual animal's level and can provide reliable information on the expression of hemocyte and other organs´ immune-related genes.

Our gene expression data in both the laboratory and the environment aqua level confirm that, the multivariate gene expression in controlled conditions while the animal is not under environmental stress, show less metabolite activity, so the animal is in tun state and the genes shows negative correlations. By increasing the temperature, the animal moves to the active stage at 28 °C which is reported as best temperature for *M. rosenbergii*. In this stage PPO, chaperon and Hsp 70 showed a positive correlation while the rest of the genes were in negative correlation or tun state. At 32 °C, the overall gene expression pattern showed fluctuations. In the early stage at 36 °C, all the studied genes showed positive correlation which shows high metabolite activity in the cells. The correlation study confirms that all the studied gene can be used as biomarkers at 32 °C. This data analysis confirms that Hsp70, Ppo1 and PpoIII can be consider as potential biomarkers for the study of *M. rosenbergii* genomic adaption under thermal stress.

The comparison between three studied organs showed that six hours after the heat shock treatment all the candidate genes show down regulations in hemolymph, hepatopancreas and muscle. Twelve hours after the heat shock treatment they show down regulations in hepatopancreas, hemolymph and muscle. It might confirm that in short term stress, hemolymph is the most affected organ while in long-term stress hepatopancreas is

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the most affected one. This study also showed that the muscle is the most stable organ in terms of gene expressions among the three studied organs.

This study may have the first report of the gene expression profile of different pathways coding genes that reveal the immune response of prawns at the heat shock response up to death. The molecular signatures identified in our study might have uncovered promising candidate genes for environmental studies. The contribution of these target coding genes in the prawn's immune response and unknown cellular pathways could be deeply explored through an in vivo approach. Since mortalities caused by heat shock can be a serious constraint to prawn production worldwide, the transcriptional signatures identified for that might also serve as immune markers of mortality events. So far, gene expression signatures have been used in diagnosis of important human diseases (Asano et al., 2001). This technique also provided predictive tools for prediction and prevention of mass mortality events in the oyster aquaculture (Chaney & Gracey, 2011). Lastly, our studied genes have the potential to clarify the molecular basis of mortality by temperature changes and elucidate Gen- environment interactions during the death process.

5.2 Physiology Discussion

Even though the IPCC reports which address the global warming impact on earth had been officially released in 2001 and 2007, 2014 (Pachauri et al., 2014), the impact of temperature on animal health still has been neglected. The environmental temperature has a direct effect on groundwater temperature. Water temperature can be a significant thermal stressor that affects the physiology of the aquaculture animal. Aquaculture animals are threatened by the fluctuations in water temperature, caused by global warming (W. Jiang et al., 2016).

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The animal's behavioural changes clearly indicated the effect of the thermal shock on treated animals in comparison with the controlled ones. The severe behavioural changes such as imperturbability, loss of balance, reduced swimming, a slower rate of food intake, convulsion was observed in high temperature. To get more understanding about the physiological health of the animal.

Hemolymph is fluid in the circulatory system of the crustacean that fills all the interior area of the animal's body and surrounds all the cells. Oxygen, hormones, nutrients, and cells are distributed in the hemolymph. This wondering liquid contains a famous copper-based protein, hemocyanin that by oxygenation turns blue in color. Hemolymph is important because of its role in the innate immunity of the prawn. It contains agglutinating materials and a role as vertebrate antibodies. In the studies on invertebrate immunity, cell-free hemolymph has been of focus because of their ability in pathogen destruction, damage repairs and regulation (Vazquez et al., 2009) (Fredrick & Ravichandran, 2012). So, hemolymph is an important fluid in the prawn's physiological system. Its characteristics parameters such as total hemocyte protein quantifications, total hemocyte cell count, hemocyte cell viability and phenoloxidase activity are important in the prawn's physiological study (Fredrick & Ravichandran, 2012; Lew, 2010; Myung & Choi, 2010).

The hemocyte color of a normal prawn is usually blue or greenish blue because of the oxygenated copper-based protein. In this study, the heat-treated prawn showed dark coloration by increasing time and temperature up to 28 °C and discoloration in 12h after the test at 36 °C. It may confirm the hemocyanin is a copper-based protein.

Degradation at a high temperature, which may cause a deficiency in oxygenation. The same situation was observed in both farms with the difference that in the bad-managed farm, this hemolymph discoloration was observed in most of the animals from the time

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that the temperature reached to 32 °C at 2 pm local time. But it was observed in a minority of animals at the same time and temperature in the well-managed farm.

In this study, Total hemocyte protein quantifications, total hemocyte count, and hemocyte cell viability assays show that the number of the hemocyte cells and hemocyte cell viability decreased by increasing the temperature and isolation time in all three environments. But degradation in a poorly-managed farm considered as severe type hemocyte cell degradation will affect the animal's survival tolerance in a high temperature and bad environmental circumstance. This may confirm that the management has a direct effect on the physiological tolerance of the animal.

In crustacean, PO activity is mainly localized in the hemolymph (Eom et al., 2014). And comparisons of intrinsic PO activity (PO enzymes activated by endogenous proteases) and the total PO activity (intrinsic PO+ PO enzymes activated by exogenous proteases) provide an important parameter used to assess immunocompetence and health status in an ecological context (Cornet et al., 2009). To get more insights about the effect of the environmental temperature on the physiological health of the prawn, a hemocyte phenoloxidase activity study had been done on both laboratory and farm samples. hemocyte, phenoloxidase activity study, confirms that by increasing the temperature, the phenoloxidase activity has severed degradation in the laboratory while in the well-managed farm it shows a linear tolerance range, and in the poorly-managed farm, it showed significant degradation in comparison with the other two environments. This may confirm that the prawns will lose their prophenoloxidase activity in the controlled condition at high temperature. But in the natural environment, under well-managed situations they will keep their prophenoloxidase activity tolerance and under poor-managed situations, they will lose their prophenoloxidase activity, which subsequently will cause sensitivity to pathogens and invader attacks. Similar negative impacts of

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negative PO activity were reported in the oyster *C. virginica* upon parasitic infection (Grizzle et al., 2008).*Vibrio parahaemolyticus* infection, happened when the PO activity of the abalone *H. diversicolor* was reduced (Cheng et al., 2004).This may be one of the results that makes *M. rosenbergii* pathogenic outbreaks in local farms.

All these observations show the important effect of the hemolymph as a circulating organ. It may also confirm that the management system has a direct effect on an animal's survival rate. It further confirms that well-managed systems will improve the animal's tolerance to environmental circumstances.

Hepatopancreas is another important organ in prawns. It's the source of nutrient absorbance and storage. It synthesizes the food digestive enzymes. Also, it is the source of lipid and energy storage. This hepatopancreatic stored energy will later be used in a starving situation, molting or reproduction, and during growth. In this study, we observed the hepatopancreatic gene expression profile as genetic-adaptation and hepatopancreatic somatic index and hepatopancreas weight and hepatopancreas Histo-architecture under different thermal conditions and environments (W. Wang et al., 2014) non-genetic adaptive parameters.

Hepatopancreas consists of four different epithelial cells, which are known as E-cells, R-cells, F-cells, and B-cells. These cells line the tubules. This organ in a crustacean is an analogous organ to the liver in vertebrates (Baticados et al., 1987; Bhavan & Geraldine, 1997) Hepatopancreas has a sensitive characteristic which makes it liable to external pressures (Lightner et al., 1996; Lightner et al., 1982). In this study, the hepatopancreas histo structure showed that the number of the R-cells which are lipid storage cells per tubule decreased subsequently in the thermal-treated animals by increasing temperature and isolation time in the laboratory, well-managed farm, and bad-managed farm. Similar observations were reported in *M. rosenbergii* (Stalin et al., 2013), *P. monodon Penaeus*

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(P Jiravanichpaisal et al., 1994), *P. vannamei P. Stylistics,* (Lightner et al., 1982). Hepatopancreas somatic index (HSI) showed the lipid storage in hepatopancreatic cells. In this study, the hepatopancreatic index decreased by increasing the temperature. In this experiment in the laboratory level by increasing the temperature prawns utilized their energy and in results they used the lipid storage of the hepatopancreas which causes significant HSI decrees in high temperature(Stalin et al., 2013). The same hist-structure pattern was observed in the bad-manged farm while in the well-managed farm, the majority of the studied animals showed normal histo structure patterns.

Muscle is another important organ of the prawn body because it's the largest organ of the body and has the main functional role in the animal´s movement. It is also the source of accumulated nutrient, which will be used as food consumption (Sriket, 2014). In this study, we looked at its gene expression as genetic-adaption, histopathology, and protein content during thermal treatment as non-genetic adaptation. The muscle has to structure pattern showed denatured muscle fiber and damaged myofilaments in high temperature in laboratory condition and bad-manage farm while the majority of the animals from well-managed farm confirmed normal muscle histo structure pattern. The same muscle histo structure pattern had been reported for *M. rosenbergii* under pathogenic infections (Baticados et al., 1987; Stalin et al., 2013).

Thermally treated groups exhibited several morphological variations such as discoloration; damaged rostrum; opaque coloration in cephalothorax; black bands and dot formation in abdomen; deformed uropods and telson in tail regions when compared with the control group. The Hepato Somatic Index reflected the severity of temperature on hepatopancreas. Histological variations in hepatopancreas and muscles of treated groups were observed. Accumulation of hemocytes in hemocoelic space, interstitial sinuses filled with abnormal infiltrated hemocytes, the tubular epithelium with ruptured basal laminae,

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abnormal and coagulated lumen, necrotic tubules, thickened basal laminae, tissue debris, necrotic hepatocytes were observed in thermally treated prawn hepatopancreas. In muscle, shrinkage of muscular fiber and necrotic musculature were observed in thermally treated prawns. These structural alterations of the organs could affect the vital physiological functions such as respiration, osmotic and ionic regulation in muscles; absorption, storage, and secretion of the hepatopancreas which in turn could adversely affect the growth and survival of freshwater prawn *M. rosenbergii*.

Water quality is decreasing due to the unstandardized husbandry in prawn ponds (Boyd, 2003; Boyd & Tucker, 2014). In this study, the impact of the water quality on the prawn's physiological tolerance had been studied. The result of the study showed that in the poorly-managemed pond, the DO level, PH, Alkalinity level are less than the normal condition. The NH3-N (mg/L), NO3-N (mg/L) are higher than the normal level. In this aquatic circumstance, the water temperature is higher than the standard water temperature and optimal temperature for the animal´s survival. In this situation, the animal shows less physiological tolerance against high temperature. While in the well-managed farm, the water temperature is the same level as the standard water temperature and near to optimal temperature for the animal´s survival. However, its physiological observation shows that this organ is losing its physiological constancy by increasing temperature in high temperature.

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5.3 Conclusion

These results highlight the sensitivity of this species to temperature and the probable consequences of heat stress to physiological tolerance of *M. rosenbergii* in the wild and open pond. Analysing the physiological performance of *M. rosenbergii* in thermal stress may help commercial farmers and researchers to make an early prediction on how ecological temperature alterations in the *M. rosenbergii* habitat may influence continuous prawn farming in the future. And improving farm management and animal husbandry is helpful to increase the farming yield.

Besides this, genome has a static characteristic, while proteome has dynamic characteristics which depend on genome reactions. Through understanding the genomic responses to stress we can predictions about proteome. Genomic biological markers or biomarker are the elements that pave the way for such understanding for scientists. A biomarker is a gene which can be used as an indicator for a diagnostic biological respond in a specific circumstance (Jones et al., 2002). Biomarkers can be used to find out the early stage of a general molecular and biological warning to an environmental stress such as temperature. The efficiency and sensitivity of a biomarker depends on the animal's adaption to the environmental circumstances (Hook et al., 2014).

The conclusion to this investigation is the first of its kind, where *M. rosenbergii* is used to assess the thermal effect shock. Thermal shocked groups showed several morphological and histological (hemocyte, hepatopancreas, and muscles) alterations. The laboratory observed results revealed that short term thermal shock induced changes are less than long term induced shocks in *M. rosenbergii*.

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Vertebrates have three stages of tolerance against environmental stress. The first stage is alarm reaction which is known as "fight-or-flight" is a reaction against any physical, chemical or environmental stress. This will cause a sudden reaction by the animal´s body to overcome the stressor. The second stage is called resistance. At-resistance, the body tries to find reasonable balance. In vertebrates, this internal procedure is called homeostasis. At this stage, the animal nerve system may think it is able to control anything because the stress alarm stage is calmed down until the stress alarm becomes fully exhausted. The third stage is exhaustion in which the biochemical systems including immune responses, metabolite reactions, and stress controller pathways are completely shut down. After long unending environmental stress, the animal´s defense system will lose tolerance and succumbs to disease either a viral or bacterial infection (Cherry, 2018).

This study revealed that even though invertebrates only have an innate immune system, but their reactions to the environmental stresses follow the vertebrate's defense pattern.

In this stage, we examined a morphological observation of the animals in the laboratory and it showed weight loss, decrease in hemocyte cell count, Ppo activity and hemocyte color and cell viability. This study may conclude that even though giant fresh water prawn doesn't show physiological tolerance to the increase in aquatic temperature, but it still genetically adapts itself under adverse circumstances and might try to pass this adaption to offspring. This adaption broad range tolerance will pave the way for this animal's genetical existence under the current global warming circumstances which are treating all phenomena existence on the earth, but the physiological abnegation to the thermal fluctuation may cause severe physiological damage which may result in this animal´s extinction. In conclusion, an increase in water temperature is a serious

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environmental issue which will cause a lot of environmental anarchies that are threatening the human life of humankind.

Lastly, this Gnomical and physiological adaptation study confirms that there is genetic adaption in the giant freshwater prawn under adverse thermal circumstances, but there is no physiological thermal tolerance to cope with the increasing water temperature in a natural environment. The giant freshwater prawn; therefore, is not able to adapt itself under adversely high thermal circumstances.

5.4 Conclusion and recommendation

Freshwater prawn, *Macrobrachium rosenbergii,* has an important economic role in Malaysian aquaculture industry. This animal has a specific genetical and physiological characteristic which makes it able to have a high survival rate under adverse environmentally circumstances through ages. Even though this animal performed a high genetical and physiological tolerance through the centuries, but the tolerance would be affected by increase in temperature and possible affect of pathogen. In this study, by comparing this animal genetical and physiological adaption and tolerance ability under three different environments, we observed that even though this animal shows some level of genetical tolerance, it doesn't show the standard level of physiological tolerance which will increase the extinction threat of this animal. Since this animal is an important aquatic specious which is widely cultured in Malaysia and on the other hands, Malaysia is a country with special climate situation which is under threat of global warming, so, the survival of aquatic animals in this country is under threaten. The initial recommendation

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of this study researcher is to find solutions to control temperature changes which subsequently will control water temperature and improve aquatic animal's habitat.

The second recommendation is improving the prawn's farm management system by increasing the technologies in animals husbandries in hatcheries. Some local improvement will help in increasing animals survival rate and quality and will improve farmers profits margins. Consequently, more environmental qualitative and quantitative researches would be required for sustainable prawn farming. In additions research in genomics areas need to be given special attention considering animal's extinction and their genetical and physiological adaption ability.

In the past decades, gene-environment interaction and adaption process of living organism have been interning subject for researchers.

However, so far there is only limited in vivo information on genetic-physiology adaption. Therefore, based on the current research following points can be focused on:

a) Isolation and characterization of proteome might help to better understanding of the protein's role in interacting environment adaption and adaption process.

b) More studies on interaction of these current studied gene with other reported genes in *M. rosenbergii.*

c) Studies on adapted animals and their off spring to the advert environmental circumstances. However, we are not sure what type of phenotypes of over expression or maybe anti-sense expression could be produced. There might be

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phenotypic differences between off spring and parents due to constitutive expression and seemingly crucial nature of the protein.

d) Studies on the nutrition content of the adapted animals to the advert climate circumstances.
e) Studies on development of the molecular markers reported under this study.

Such this studies that report gene-environment interaction under different circumstance, contribute to make more insights into the role of the genes, proteins and cell structures in living organisms' adaption to the advert climate circumstances. Along with different gene cluster some genes have the potential to be biomarkers candidate. This biomarker can be later on use as diagnostic gene to screen a genetical tolerance or adaption and finally, predict animal's survival and adaption rate. This biomarker is also involved in regulation of transcriptional activities and they are reported to be required for specific gene regulator process. Therefore, developing molecular markers will help in clear understanding of their function in vivo and will benefit work on environmental effects.

Furthered more developing of understanding the DNA regulation structure will pave the way of molecular science in fast growing field such as nanotechnology, biophysics and material science. Hence, more work is needed to elucidate the gene expression understandings in gene-environment interaction and genetic and physiologic adaption.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PAPER PRESENTED

1. Alinejad, T., Bhassu, S., Othman, R. Y. (2015), Hepatopanceras Gene expression profiling of *Macrobrachium rosenbergii* succumbing to heat shock. (International Fisheries Symposium 1-4 Dece2015, Penang, Malaysia).

2. Alinejad, T., Bhassu, S., Othman, R. Y. (2017), Hemolymph Gene expression profiling of *Macrobrachium rosenbergii* succumbing to heat shock. (The 22nd Biological Sciences Graduate Congress (BSGC2017) UTown 19–21 Dec 2017, National University of Singapore).

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