MOLECULAR CHARACTERISATION OF HSP70 GENE EXPRESSION, HISTOPATHOLOGY AND IMMUNE SYSTEM OF SNAKEHEAD FISH, *Channa striata*, UNDER TEMPERATURE AND BACTERIAL STRESS

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GENE ADAPTATION, GENE EXPRESSION AND HISTOPATHOLOGY OF SNAKEHEAD FISH, *Channa striata*, UNDER TEMPERATURE AND BACTERIAL STRESS

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MOLECULAR CHARACTERISATION OF HSP70 GENE EXPRESSION, HISTOPATHOLOGY AND IMMUNE SYSTEM OF SNAKEHEAD FISH, *Channa striata*, UNDER TEMPERATURE AND BACTERIAL STRESS

ABSTRACT

Snakeheads are air-breathing freshwater fishes containing two genera, *Channa* (Asia) and *Parachanna* (Africa). The Asian genus is dominated by *Channa striata* which is found in southern and Southeastern parts of the continent. In this research, environmental and bacteria stressor to *Channa striata* were investigated. HSP70 gene was studied as an environmental stress gene (ChHSP70). Results showed a cDNA sequence of the gene to be (~918bp). It was also shown the gene is highly similar to that of other fish species. Constructing a phylogenetic tree of the gene across vertebrate species revealed six main clusters; same class remained conserved by showing high sequence similarity except for fish which showed two groups. One possible reason for this diversity might be the gradual global warming. Of the two fish groups, polymorphism was found to be higher in one of them, indicating clear bias in their mutation rates, which may result in new speciation. The clear separation between the two fish groups also suggests presence of undiscovered functional differences. Genes that are phylogenetically stable have conserved their core functions, while those that display a much more varied or unstable phylogeny have much more varied functions. This diversity in fish species may be due to the environmental adaptation of this gene to environmental stresses that are more pronounced for aquatic organisms than higher, terrestrial vertebrates. HSP70 expression has been shown to be stimulated by a wide variety of physiological and environmental stresses, including thermal shock, heavy metals, free radicals and microbial infection. Responses of HSP

gene can vary according to tissue, HSP family, and stressors. The sensitivity of HSP gene expression can also vary according to species, developmental stage and season. Higher expression levels of HSP70 due to thermal stress indicates better tolerance to heat stress. In this study the expression of the gene at 32°C in the stomach and fin was higher than that of the gills. The study showed the sustainability of *C. striata* in term of hydrologic regime and global warming might cause mortality or even extinction of this fish. Another factor that may have affected the survival of this fish was bacterial disease. A strain of bacteria was isolated from an infected fish. Identification of the bacterium was based on 16S rRNA gene sequence of 1160bp which showed the closest match to *Enterobacter soli* strain LF7a (99.31% similarity). The sequences obtained designated as *Enterobacter soli* Es2. The current study is the first to report *Enterobacter soli* Es2 as a disease causative agent in fish. The main clinical signs of the disease were skin ulcer, dullness, loss of appetite and abnormal swimming behavior. Histopathologically, hepatic lesions including blood congestion were observed. The latter hinders blood movement from the hepatic portal vein and hepatic artery into the central vein resulting in dilation of sinusoids and rupture of blood vessels. Moreover, expression of galectin 8 (CsGale), glutathioneS-transferase (CsGlut), h2Calponin (CsCalp) and cytochromeb5 (CsCyto) genes, which are known as immune-related genes, were tested after challenging the fish with *Enterobacter soli* Es2. The tested genes showed different Patterns of expression in different tissue types.

Keywords: Snakehead fish, *Enterobacteriaceae*, Gene expression

MOLECULAR CHARACTERISATION OF HSP70 GENE EXPRESSION, HISTOPATHOLOGY AND IMMUNE SYSTEM OF SNAKEHEAD FISH, *Channa striata*, UNDER TEMPERATURE AND BACTERIAL STRESS

ABSTRAK

Ikan haruan adalah ikan air tawar yang bernafas udara yang mengandungi dua genera, *Channa* (Asia) dan *Parachanna* (Afrika). Genus Asia dikuasai oleh *Channa striata* yang terdapat di bahagian selatan dan tenggara di benua itu. Dalam kajian ini, stres alam sekitar dan bakteria kepada *Channa striata* disiasat. Gen HSP70 dikaji sebagai gen tekanan alam sekitar (ChHSP70). Keputusan menunjukkan urutan gen DNA (~ 918bp). Ia juga menunjukkan gen sangat serupa dengan spesies ikan lain. Membina pokok phylogenetic gen di seluruh spesies vertebrata mendedahkan enam kelompok utama; kelas yang sama tetap dipelihara dengan menunjukkan persamaan turutan yang tinggi kecuali ikan yang menunjukkan dua kumpulan. Satu sebab yang mungkin untuk kepelbagaian ini adalah pemanasan global yang beransur-ansur. Daripada kedua-dua kumpulan ikan, polimorfisme didapati lebih tinggi di salah satu daripadanya, menunjukkan kecenderungan yang jelas dalam kadar mutasi mereka, yang mungkin mengakibatkan spekulasi baru. Pemisahan yang jelas antara dua kumpulan ikan juga menunjukkan adanya perbezaan fungsi yang tidak dapat ditemui. Gen yang stabil secara phylogenetikal telah memelihara fungsi terasnya, sementara yang memaparkan filogeni yang lebih pelbagai atau tidak stabil mempunyai fungsi yang lebih pelbagai. Kepelbagaian spesies ikan ini mungkin disebabkan penyesuaian alam sekitar gen ini terhadap tekanan alam sekitar yang lebih ketara untuk organisma akuatik daripada vertebrata terestrial yang lebih tinggi. Ekspresi HSP70 telah dirangsang oleh pelbagai tekanan fisiologi dan persekitaran, termasuk kejutan haba, logam berat, radikal bebas dan jangkitan mikrob. Tanggapan gen

HSP boleh berbeza mengikut tisu, keluarga HSP, dan tekanan. Kepekaan ekspresi gen HSP juga boleh berubah mengikut spesies, peringkat perkembangan dan musim. Tahap ekspresi tinggi HSP70 disebabkan oleh tekanan haba menunjukkan toleransi yang lebih baik untuk tekanan haba. Dalam kajian ini, ungkapan gen pada 32 ° C dalam perut dan sirip lebih tinggi daripada insang. Kajian menunjukkan kemampanan *C. striata* dari segi rejim hidrologi dan pemanasan global mungkin menyebabkan kematian atau kepupusan ikan ini. Satu lagi faktor yang mungkin menjejaskan survival ikan ini ialah penyakit bakteria. Ketegangan bakteria telah terisolasi daripada ikan yang dijangkiti. Pengenalpastian bakteria adalah berdasarkan urutan gen 16S rRNA 1160bp yang memperlihatkan perlawanan terdekat dengan *Enterobacter soli* strain LF7a (99.31% kesamaan). Kajian semasa adalah yang pertama untuk melaporkan *Enterobacter soli* Es2 sebagai agen penyebab penyakit dalam ikan. Tanda-tanda klinikal utama adalah ulser kulit, kebodohan, kehilangan nafsu makan dan kelakuan berenang yang tidak normal. Secara histopatologi, lesi hepatik termasuk kesesakan darah diperhatikan. Yang terakhir menghalang pergerakan darah dari vena portal hepatic dan arteri hepatic ke dalam vena sentral yang menyebabkan pelepasan sinusoid dan pecahnya pembuluh darah. Selain itu, gen ekspresi galectin 8 (CsGale), gen glutathioneS-transferase (CsGlut), h2Calponin (CsCalp) dan cytochromeb5 (CsCyto), yang dikenali sebagai gen yang berkaitan dengan imuniti, telah diuji selepas mencabar ikan dengan *Enterobacter soli* Es2. Gen yang diuji menunjukkan Corak ungkapan yang berbeza dalam jenis tisu yang berbeza.

Kata kunci: Ikan haruan, *Enterobacteriaceae*, Ekspresi gen

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I first say, “All the praises and thanks are to Allah, the Lord of the Âlalamîn”. Allah almighty said: “And not alike are the two bodies of water. One is fresh and sweet, palatable for drinking, and one is salty and bitter. And from each you eat tender meat and extract ornaments which you wear, and you see the ships plowing through [them] that you might seek of His bounty; and perhaps you will be grateful, (Surat Fatir- the Holly Quran).

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LIST OF SYMBOLS AND ABBREVIATIONS

<	:	Less than
>	:	Greater than
µl	:	Microliter (10–6 Liter)
ºC	:	Degree Celsius
MS-222	:	Tricaine methanesulfonate
A	:	Adenine
ANOVA	:	Analysis of variance
BLAST	:	Basic local alignment search tool
bp	:	Base pair
cm	:	Centimeter
cDNA	:	Complementary deoxy-ribonucleic acid
CFU	:	Colony forming unit
C	:	Cytosine
d.f	:	Degrees of freedom
DNA	:	Deoxy-ribonucleic acid
dNTP	:	Deoxy nucleoside triphosphate
DO	:	Dissolved Oxygen
FST	:	Fixation Index
g	:	Gram
G	:	Guanine
HSP	:	Heat shock protein
Kg	:	Kilogram
L	:	Liter
LD50	:	Lethal dose 50

M	:	Meter
MANOVA	:	Multivariate analysis of variance
Mega	:	Molecular evolutionary genetics analysis (software)
Mg Cl2	:	Magnesium chloride.
mRNA	:	Messenger RNA
NCBI	:	National center for biotechnology information
ORF	:	Open reading frame
PCR	:	Polymerase Chain Reaction
RNA	:	Ribonucleic acid
T	:	Thymine

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Snakeheads (family *Channidae*) are air-breathing freshwater fish comprising of two genera: Genus *Channa* which is native to tropical Asia and *Parachanna* which is widespread in tropical Africa. *Channa striata* (commonly known as Haruan in Malaysia) is the most common species in the Asian snakehead genus. Its natural geographical distribution covers most of southern and Southeastern Asia spreading from Sri Lanka in the west to Borneo and Sumatra in the Southeast (Courtenay & Williams, 2004). It also has a wide variety of habitats ranging from lakes, rivers, ponds, swamps, canals and rice fields.

The demand for *C. striata* in Malaysia is momentously expanding due to its profitable value; agreeable flavor as local food (Hossain et al., 2008). Moreover, traditionally the fish is known to be purposely consumed by women after parturition for its healing properties (Jais A et al., 1997). Consequently, it is generally believed to enhance wound healing and reduce post-operative pain and discomfort (Jais A et al., 1997). So, it has been extensively used for nutraceutical and pharmaceutical purposes. Precisely, its fresh fillet was reported to contain an appreciable amount of arachidonic acid which is a precursor of prostaglandins (Jais A et al., 1998) that plays a role in blood clotting and tissue growth (Bowman & Rand 1980). Its fresh fillet also contains high glycine (Jais, A et al., 1998; Zakaria et al., 2007), an amino acid required for collagen formation in human skin. Because of its apparent abundance, production in Malaysia has been largely through catching from the wild, but recognizing the risk of sole dependence on wild populations and the importance of attaining sustainable production, a shift towards aquaculture has started (Sharifuddin, & Azizah, 2014) as the business has grown very fast in the Asia-Pacific region (Caipang et al., 2014) as a whole.

One of the environmental conditions that break host-pathogen equilibrium is heat. More than 99% of species on Earth (including fish) are ectothermic - that means they rely primarily on external sources for their body heat. Temperature variations ultimately affects organisms' metabolism (Atkinson & Sibly, 1997; Berg et al., 2010). In combination with hydrological regime, temperature changes are observed to affect fish physiology (Ashoka & Macusi, 2012). These conditions challenged their survival and reproduction (Jesus et al., 2013). Recently, significant heat rises were detected in the annual number of hot days and warm nights; these trends in extreme temperatures showed substantial steadiness in Southeast Asia (Manton et al., 2001). This kind of increments may precisely affect fish in captivity as usually water depth is not so deep leading to stratification of water temperature. This may pose substantial challenges to survival and reproduction of farmed fish (Jesus et al., 2013). Fish has shown varying abilities to adjust their upper temperature tolerance limits with increased acclimation to temperature changes (Roessig et al., 2004). Most importantly, minor rises in temperature (± 1 to 2°C) may be enough to have sub-lethal effects on tropical fish physiology principally reproduction. This condition may cause an increment of Heat Shock Proteins production (HSPs) in response.

In the last three decades, there has been an exponential increase in research activity concerning description, classification and functional significance of these proteins. However, heat shock protein studies in fish are still not intensive if compared with those in bacteria, yeast and mammals (Alak et al., 2010). Within fish species, teleost that represents nearly half of all living vertebrates, display an amazing level of diversity in morphology, behavior, physiology, and environments that they occupy. Strategies for surviving with diverse environmental stresses have progressed in different teleost species. Therefore, teleost fish are considered to be good models for investigating the adaptation and response to many natural environmental stressors (Van der Meer et al., 2005; Eid et

al., 2016). Recent genome-sequencing projects in several fish have provided insights into the molecular and genetic mechanisms underlying their responses to some environmental stressors (Schartl, et al., 2013; Chen et al., 2014).

Protein expressions of fish toward global climate changes in tolerating temperature (including lethal limits) as well as the rates of fish thermal acclimation provided important information for aquaculture. Adaptations are changes that an organism undergoes to fit different surroundings. Thus, adaptation is an important survival skill in all species. If an organism is not able to evolve over time to fit itself to changes of environmental conditions, it may ultimately become extinct. When alterations occur in the environment, populations may (i) escape by migrating to unaffected habitats, leading to local extinction but continue existence elsewhere; (ii) adjust to the new conditions through phenotypic plasticity without altering the genetic constitution; (iii) adapt to the changed conditions through genetic changes (Russo et al., 2010). Different species of fish showed varied levels of ability in adjusting their upper temperature tolerance limits with increased acclimation temperatures (Roessig et al., 2004).

Heat shock proteins (HSP) which are also known as stress proteins, are a group of proteins that response to external stressful conditions. This group of proteins protects cells from extreme physiological, pathological and environmental conditions. They play roles in correcting protein misfolding and preserving immature polypeptides from aggregation under stresses. Consequently, enabling fish to adapt to environmental stressors (Walia & Balkhia, 2016). The first type of HSP discovered in Drosophila, fruit flies. The protein is a subset of cellular proteins that are induced upon heat shock. Several researches on HSP were also carried out on various organisms and its functions in stress tolerance (Feder & Hofmann, 1999). This HSP was found in all studied living beings, from bacteria to humans, and is considered to be a highly conserved gene across organisms (Molina et al.,

2000). Furthermore, there is exponential increase in the level of interest and research activities concerning the description, classification and functional significance of this HSP protein.

Turning back to the interaction between host pathogen and environment. The aquatic environment supports their pathogens as well, which may reach densities sufficient to cause disease or to render them immunocompromised. In addition, overstocking or poor rearing circumstances contribute significantly in abolishing "host–pathogen environment" equilibrium and, eventually, lead to disease outbreak (Das et al., 2008). The annual economic loss to the aquaculture industry through diseases is estimated to be billions of USD worldwide (Pridgeon & Klesius, 2013). Major pathogens that affect the industry include bacteria (Frans et al., 2011; Wang, 2011), fungi (Public Health England, 2015), viruses (Vega-Heredia et al., 2012) and parasites (Brooker et al., 2007). Precisely, they cause infectious diseases in the order of 54.9%, 22.6%, 19.4% and 3.1% for bacteria, viruses, parasites and fungi respectively (Martin & Król, 2017). Bacteria are found everywhere; in air, water, soil and on our own bodies. In fact, bacteria are pathogens that can affect this equilibrium. There is growing evidence that climate change will increase the prevalence of harmful bacteria (Turner et al., 2016). Some fish, including non-migratory species, are being exposed to novel pathogens as a result of climate change (Martin & Król, 2017). Bacterial diseases in fish are a serious threat to aquaculture systems that cause severe damage and mortality (Hassan et al., 2012).

Several types of bacteria are opportunistic pathogens; thus, can lead to mortalities when fish is under severe condition of stress (Vega-Heredia et al., 2012).

Enterobacteriaceae is extensively spread in nature. It is found in soil, water, feces of humans and animals, insects, plants, plant materials, and dairy products (Neto et al., 2003). It comprised 50% of the microbes recovered from both water and fish of earthen

pond fertilized with animal fecal waste (Ogbondeminu & Okaeme, 1989). *Enterobacteriaceae* species were found to be involved in the mortality of different fish species such as *Pangasianodon hypophthalmus* (Kumar et al., 2013). *Mugil cephalus* (Sekar et al., 2008) and *O. niloticus* (Hassan et al., 2012). It has also been recognized as an important human pathogen because of its ability to develop resistance to antibiotics (Sekar et al., 2008).

As *Channa striata* is an airbreathing fish that prefers stagnant and shallow waters. It naturally remains in bottom mud during dry periods till situation is suitable (Muntaziana, et al., 2013). This habitat frequently exposes the fish to soil bacteria such as *Enterobacter soli* strain LF7a which is first isolated from soil by Manter et al. (2011) which could be an indication of sewage pollution. This bacterium belongs to the genus *Enterobacter*, order *Enterobacteriales*, and family *Enterobacteriaceae* accommodates a number of species of heterotrophic, Gram-negative bacteria. Actually, it has been reported as opportunistic pathogen in fish (Hassan et al., 2012). Enteric bacteria are not the normal flora in the intestinal tract of fish and hence their presence in infected fish may be as a result of the association of fish with the polluted waters. Therefore, the infection of fish by these microbes has been related to the feeding habits of fish. For example, bacterial infections in the bottom feeding brown bull head catfish might probably be due to its habit of ingesting the human flocculation deposits containing bacteria (Troast, 1975). This may be true for other bottom feeders such as *Channa striata*.

Bacterial infection on any type of tissue can be examined through histopathology. Histopathology is the diagnosis of disease by visual examination of tissues under the microscope and observing tissue abnormalities. In order to examine tissue sections (which are virtually transparent), they will be prepared using coloring histochemical stains that bind selectively to cellular components (Khan et al., 2014). The careful study

of pathology has delivered an understanding of disease sufficient to guide effective treatment, and recognize ineffective treatment, so important to individual's enhanced life expectancy. This model has been particularly successful in providing insights for treatment of acute diseases; however, in the setting of chronic disease, progress towards effective therapy has been slow (Perry et al., 2008).

Furthermore, bacterial infection affects fish immune system. In fish as in other vertebrates, immunity is typically divided into two distinct components: the innate immune response and the adaptive immune response. Innate immunity is the first line of defense against infection and it includes both physical barriers as well as humoral and cellular responses. The adaptive immune response also relies on humoral and cellular mechanisms and is characterized by a specific antigen recognition that drives a stronger and faster secondary pathogen-specific immune response. Recent advances in general and fish immunology have demonstrated that many cells and molecules are considered unique to either the innate or adaptive systems play specific roles in both of them, they co-operate in the maintenance of homeostasis (Martin & Król, 2017). Really, the majority of what we know today about the composition, function, and modulation of immune systems are mainly derived from works on mammals. To understand the evolutionary history of immune systems in vertebrates as a whole, work on non- mammal vertebrates is essential too (Zhu et al., 2013). Immunology in fish was traditionally less understood, largely due to the lack of model organisms for genetic manipulation. The lack of sufficient knowledge in fish immunity limits the investigation of immune system evolution, the development of vaccines, and the selection of disease-resistant breeds. More recently, the emergence of zebrafish (*Danio rerio*) as a new model organism and the advancement in genome sequencing technology and bioinformatics have greatly expedited the discovery and functional delineation of genes associated with immunity in fish (Lieschke & Trede, 2009; Zhu et al., 2013).

Variations in the immune response of individuals can be measured using real-time PCR to quantify the expression of genes and functional differences. The exact quantification of biologically relevant gene expression levels is usually difficult, as gene expression can depend on the genetic background of an individual, its developmental stage and the tissue type that is being used for extraction, not to mention technical cautions of working with the more fragile RNA molecules (Whitehead & Crawford, 2006). Even more importantly, it is well established that environmental effects can significantly affect expression of genes (Eid et al., 2016).

This latter fact is the reason why many studies on differential gene expression are done under controlled conditions in the laboratory, in order to minimize environmentally induced changes in gene expression and thus to maximize the possibility to find actual significant differences in expression levels between groups of individuals (Lenz, 2015).

Phenotypic evolution occurs both by changes to RNA and protein sequences and by changes in the level at which these molecules are expressed within cells. Evolution of gene regulation was hypothesized and has been demonstrated to constitute an important component of divergence among species and variation within populations (Meiklejohn et al., 2014).

Mostly, the increase in the expression of immune genes is usually considered as a sign for immune stimulation or enhanced immune response (Abo-Al-Ela, 2018). Usually Pathogens induce changes in gene expression in host cells during the course of infection. These changes may be long lasting on many types of tissues or just momentary. Defining the way, reason and process of bacteria to infect cells represents a stimulating challenge that opens up the opportunity to grasp the essence of pathogenesis and its molecular facts (Tran Van Nhieu & Arbibe, 2009).

Bringing the fish from the wild to captivity will exert some stresses on the fish. Moreover, climate change was observed to affect fish hence the study objectives are the followings:

1.1 Objectives:

1. To investigate the effects of global climate change mainly temperature stress on the HSP70 gene expression in *Channa striata* exposed to high and low temperatures comparing its patterns of expression in different organs of the fish.

2. To investigate the association of *Enterobacteriaceae* bacteria with the mortality of *Channa striata* in Malaysia.

3. To determine the expression of immune related genes in different tissues of the fish challenged with *Enterobacter soli* Es2.

Accordingly, the structure of my thesis sections are as follows:

Chapter Two: The aim is to give an overview of the biology and taxonomy of *Channa striata* and to shed light on the impact of pathogen and environment on it. Essentially, this chapter is a review of work conducted on bacterial pathogen and heat stress on this fish beside a review about the immune system and gene expression procedure.

Chapter three: This section of the thesis gives a detailed account of the procedure that was followed in completing the experiments. It gives enough information about the material and method used to conduct the experiments and also the statistical analysis carried out.

Chapter four: The aim of this chapter is to report the findings of the research presenting the data obtained in appropriate figures and tables. It gives the results and observations of the experiments accomplished.

Chapter five: The aim of this chapter is to interpret and describe the significance of the results obtained in light of what was already known about the research problem being investigated. It discusses and justifies the findings contradiction with other published works.

Chapter six: The aim of this chapter is to provide a general conclusion for issues raised in chapter three, four and five and to draw the study conclusions and recommendations.

CHAPTER 2: LITERATURE REVIEW

2.1 Taxonomy of *Channa striata*

The *Channidae* are represented by 26 species of which 23 occur in Asia and the rest in Africa. (Musikasinthorn, 2000). The snakehead murrel, *Channa striata*, known as "Haruan" in Malaysia, its classification is:

Kingdom:	*Animalia*
Subkingdom:	*Bilateria*
Infra kingdom:	*Deuterostomia*
Phylum:	*Chordata*
Subphylum:	*Vertebrata*
Infraphylum:	*Gnathostomata*
Superclass:	*Osteichthyes*
Class:	*Actinopterygii*
Subclass:	*Neopterygii – neopterygians*
Infraclass:	*Teleostei*
Superorder:	*Acanthopterygii*
Order:	*Perciformes*
Suborder:	*Channoidei*
Family:	*Channidae* – snakeheads
Genus:	*Channa Scopoli*, 1777 – Asian snakeheads
Species:	*Channa striata* (Bloch, 1793) – Chevron snakehead, striped snakehead

http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=166667

2.2 Interactions between host, pathogen, and environment

Factors that control the spread of diseases of humans and other mammals should, with modification, be applicable to the study of infectious diseases in fishes. Disease in populations is a dynamic phenomenon; fluctuations in occurrence and impact are dependent on the interactions between host, pathogen, and environment. The development of disease is a complicated process, dependent not only on bacteria being capable of causing health disorders, but also on the immune status of fish, environmental conditions, and virulence of the disease agent. Therefore, changes occurring in freshwater ecosystems seem to be fundamental in the development of any disease (Pękala-Safińska, 2018).

By understanding the multiple factors that contribute to the occurrence of pathogens affecting fish more broad ecosystem management plans could be developed to protect and restore fisheries. Improving environmental conditions will make them less disposed to diseases infections.

2.2.1 Snakehead fish and environment

Freshwater taxa present a unique opportunity to investigate the link between genealogy and landscape evolution because, unlike many terrestrial taxa that can scatter via wind currents, floating debris, or actively by walking or flying, most obligate freshwater taxa can only disperse among freshwater environments when geographical landscapes provide direct freshwater connections (Briggs, 2003). This condition makes difficult for the aquatic organisms to migrate to a better environment to survive.

Snakeheads are primary freshwater fishes and have little or no tolerance for seawater (Mirza, 1994). Habitat of *Channa striata* is freshwater ponds and streams, usually in stagnant muddy waters; primarily found on plains in India (Talwar & Jhingran, 1992),

also can occur in reservoirs. Nevertheless, in Malaysia, this species is reported to exist in rivers, lakes, swamps, rice paddies, mining pools, and roadside ditches (Lee & Ng, 1991). All members of the family *Channidae* can tolerate hypoxic conditions because they are air-breathers from late juvenile stages; they acquired the capacity for gas exchange with water in their gills and skin, as well as with air, through their supra-branchial chamber (Chandra & Banerjee, 2004). Some channids, perhaps all, have a daily rhythm in frequency of oxygen uptake. *Channa marulius*, for example, showed a peak in oxygen uptake at night. *C. striata* and *C. gachua* peaked in early night hours, and *C. punctata* at dusk. These rhythms are attributed to growing in swamp ecosystems (Munshi & Hughes, 1992). *Channa striata* can bury themselves in mud during times of drought, they are known to secrete mucus that helps to reduce dryness and facilitates cutaneous breathing (Courtenay & Williams, 2004).

2.2.1.1 Temperature as one of the environmental stressors

Temperature stress, particularly cold temperatures, can completely halt the activity of the immune system, eliminating this defense against invading disease organisms. Excessively high temperatures are also extremely detrimental to the fish's ability to withstand infections. High water temperature may favor rapid population growth of some pathogens and boost the rate of disease spread (Karvonen et al., 2010). High temperature also reduces the ability of the water to hold oxygen and increases the metabolic rate and resulting oxygen demand of the fish (Rottmann et al., 1992).

All teleostean species have developed their own specific adaptive mechanism, both behavioral and physiological, to cope up with temperature fluctuations. Das et al. (2004) tested three species *Cirrhinus mrigala, Labeo rohita* and *Catla catla*, he found that *C. mrigala* shows remarkably high thermal adaptation owing to their specific ecological niches.

Adaptations of temperature-tolerant species are of a particular interest. The goldfish, *Carassius auratus*, is an eurythermal species being able to survive static temperatures between about 0 and 41°C and short-term exposures to temperatures close to 44°C (Lushchak & Bagnyukova, 2006).

Previous research has indicated that *Cyprinus carpio* can tolerate more temperature fluctuations than *L. rohita*. It is worth mentioning the cold tolerance ability of *C. carpio* over *L. rohita*. This must be due to the difference in ecological places (bottom feeding and cosmopolitan distribution) of *C. carpio* in comparison to *L. rohita* (column feeding and tropical distribution) (Chatterjee et al., 2004).

2.2.1.2 Heat shock proteins (HSP)

The heat shock response was first reported by a researcher who observed a pattern of Drosophila salivary gland chromosome puffs that were induced in response to transient exposures to elevated temperatures (Hochachka & Somero, 2002). This family of proteins is highly conserved, displaying high sequence homology between different species. The highly conserved nature of HSPs is a reflection of their essential role in protective mechanisms from stress conditions (Kilic & Mandal, 2012). They have been classified into several families based on their molecular weight such as Hsp90 (85-90 kDa), Hsp70 (68-73 kDa), Hsp60, Hsp47, and small Hsps (12-43 kDa) (Park et al., 2007).

The importance of Hsps in the protein folding pathway is reflected in the fact that a number of heat shock genes are expressed at high levels during normal cell growth. Oxygen radicals, toxicants, and inflammatory stress enhance the synthesis of Hsps and often give rise to an accumulation of denatured and aberrantly folded proteins within the cell. Thus, the interaction of Hsps with abnormal proteins during stress is thought to be an extension of their role under normal, non-stress conditions (Kayhan & Duman, 2010).

They develop following up-regulation of specific genes, whose transcription is mediated by the interaction of heat shock factors with heat shock elements in gene promoter regions. HSPs function as helper molecules or chaperones for all protein and lipid metabolic activities of the cell, and it is now recognized that the up regulation in response to stress is universal to all cells and not restricted to heat stress. Thus, other stressors such as anoxia, toxins, protein degradation, hypoxia, acidosis and microbial damage will also lead to their up-regulation. They play a fundamental role in the regulation of normal protein synthesis within the cell. HSP families, such as HSP90 and HSP70, are critical to the folding and assembly of other cellular proteins and are also involved in regulation of kinetic partitioning between folding, translocation and aggregation within the cell. HSPs also have a wider role in relation to the function of the immune system, apoptosis and various facets of the inflammatory process. In aquatic animals, they have been shown to play an important role in health, in relation to the host response to environmental pollutants, to food toxins and in particular in the development of inflammation and the specific and non-specific immune responses to bacterial and viral infections in both finfish and shrimp (Roberts et al., 2010).

The Hsp response can vary according to tissue (Cheng et al., 2007), distinct Hsp families (Smith et al., 1999) and stressors (Airaksinen et al., 2003). Also, the sensitivity of Hsp expression may vary with the species (Basu et al., 2002) developmental stage of the animal (Alak et al., 2010), and season of the year (Encomio & Chu, 2005).

2.2.1.3 HSP70

HSP70s are a family of widely expressed heat shock proteins. It is found in prokaryotes and eukaryotes and is mainly localized in the cytosol, mitochondria and endoplasmic reticulum and exhibit constitutive and inducible regulation (Trivedi et al., 2010).

The HSP70 in a normal cell act as a protein quality control system or the Folding Refolding Degradation machinery and, depending on the state of the protein, sends the protein either for re-folding or for degradation. So, a cell maintains protein homeostasis. The HSP70 also controls the activity of key signaling proteins by maintaining these proteins in an inactive or active state by regulating their levels and by intracellular transport (Malyshev, 2013).

Decreasing the levels of functional Hsp90 in Drosophila by genetic mutation or by treatment with an Hsp90 inhibitor geldanamycin causes developmental abnormalities. Likewise, increasing the levels of Hsp70, by gene transfer mediated overexpression or by heat shock, has growth-inhibitory effects on mammalian tissue culture cells and in Drosophila salivary gland cells, whereas expression of a dominant-negative form of Hsp70 causes developmental defects in Drosophila (Nollen & Morimoto, 2002).

HSP70 in fish thought to have a molecular chaperone function such that it transiently binds to nascent polypeptides and unfolded proteins, thereby preventing intramolecular and intermolecular interactions that can result in misfolding or aggregation of these substrate proteins (Yamashita et al., 2010).

HSP70 cDNA sequences have been isolated from several fish species that include seabream, rainbow trout, Wuchang bream Pacific abalone and tilapia. Some efforts were made to examine gene expression of Hsp70 under different stimuli stress including thermal effect, environmental pollution, heavy metals, crowding stress and insecticidal toxicity effects. Recently, a few studies reported that mRNA levels of Hsp70 were increased in response to bacterial infection in fish, which sparked the involvement of this gene in fish immunity (Bakiu et al., 2014). Based on the considerations mentioned above Hsp70 could be thought as sensitive biomarkers for these environmental and bacterial pressures.

2.2.2 Biological adaptation

The substantial variation observed in the world is a result of three components: DNA replication, mutations and environmental structure so that groups do not completely overlap (Hey, 2001). This divergence, in either regulatory or protein-coding sequences can result in quite different in biological functions for even closely related genes (Greer et al., 2000).

Different groups share traits that are a result of a similar history of genetic drift and the adaptive fixation of useful mutations. Different categories represent groups of organisms where these processes are shared to a certain degree. Depending on the resolution, or the degree of shared processes, the boundaries can be drawn at varying levels of inclusiveness and therefore define diversity differently. Smaller boundaries represent groups that have more traits in common, while more inclusive groupings share only a few key traits. The continuum of biodiversity is dynamic and responsive, interacting not just within itself and the abiotic and geophysical diversity, but also with the diversities of human cultures (Gavin et al., 2015).

Tong et al. (2017) studied Tibetan *Schizothoracinae* fish to understand evolutionary scenarios occurring under environmental changes during the elevate of the Tibetan Plateau area. They found that the potential new functionalization of genes may contribute to the adaptation to the extreme environment in Lake Qinghai. Adaptive evolution occurred in genes involved into metabolism, immune system and transport functions, and helps the functional adaptation to the chronic cold, extreme alkaline and saline, lighter load of pathogens environment in Lake Qinghai.

2.2.3 Snakehead fish and bacterial diseases

The study of fish diseases has concentrated on problems in fish farms (aquaculture), where outbreaks either begin suddenly, progress rapidly often with high mortalities, and disappear with equal rapidity (acute disease) or develop more slowly with less severity, but persist for greater periods (chronic disease). The disease is usually the outcome of an interaction between the host (fish), the disease-causing situation (pathogen) and external stressors (unsuitable changes in the environment; poor hygiene; stress). Before the occurrence of clinical signs of disease, there may be obvious damage to the weakening of the host. Yet all too often, the isolation of bacteria from an obviously diseased fish is taken as evidence of infection (Austin & Austin, 2007).

It is apparent that fish are continuously exposed to the microorganisms present in water and in sediment including the contaminants in sewage/feces. These organisms will undoubtedly influence the microflora on external surfaces, including the gills, of fish. Similarly, the digestive tract will receive water and food that is populated with microorganisms. Certainly, colonization may well start at the egg and/or larval stage and continue with the development of the fish. Thus, the numbers and range of microorganisms present in the eggs, on food, and in the water, will influence the microflora of the developing fish. From the published literature, it may be deduced that there are three likely scenarios for the fate of bacteria coming into contact with fish:

1. The organisms from the environment around the fish may become closely associated with and even colonize the external surfaces of the fish. There may be an accumulation of the organisms at sites of damage, such as missing scales or abrasions.

2. The organisms may enter the mouth with water or food and pass through and/or colonies the digestive tract.

3. The organisms coming into contact with fish surfaces may be inhibited by the resident microflora or by natural inhibitory compounds present on or in the fish (Austin, 2006).

2.2.3.1 *Enterobacteriaceae*

Members of the *Enterobacteriaceae* are small Gram-negative, non-spore forming straight rods. Some genera are motile by means of flagella. They are facultatively anaerobic and most species grow well at 37°C, although some species grow better at 25-30°C. Members of the family are responsible for causing foodborne disease and some also cause food spoilage and therefore contribute to substantial economic losses and food wastage (Baylis et al., 2011).

Enterobacter spp. can be found on human skin and plants as well as in soil, water, sewage, intestinal tracts of humans and animals, and some dairy products. However, some species of *Enterobacter*, such as *Enterobacter sakazakii*, are opportunistic human pathogens. *Enterobacter cloacaeA*-11 and similar bacteria can be found on cucumber and radish seeds as well as peas, soybeans, sunflowers, and sweet corn seeds.

Cell contain a characteristic antigen, called the enterobacterial common antigen. The outer membrane lipopolysaccharide (LPS) is called the O antigen. Its antigenic specificity is determined by the composition of the sugars that form the long terminal polysaccharide side chains linked to the core polysaccharide and lipid A. Cell surface polysaccharides may form a well-defined capsule or an amorphous slime layer and are termed the K antigen. Motile strains have protein flagella, which extend well beyond the cell wall and are called the H antigen. Many members of the *Enterobacteriaceae* have surface pili, which are antigenic proteins but not yet part of any formal scheme (Ryan & Ray, 2004).

Enterobacteriaceae are often take advantage of their common presence in the environment and normal flora to produce disease when they gain access to normally sterile body sites. Surface structures such as pili are known to aid this process for many species. Once in deeper tissues, their ability to persist and causes injury is little understood except for the action of LPS endotoxin and the species known to produce exotoxins (Ryan & Ray, 2004).

2.2.3.2 Classification of *Enterobacteriaceae*

The bacterial family *Enterobacteriaceae* has 53 genera (and over 170 named species) and they include *Arsenophonus, Biostraticola, Brenneria, Buchnera, Budvicia, Buttiauxella, Calymmatobacterium, Cedecea, Citrobacter, Cosenzaea, Cronobacter, Dickeya, Edwardsiella, Enterobacter, Erwinia, Escherichia, Klebsiella, Kluyvera, Leclercia, Leminorella, Levinea, Salmonella, Samsonia, Serratia, Shigella, Shimwellia, Sodalis, Xenorhabdus, Yersinia* and *Yokenella*. Of these, 26 genera are known to be associated with infections in humans. The nomenclature of the *Enterobacteriaceae* is complicated and has been based on biochemical and antigenic characteristics. Recently, the application of new technologies such as DNA hybridization has resulted in numerous changes in classification of the *Enterobacteriaceae*. Many new genera and species have been discovered, some unusual and rare, and many species have also been reclassified to other genera e.g. the transfer of *Enterobacter sakazakii* to the *Cronobacter sakazakii* (Public Health England, 2015). The genus *Enterobacter* has 19 species (excluding *E. aerogenes*) that making it one of the largest genera within the family.

2.2.3.3 Genus *Enterobacter*

The genus *Enterobacter* is also one of the most rapidly expanding genera within this family, with 50% of the novel species descriptions taking place in the last decade. The

older species were assigned to the genus based on DNA-DNA hybridization values and phenotypic data, whereas the more recently described taxa rely on 16S rRNA gene sequence. At a higher taxonomic level, the *Enterobacter* species are divided into five different clades (MLSA groups A to E: MLSA directly measures the DNA sequence variations in a set of housekeeping genes and characterizes strains by their unique allelic profiles) dispersed among their phylogenetic relatives *Cronobacter, Raoultella, Citrobacter, Kluyvera, Klebsiella* and *Yokenella.* The majority of *Enterobacter* species cluster in MLSA group A, referred to as the "core" *Enterobacter* group, containing *E. cloacae, E. asburiae, E. mori, E. ludwigii, E. hormaechei, E. cancerogenus* and *E. soli* (Brady et al., 2013).

2.2.4 Identification of bacteria using 16S rRNA gene sequence

Some bacteria may be difficult to be identified due to fastidious growth, morphological variations, unusual biochemical reactions, lack of previous recognition, or a combination of these. Subculture failure, though it rarely happens, virtually makes routine identification impossible. Fortunately, technological advances have largely overcome these limitations for bacterial identification. One of the advances realized in the past decade or so has been the analysis of the nucleotide sequences of the 16S ribosomal RNA gene (16S rDNA), which has emerged as the single best method to identify bacteria (Kolbert & Persing, 1999; Drancourt et al., 2000).

The first bacterial 16S rRNA gene was sequenced by Ehresmann et al. (1972) for *Escherichia coli*. This first 16S rRNA (GenBank accession no. J01859) contains 1542 nucleotides. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random

sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1500 bp) is large enough for informatics purposes (Patel, 2001). As well as its conservation and divergence reflect bacterial evolution. Therefore, 16S rRNA gene sequencing became a tool for studies of bacterial Phylogeny (Tang & Stratton, 2006).

Conserved regions of 16S rRNA gene allow design of highly conserved primers for nearly universal amplification of most bacterial species (Han et al., 2002). The nucleotide sequences of the amplicon are determined, which, when compared with a database, yield homology matches and consequent identification of a particular bacterium. It is the variable regions of 16S rRNA gene that give discriminatory power. The longer the sequences are determined, the more accurate the identification is. Generally, at least 200 bp are required to yield meaningful results. A comprehensive and accurate database is essential for homology matches and identification of bacteria. There are multiple public and private databases available, such as GenBank, Ribosomal Database Project (RDP), Ribosomal Differentiation of Medical Microorganisms (RIDOM), and others.

Ten years ago, researchers reviewed the first decade of bacterial genome sequencing (Binnewies et al., 2006). At that time, there were about 300 sequenced bacterial genomes and only two published metagenomic projects; this represented a growth of more than 100-fold from the mere two genomes sequenced in 1995. The number of sequenced genomes has continued to increase dramatically in the last 10 years growing another hundred-fold. There are more than 88,000 sequenced bacterial genomes currently publicly available in 2017 (NCBI, 2017).

In 1980 in the Approved Lists, 1,791 valid names were recognized at the rank of species. Recently this number has ballooned to 8,168 species, a 456% increase. Although it has been demonstrated that 16S rRNA gene sequence data on an individual strain with a nearest neighbor exhibiting a similarity score of 97% represents a new species, the

meaning of similarity scores of 97% is not as clear. This latter value can represent a new species or, alternatively, indicate clustering within a previously defined taxon (Janda & Abbott, 2007).

After identification of bacteria by molecular techniques it is important to identify the effect of this pathogen on the tissues of the infected organism. One of the most important ways to study changes in the tissue is the histopathological examination.

2.2.5 Histopathological effect of bacteria

Histology is a branch of biology that involves the microscopic examination of thin, stained tissue sections in order to study their structure and function and, in the case of histopathology, to determine changes which may be due to pathogens and disease. Histology has a central role in disease diagnosis.

Tissues can be classified into four basic types according to structure and function as stated by Patton, (2015):

1. Epithelial tissue covers body surfaces and lines hollow organs, body cavities, and ducts. It also forms glands.

2. Connective tissue protects and supports the body and its organs. Various types of connective tissue bind organs together, store energy reserves as fat, and help provide immunity to disease-causing organisms.

3. Muscular tissue generates the physical force needed to make body structures move and generates body heat.

4. Nervous tissue detects changes in a variety of conditions inside and outside the body and responds by generating action potentials (nerve impulses) that activate muscular contractions and glandular secretions.

Stained fish tissue sections prepared and examined by light microscopy. The tissue sections can be stained using biological stains or dyes. The most widely used histological stain is Hematoxylin and eosin (H&E) stains which have been used for at least a century and are still essential for recognizing various tissue types and the morphologic changes for diagnosis. The stain has been unchanged for many years because it works well with a variety of fixatives and displays a broad range of cytoplasmic, nuclear, and extracellular matrix features. because of its ability to reveal a wide range of different tissue components (Fischer et al., 2008).

2.3 Fish immune system

Like other animals, fish can suffer from a wide variety of diseases and parasites. To prevent disease, they have a variety of non-specific defenses and specific defenses. Non-specific defenses include the skin and scales, as well as the mucus layer secreted by the epidermis that traps microorganisms and inhibits their growth. Should pathogens break these defenses, fish can develop an inflammatory response that increases the flow of blood to the infected region and delivers the white blood cells that will attempt to destroy the pathogens. Specific defenses are specialized responses to particular pathogens recognized by the fish's body, in other words, an immune response (Prabhakar, 2010).

Various internal and external factors can influence innate immune response parameters. Temperature changes, stress management and density may have suppressive effects on this type of response, while several food additives and immuno-stimulants can enhance their efficiency (Uribe et al., 2011). As fish comprise the largest vertebrate class, they are

very diverse in evolutionary terms and may be divided into jawless fish (such as the lampreys) and jawed fish. The latter can be further subdivided into cartilaginous fish (e.g. sharks) and bony fish (e.g. teleosts).

The immune system of Teloests are the most studied fish, so, the facts mentioned below particularly concerns them unless otherwise stated.

2.3.1 Some immune organs

2.3.1.1 The Liver

The liver is a large organ located in the anterior portion of the peritoneal cavity and also performs many functions similar to those known in mammals (Powell, 2000). The liver is furthermore involved in presentation of particulate antigens; evidence for this is the presence of phagocytic mononuclear cells in the liver sinusoids (Powell, 2000).

Recently Heymann & Tacke, (2016) stated that the liver has been clearly put forward as a central immunological organ with a high exposure to circulating antigens and endotoxins from the gut microbiota, particularly enriched for innate immune cells (macrophages, innate lymphoid cells, mucosal-associated invariant T cells).

2.3.1.2 The Intestine

The intestine has been shown to be an important area of antigen uptake and processing (Powell, 2000). It was already known for many years that leucocytes are abundantly present in lamina propria and intestinal epithelium (Rombout et al., 1989).

In the variety of enteric infections, the intestinal tracts often exhibit characteristics signs of diseases for example bacterial infections with *Yersinia ruckeri* and *Aeromonas hydrophila* results in petechial hemorrhaging in the small and large intestine. In trout, it is located along the lowest portion of the posterior peritoneal cavity. In contrast to

mammals, the fish intestine does not contain aggregates of lymphocytes. The granular cells of the stratum granulosum have, however, been implicated in mucosal immunity (Ostrander, 2000).

2.3.1.3 The Spleen

The spleen, as in other vertebrates, is the major filter of blood-borne antigens but it also performs immunopoietic (activate immune system) functions (Anderson & Zeeman, 1995). In teleost fish, it is involved in hematopoiesis and may have immune functions that are comparable to lymph nodes in mammals (fish do not have lymph nodes) (Powell, 2000). It is situated in the lower posterior abdominal cavity, has a smooth texture and a dark red color. The spleen has an outer capsule consisting of connective tissue and a pulp matrix. The pulp contains both hematopoietic red pulp and lymphopoietic white pulp. The spleen may keep a large number of mature erythrocytes, which can be released into the circulation when needed. it is the major site of thrombocyte production (Pastoret et al., 1998).

2.3.2 Nonspecific immune system

Aquatic environments, in which fish live, are very encouraging to the transmission of disease-bearing organisms (Anderson & Zeeman, 1995). Hence, they possess a well-developed nonspecific immune system. The skin, lateral line, and gills are the first line of defense against pathogens (Powell, 2000). Fish skin is coated by mucus, which is continuously secreted by goblet cells and contains antibodies as well as lysozyme. The epidermal cells are the next barrier followed by the scales (Anderson & Zeeman, 1995). Fish are often more vulnerable in areas not covered by mucus or scales, such as the gills; macrophages may then be found on gill surfaces. Furthermore, it has been shown that antigens originating from pathogens such as *Yersinia ruckeri* bacteria, given as

suspension in water, are taken up very effectively by rainbow trout and trigger strong immune responses (Powell, 2000). Other pathogens such as *Aeromonas salmonicida* do not produce such a strong response. Fish are capable of inflammatory reactions (Pastoret et al., 1998), which involve migration of neutrophils, eosinophils, basophils (not in all species), macrophages, and lymphocytes to the site of infection; even slight increases in temperature have been detected (Anderson & Zeeman, 1995).

2.3.3 Specific immune systems

Fish are not only able to take up antigens in the water through their skin, the gills, the lateral line but they can orally perform that. The major histocompatibility complex (MHC) is a set of cell surface proteins essential for the acquired immune system to recognize foreign molecules in vertebrates. The main function of MHC molecules is to bind to antigens derived from pathogens and display them on the cell surface (Janeway et al., 2001). After antigen recognized by major histocompatibility complex (MHC), T and B cells are activated, produce cytokines and thus induce plasma cells to produce antibodies. The production of antibodies in fish is largely temperature dependent and immunizations as well as cultivation of leukocytes should be carried out at the optimum temperature for the species, e.g. 15–20°C for trout (Hitzfeld, 2016).

2.4 Gene expression

Generally, the term gene expression refers to the process by which the information encoded in a DNA sequence is translated into a product that has an influence on at least some cells of an organism (Alberts et al., 2013). It is a multistep process that involves the transcription, translation and turnover of messenger RNAs that dictates a certain protein to be synthesized (Schwanhäusser et al., 2011).

2.4.1 Role of DNA in gene expression

DNA acts as a control room for gene expression. So, when a particular protein is needed by the cell, the nucleotide sequence of the appropriate section of an immensely long DNA molecule in a chromosome (i.e the gene) will first be copied into another type of nucleic acid-RNA (ribonucleic acid). These RNA copies of short segments of the DNA are then used to direct the synthesis of the protein. Therefore, the flow of genetic information in cells is from DNA to RNA to protein. This process is universal for living organisms, from bacteria to humans, thus it has been termed as the central dogma of molecular biology (Alberts et al., 2013).

Synthesis of many identical RNA copies will be induced by the same gene because each cell contains only one or two copies of any particular gene and each RNA copy can be used to simultaneously produce a large number of molecules of that certain protein. By controlling the efficiency of transcription and translation of the different genes, a cell can produce different proteins at different concentrations. Meaning that at a particular point of time, a cell can temporally change gene expression according to its priority need for a particular protein (Datta et al., 2006).

2.4.2 Transcription

Many identical RNA copies can be made from the same gene (Figure 2.1) because each cell contains only one or two copies of any particular gene and each RNA copy can be used to simultaneously produce many identical protein molecules. By controlling the efficiency of transcription and translation of the different genes, a cell can produce small amounts of some proteins and large amounts of others. In addition, a cell can change gene expression in response to the temporally changing needs for a particular protein (Datta et al., 2006).

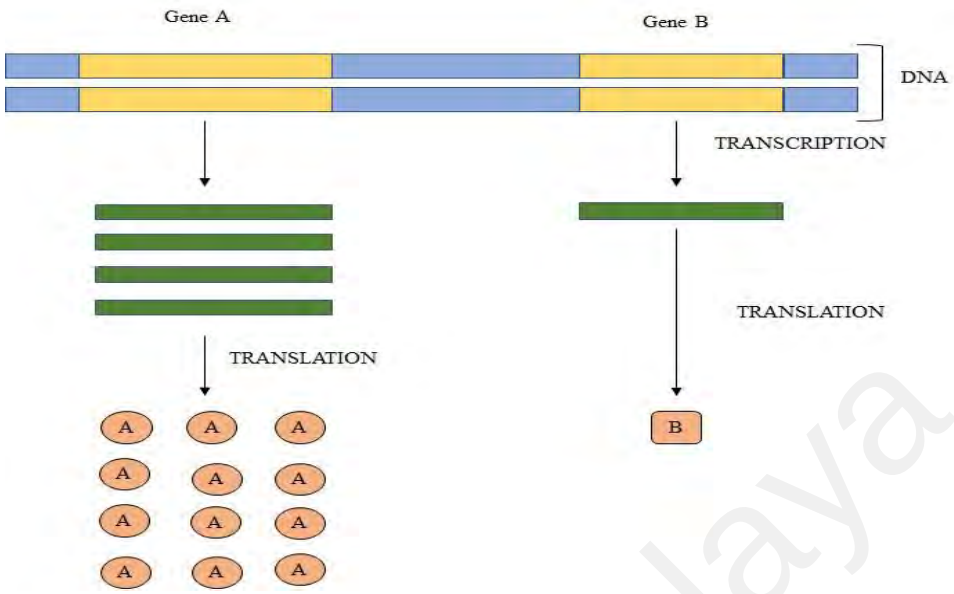

Figure 2.1: Schematic draw explaining that genes can be expressed with different efficiencies. Gene A is transcribed and translated much more efficiently than gene B. this allows the amount of protein A in the cell to be much higher than that of protein B

All the RNA in a cell is made by transcription. It initiates with the opening and unwinding of a small portion of the DNA double helix to expose the bases on each DNA strand. One of the two strands of the DNA double helix then act as a template for the synthesis of RNA. Ribonucleotides are added, one by one, to the growing RNA chain, the nucleotide sequence of the RNA chain is determined by complementary base-pairing with the DNA template. When a good match is made, the incoming ribonucleotide is covalently linked to the growing RNA chain in an enzymatically catalyzed reaction. The RNA chain produced by transcription (the transcript) is therefore elongated one nucleotide at a time and has a nucleotide sequence exactly complementary to the strand of DNA used as the template. The almost immediate release of the RNA strand from the DNA as it is synthesized means that many RNA copies can be made from the same gene in a relatively short time; the synthesis of the next RNA is usually started before the first

RNA has been completed. At any given time, there could be as many as 15 polymerases speeding along this single stretch of DNA (Alberts et al., 2013).

2.4.3 Initiation of transcription

The initiation of transcription is a principally critical process because it is the main point at which the cell can dictates which proteins or RNAs are to be produced and at what rate.

To begin transcription, Eukaryotic RNA polymerase requires a set of general transcription factors which are called TFIIA, TFIIB, and so on. They accumulate on the promoter, where they position the RNA polymerase, pull apart the double helix to expose the template strand, and launch the RNA polymerase, to begin transcribing.

The first step in transcription is initiation, when the RNA polymerase binds to the DNA upstream (5′) of the gene at a specialized sequence called a promoter. Many promoters contain a DNA sequence called the TATA box (a short double-helical DNA sequence primarily composed of T and A nucleotides) that serves as the location of transcription initiation, (Clancy, 2008).

The general transcription factor TFIID bind TATA box, upon binding to DNA, TFIID causes a dramatic local distortion in the DNA which helps to serve as a landmark for the subsequent assembly of other proteins at the promoter. The TATA box is a key component of many promoters used by RNA polymerase, and it is approximately located 25 nucleotides upstream from the transcription start site. Once the first general transcription factor has bound to this DNA site, the other factors are assembled, along with RNA polymerase, to form a complete transcription initiation complex. After RNA polymerase, has been bound to the promoter DNA in the transcription initiation complex, it must be released from the complex of transcription factors to begin its task of making an RNA molecule. A key step in this release is the addition of phosphate groups to the

'tail' of RNA polymerase, an act performed by the general transcription factor TFIIH, which contains a protein kinase enzyme as one of its subunits. This phosphorylation is thought to help the polymerase disengage from the cluster of transcription factors, allowing transcription to commence. Once transcription has begun, most of the general transcription factors are released from the DNA so that they are available to initiate another round of transcription with a new RNA polymerase molecule. When RNA polymerase finishes transcribing, it is released from the DNA, the phosphates on its tail are stripped off by phosphatases, and it can reinitiate transcription. Only the dephosphorylated form of RNA polymerase can initiate RNA synthesis at a promoter (Alberts et al., 2013).

2.4.4 Transfer of mRNA from the Nucleus

The total mRNA that is synthesized, only a small fraction of the mature mRNA is useful to the cell. The remaining RNA fragments-excised introns, broken RNAs, and aberrantly spliced transcripts-are not only useless but could be dangerous to the cell if not destroyed. How, then, does the cell distinguish between the relatively rare mature mRNA molecules it needs to keep and the overwhelming amount of debris generated by RNA processing? The answer is that the transport of mRNA from the nucleus to the cytoplasm, where it is translated into protein, is highly selective: only correctly processed RNAs are allowed to pass. This reliance on proper processing for RNA transport is mediated by the nuclear pore complex, which recognizes and exports only completed mRNAs. These aqueous pores connect the nucleoplasm with the cytosol, they act as gates that control which macromolecules can enter or leave the nucleus. To be export, an mRNA molecule must be bound to an appropriate set of proteins, each of which signals that the mRNA has been correctly processed. These proteins include poly-A-binding proteins, a cap-binding complex, and proteins that mark completed RNA splices (Figure 2.2). It is presumably

the entire set of bound proteins, rather than any single protein, that ultimately determines whether an RNA molecule will leave the nucleus. The 'waste RNAs' that remain behind in the nucleus are degraded, and the building blocks are reused for transcription (Albert et al., 2013).

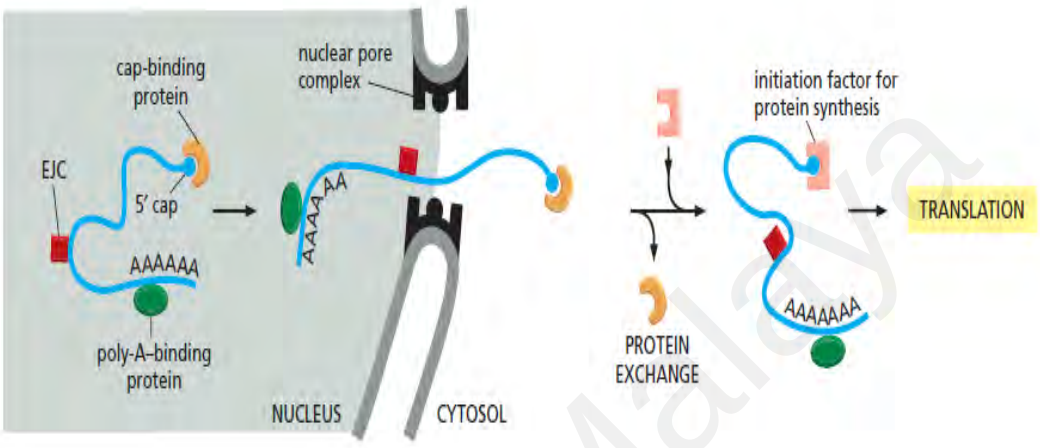

Figure 2.2: A specialized set of RNA-binding proteins signal that a matur mRNA is ready for export to the cytoplasm. As indicated on the left, the cap and poly-a tail of a mature mRNA molecule are 'marked' by proteins that recognize these modifications. In addition, a group of proteins called the exon junction complex (EJC) is deposited on the mRNA after successful RNA splicing has occurred and guides it through the nuclear pore. Once in the cytosol, the mRNA can shed previously bound proteins and acquire new ones. Reproduced from Albert et al. (2013)

2.5 Reverse transcription quantitative PCR

Reverse transcription quantitative PCR (RT-qPCR) distinguishes itself from other methods available for gene expression in terms of accuracy, sensitivity, and fast results. Because of this, the technology has established itself as the golden standard for medium throughput gene expression analysis. Due to its apparent simplicity, also inexperienced users can rapidly produce results (Derveaux et al., 2010).

However, quantification of RNA expression levels serves as a major indicator of the physiological status of a cell or tissue and plays a central role in a wide variety of life science studies. The purity and integrity of RNA samples were shown to have a direct influence on the outcome of gene expression experiments and may strongly compromise the accuracy of any RNA profile, irrelevant of the method by which it was obtained (Vermeulen et al., 2011) RNAs are very sensitive molecules, especially compared to DNAs, and are easily fragmented by heat, UV, or the ubiquitous occurring nucleases. In addition, contaminants introduced through sloppy lab handling and ineffective sampling or extraction procedures, were proven to weaken downstream reactions and overall affect quantitative gene expression results (Nolan et al., 2006).

2.6 Studied genes

2.6.1 Galectin 8

Galectins, a family of *β*-galactoside-binding developmentally regulated proteins formerly known as S-type lectins, have been proposed to participate in a variety of biological functions, including cell–cell and cell–extracellular matrix interactions that mediate developmental processes. Unlike other lectin families, galectins are a relatively homogeneous, evolutionarily conserved and ubiquitous group, with representatives identified in most animal taxa (Cooper, 2002). Furthermore, from the biochemical, structural, and genetic standpoints, galectins constitute one of the best-characterized lectin families (Ahmed et al., 2004).

A total of 15 major lectin families have been identified in animals, among which C-type lectins, galectins, F-type lectins, rhamnose-binding lectins, and intelectins have also been demonstrated to exist in fish species. Lectin refers to a group of sugar-binding proteins that can specifically bind to these carbohydrate structures independent of enzymes and immunoglobulins, and in turn agglutinates various cells to exert its

biological function. Lectins are an enormous superfamily that consists of a great number of members throughout almost all living creatures, including virus, bacteria, fungi, protists, plants, and animals. In animals, the lectins are pivotal components of innate immune response by inducing phagocytosis, activating platelet, initiating complement system, and enhancing the natural killer cell activity. With the emergence of adaptive immunity in vertebrates, the lectins have corresponding functions as regulators of adaptive immune responses by recognizing bacterial or viral components on dendritic cells (antigenic cells), promoting signals that initiate or modulate cytokine responses and inducing lymphocyte maturation, and separation of the invading pathogens (Zhu et al., 2013).

Galectins are distinct in that they can regulate cell death both intracellularly and extracellularly. Extracellularly, they cross link glycans on the outside of cells and transduce signals across the membrane to directly cause cell death or activate downstream signaling that triggers apoptosis. Intracellularly, they can directly regulate proteins that control cell fate (Hernandez & Baum, 2002).

It was found that galectin-8 induced firm and reversible adhesion of peripheral blood neutrophils in vitro. Neutrophils play a central role in innate immunity to bacterial infection. Recruitment of circulating neutrophils to an affected site proceeds through several defined steps, namely, attachment to, rolling on, and firm adhesion to endothelial cells and then trans-endothelial migration (Nishi et al., 2003).

2.6.2 Glutathione S-transferase

Glutathione S-Transferase (GST) is an important enzyme which detoxifies the toxic molecules and hence lowers the oxidative stress. GST is present in cytoplasm and mitochondria of almost every cell. This enzyme belongs to a multigene family of

isoenzymes that catalyze the conjugation of reduced Glutathione to a variety of electrophilic compounds as the first step in a detoxification pathway. It also detoxifies toxic metabolites produced within the cell and protects cells from oxidant injury (Pahwa et al., 2017). GST are regulated by a structurally diverse range of xenobiotics and, at least 100 chemicals have been identified that induce GST; a significant number of these chemical inducers occur naturally and, as they are found as non-nutrient components in vegetables and citrus fruits (Hayes & Pulford, 1995).

The high intracellular concentrations of GSTs coupled with their cell-specific cellular distribution allows them to function as biomarkers for localizing and monitoring injury to defined cell types. For example, hepatocytes contain high levels of alpha GST and serum alpha GST has been found to be an indicator of hepatocyte injury (Loguercio et al., 1998).

Previous studies have reported that the measurement of antioxidant enzymes such as GST in aquatic organisms can be used as sensitive biomarkers for the biomonitoring of polluted marine areas containing contaminants, such as pesticides and heavy metals (Tebourbi et al., 2011). whereas other studies do not support the use of total GST expression as diagnostic cellular markers of environmental carcinogenesis in bullheads fish. GST activity and GST protein expression in chemical-induced hepatic lesions in fish appears to vary according to species (Henson & Gallagher, 2004).

2.6.3 Cytochrome b5

Cytochrome b5 has been proposed to modulate the activity of cytochrome P450 enzymes, including those involved in endogenous processes such as cholesterol and steroid hormone synthesis/breakdown, as well as the metabolism of exogenous xenobiotics, chemical toxins and drugs (Finn et al., 2011).

Cytochrome b5 plays a key role in a wide range of metabolic processes of central importance to both nutrition, drug metabolism and a range of human diseases and further demonstrate the importance of de novo unsaturated fatty acid biosynthesis in human health (McLaughlin et al., 2010).

Cytochrome b5 is needed for the successful desaturation of fatty acids as well as for many other oxidative reactions in the cell. It is a small heme-binding protein that appeared very early in evolution and is usually associated with the endoplasmic reticulum of higher plants, fungi and animals (Gostinčar, et al., 2010).

2.6.4 h2 Calponin

Calponin is an actin filament-associated protein expressed in both smooth muscle and non-muscle cells and exists in three distinct isoforms, h1 (basic), h2 (neutral), and h3 (acidic) (Hines, et al., 2014). Compared to the more restricted tissue distribution of h1 (basic) and h3 (acidic) calponin isoforms, h2 (neutral) calponin is expressed broadly in various tissue types, including developing and remodeling smooth muscles, epidermal keratinocytes, fibroblasts, lung alveolar cells, endothelial cells, and white blood cells (Wu & Jin, 2008). The lack of h2-calponin also resulted in significantly increased phagocytic activity, suggesting a mechanism in the regulation of macrophage function (Huang et al., 2008).

CHAPTER 3: MATERIALS AND METHODS

3.1 Tolerance of *Channa striata* to temperature stress

3.1.1 Experimental fish

Snakehead fish (*Channa striata*) weighing between 70 to 130g were obtained from the Perak River, Malaysia, and transferred to laboratory in 30 L aquaria. The aquaria were equipped with dechlorinated freshwater recirculation and maintained at 28±1°C (mean temperature observed during sampling). All the fish were acclimatized for 1 week before being exposed to heat stress. A maximum of 10 fish per aquarium were maintained during the experiment.

3.1.2 Sequence identification

A partial length of ChHSP70 gene was identified from DNA sequencing of the muscle of *Channa striata* using Illumina Solexa sequencing technology. This gene was identified as heat shock protein 70 (ChHSP70) through a BLAST homology search against the NCBI database. The sequence was compared with other fish sequences which are available on the NCBI database. The similarities of these genes were analysed. The open reading frame (ORF) and amino acid sequence of ChHSP70 were obtained by an ORF finder program available online (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Furthermore, the mRNA sequences of ChHSP70 from 54 different vertebrates were downloaded from the NCBI GenBank database. The sequences were aligned using online available ClustalO program (http://www.ebi.ac.uk/Tools/msa/clustalo/). A neighbour-joining phylogenetic tree was then constructed for the sequences, based on pairwise differences, by Mega v.6 software (Tamura et al., 2013) and using the maximum composite likelihood calculated with a bootstrap of 1000 replicates. Arlequin v.3.5 (Excoffier & Lischer, 2010) was applied to test the partitioning of genetic variations

within and between the clustered groups generated by the phylogenetic tree, using an analysis of molecular variance (AMOVA) by computing conventional F-statistics from sequences with 16000 permutations. The fixation index (FST) between different sequence classes was also calculated.

3.1.3 Temperature stress

The experimental fish were exposed to a natural photoperiod (11 h light: 13 h darkness) and fed once daily with squid. They were acclimated to these conditions for at least 2 weeks prior experiments. For temperature stress challenge test, the fish were divided into two groups, six individuals each, one day before experiment. One group was exposed to cold conditions, 16°C; and the other was exposed to hot conditions 32°C. These temperatures were chosen as temperature ranges from 20 to 32°C was reported for the experimental laboratory fish platyfish *Xiphophorus maculatus* and zebrafish *Danio rerio* (Yamashita et al., 2010). The fish were maintained at the respective chosen temperatures for 1 hour. At the end of experiments, tissue samples from the liver, kidney, stomach, gills, fins and muscle were removed, immediately frozen with liquid nitrogen and stored at -80ºC until use.

3.1.4 RNA extraction and cDNA synthesis

Total RNA was isolated from tissues using TRIzol® Reagent according to the manufacturer's recommendation (Invitrogen, USA) (Appendix A). RNA pellets were re-suspended in TE buffer (Tris-EDTA, pH 8.0). To eliminate possible genomic DNA contamination, the RNA samples were treated with a DNase I (Sigma) in accordance with the manufacturer's instructions. All tissues extracted RNA were checked using a Nanodrop-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The purity of them were determined with the ratio of 260/280 nm absorbance. All samples had

260/280 nm ratios >1.6, these were the acceptable level. Concentrations of the samples were also determined to ensure sufficient amounts of homogeneous RNA for complementary DNA (cDNA) synthesis. (Appendix B)

For first strand cDNA synthesis, total RNA (1µg) was added to a reaction mix (20 µl) containing 0.5 µg oligo DT primer (Pharmacia LKB, Sweden), 2 µl dithiothreitol (0.1M), 1 µl dNTP mix (Pharmacia LKB; 10 mM), 4 µl reaction buffer and 1 µl reverse transcriptase (Gibco-BRL; 200 U/µl). First strand cDNA synthesis proceeded at 42ºC for 1 h, after which the reaction was incubated at 70ºC for 10 minutes and then stored at -20ºC for future analyses.

3.1.5 Quantitative reverse transcription polymerase chain reaction

The relative expressions of ChHSP70 in the liver, kidney, stomach, gills, fins and muscle were measured by quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was carried out using a ABI 7500 Realtime Detection System (Applied Biosystems) in 20 µl reaction volume containing 4 µl of cDNA from each tissue, 10 µl of Fast SYBR Green Master Mix, 0.5 µl of each primer (20 pmol/ µl) and 5 µl dH2O. The qRT-PCR cycle profile was 1 cycle of 95°C for 10 s, followed by 35 cycles of 95°C for 5 s, 58°C for 10 s and 72°C for 20 s, and finally 1 cycle of 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. The same qRT-PCR cycle profile was used for the internal control gene, ß-actin. The details of the gene specific primer (ChHSP70) and internal control (ß-actin) are presented in Table (3.1).

After the PCR program, the data were analyzed with ABI 7500 SDS software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to analyze the expression level of ChHSP70 (Livak & Schmittgenm, 2001).

Table 3.1: List of primers used in the study

Name	Target	Sequence (5'-3' direction)
ChHSP70 (F1)	Amplification	TGAGGGCATTGACTTCTACAC
ChHSP70 (R2)	Amplification	TGCTGGTACTCCTCTGTATCT
ß-actin (F3)	qRT-PCR internal control	ACCACCGAAATTGCTCCATCCTCT
ß-actin (R4)	qRT-PCR internal control	ACGGTCACTTGTTCACCATCGGCATT
ChHSP70 (F5)	qRT-PCR amplification	CACCATTCCCACCAAACAAAC
ChHSP70 (R6)	qRT-PCR amplification	CCATCCTCTCAATCTCCTCTCT

3.2 Determining the infectious bacteria

3.2.1 Isolation of bacteria

The occurrence of selective mortality of *Channa striata* was observed in a fish farm in Selangor area, Malaysia. About 50 samples of fish caught from ponds were transported alive in containers partly filled by the same water of the fishpond to the laboratory of genomic and evolutionary biology at the University of Malaya, and they were kept in precleaned and sterilized aquaria filled with dechlorinated tap water. A randomly chosen sample of five sluggish moribund fish showed lethargy, reduced feeding, skin white lesions was taken. Fish were rinsed with de-ionized water then slayed and dissected inside a biosafety cabinet Class II. A swab from liver and deeply infected areas of the skin (after removing the scales) were taken and inoculated onto Tryptic soy agar (TSA) supplemented with 1% (w/v) Na Cl and nutrient agar. These media were chosen because they are universal enrichment media to ensure growth of the unknown bacteria that caused the selective mortality of fish. Plates were incubated at 30°C for 24 hours. After that different colonies were sub-cultured to obtain pure isolates. Colonies selected from the

last (third or fourth) quadrants of the agar plate to minimize the chance of a contaminant being carried over in the final step of purification. Accordingly, six isolates of bacteria were purified. However, only one of them gave typical symptoms of the disease observed in the fish farm which was identified using molecular technique. This bacterium was used in the LD50 and pathogenicity of the disease. Clinical and histopathological changes of healthy fish samples and challenged fish were reported following the Koch's postulates to confirm the causative agent.

3.2.2 16S rRNA gene amplification and sequence analysis

The isolated bacteria were grown in nutrient broth supplemented with 1% (w/v) NaCl overnight at 30°C. Genomic DNA of the bacteria was extracted from the isolates described above by a Qiagen kit following the manufacturer's protocol. The 16S rRNA gene was amplified by a forward primer (27F 5'AGAGTTTGATCCTGGCTCAG3') and a reverse primer (1492R 5'TACGGTTACCTTGTTACGACTT3'). Amplification was achieved in a thermal cycler (Eppendorf Master cycler gradient; Eppendorf, Hamburg, Germany), using the following programs: at 94°C for 5 minutes (1 cycle), at 94°C for 40 seconds (35 cycles), 40 seconds at 53°C, 40 seconds at 72°C and a final 1 cycle for 5 minutes at 72°C. The products were checked by gel electrophoresisusing 1% (w/v) agarose in TBE (Tris-borate EDTA) buffer. The PCR product was purified with Qiagen Gel purification kit (Germany) and sequenced with an ABI PRISM 3730xl Genetic Analyzer of Applied Biosystems, USA with Big Dye® Terminator v3.1 Cycle Sequencing Kit. The 16S rRNA gene sequences were analyzed using NCBI BLAST (Johnson et al., 2008) and Ez Taxon database (Kim et al., 2012).

3.2.3 LD50 experiment

It is the number of bacteria that kills 50% of experimentally infected organisms. To prepare LD50 solution, an amount of 3 full loops of the fresh pure culture of the bacteria was taken and mixed with 1 ml PBS. This dilution (10 mg in 1 ml) was used as the base for the serial dilutions. Then 10-fold dilutions were prepared via the serial dilution method. Four of them, with approximately10^7, 10^6, 10^5 and 10^4 CFU/ml, were used for the (LD50) experiment. From each of the above-mentioned dilutions, 0.2 ml bacterial suspension was injected intramuscularly to each individual fish of the 4 groups (n=10) which were previously stocked in separate tanks. Tricaine methanesulfonate (MS-222) at a concentration of 150 ppm was used to anesthetize fish prior to injection. Each group was kept in a separate aquarium labeled properly to recognize the dose. The experimentally infected fish were observed up to 14 days. Unlike direct microscopic counts where all cells, dead and living, are counted, CFU measures only viable cells. CFU/ml was worked out as shown below provided that: the dilution factor identified was 10^6, the number of colonies in the plate that killed half of the population (LD50) was 32 colonies (Appendix E) and the amount of bacteria suspension used was 200 µl.

$$\text{CFU/ml} = \frac{\text{number of colonies X dilution factor}}{\text{volume of culture plate}}$$

$$= \frac{32\text{x}1.0\text{x}10^6}{0.2} = 16\text{x}10^7 \text{ cfu/ml}$$

This study precisely presented the official ethical review board (UM ICUCA) for approval [Ethic No. ISB/13/03/2015/IE (R)].

3.2.4 Pathogenicity experiment

The intramuscular injection technique, a method applied by several investigators (Sarkar & Rashid, 2012; Hossain et al., 2013; Neely et al., 2002) was used to detect both the effectiveness of the method in initiating the disease and the pathogenicity of the bacteria. One ml of sterile and disposable insulin needles was used for intramuscular injection. A total of 30 fish was injected with 0.2 ml of LD50 (16×10^7 CFU/fish) bacterial dosage below the fore part of the dorsal fin. Disinfection by cotton containing 70% (v/v) alcohol was used before injection. Another group of fish (n=10) was injected by the same dose of the PBS as a control. The fore part of the dorsal fin was selected for injection because it contains the deep muscle. These two groups of experimented fish were realized in separated aquaria, and they were noticed up to 15 days of the experiment length for any changes in the clinical appearances, and they have been recorded appropriately.

Every morning about 70% of the water is changed and the fish is provided with pellet feed. The clinical signs observed were lethargy, loss of appetite, lesion of the skin and mortality. Liver and muscle samples were taken from moribund fish and kept in 10% (v/v) phosphate-buffered formalin solution for histopathological examination.

3.3 Gene expression of challenged *Channa striata*

3.3.1 Experimental fish

Healthy *Channa striata* fish (5 inches length) were purchased live from a commercial farm in Selangor, Malaysia. The fishes were maintained in a flat-bottomed glass tank (300 L) with dechlorinated tap water at 29 ± 2 °C in the laboratory. All of the fishes were acclimatized for a week before the experiments commenced. A maximum of 15 fishes per tank was maintained during the experiment. Fish were injected with *Enterobacter soli* Es2 bacteria as described in 3.2.4 and tissues from the gill, liver, spleen, muscle, stomach,

intestine and fin were collected at different timing points (1, 3, 7, 10 and 14 days) for gene profiling distribution (3.3.3).

3.3.2 Experimental bacterial infection and tissue collection

The immune response was investigated in a group of 50 fish injected intramuscularly by 200 µL of *Enterobacter soli* Es2 (16×10^7 CFU/fish) suspension at a point below the fore part of the dorsal fin. Tissues including intestine, muscle, gill, fin, liver, stomach and spleen were collected at different timing points (1, 3, 7, 10 and 14 days) as well as samples from the already diseased fish. Bacteria were isolated and identified from samples of infected *C. striata*, as described by Dhanaraj et al. (2008). The controls were instead injected with 200 µL PBS. All the different tissues samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C for total RNA isolation. RNA extraction and cDNA synthesis were done as described in 3.1.4.

3.3.3 Quantitative real-time PCR

The expression patterns of mRNA of four genes in different tissues were determined using a quantitative real-time PCR as described in experiment 1 after challenge with *Enterobacter soli* Es2, with some modifications as follows: The qrtPCR was performed in a reaction mixture of 20 µL, composed of 4 µL of 50 ng/µL cDNA template, 10 µL SYBR Green qPCR Master Mix (Applied Biosystems, USA), and 0.3 µM of each of the primers. The qrtPCR cycle profile was 1 cycle of 95 °C for 4 minutes, followed by 40 cycles of 95 °C for 5 seconds, 60 °C for 10 seconds. The β-actin of the *C. striata* primer pairs were designed from the sequence of GenBank Accession No. EU570219. The primers details of the gene specific primers and the internal control (β-actin) are shown in Table 3.2.

Table 3.2: Sequences of primers used in the study

qReal-time PCR	**Sequence (5'-3')**
CsCalp Forward	TGCCAAGTACTGTCCTAAAGC
CsCalp Reverse	CATCTTGGTACGTCTGCTCTTC
CsCyto Forward	GGGACAAAATGGGTGAAAAGG
CsCyto Reverse	CCTCCAGAAACTTAGTCACATCG
CsGlut Forward	CTTGCTGTGAACGAGTCCTAC
CsGlut Reverse	CATTTTCTGGGTCAGCGTTTG
CsGale Forward	GAGAAACCAATCAAAACGCCC
CsGale Reverse	CCCCAACAGTTAGACAGGAAG
β-actin Forward	TCTTCCAGCCTTCCTTCCTTGGTA
β-actin Reverse	GACGTCGCACTTCATGATGCTGTT

In order to compare relative mRNA expressions, a statistical analysis was performed using a one-way ANOVA. Mean comparisons were made by Duncan multiple rang test, using SPSS 11.5 at the 5 % significance level. Type of tissues organ and time passed after injection were used as independent variables.

CHAPTER 4: RESULTS

4.1 Results of tolerance of *Channa striata* to temperature stress

4.1.1 Sequence characterization and phylogenetic analysis

The partial DNA sequence of ChHSP70 (Appendix C & D) was ~ 918 base pairs (bp), which consisted of an open reading frame of 854 bp (encodes 290 amino acids (aa) residues). The identity of the amplicon was confirmed by sequencing the gene and verified using NCBI BLAST. As shown in Table 4.1, the amplified sequence of ChHSP70 showed high similarity to other fishes, including *Siniperca chuatsi* (91% identity; accession number: KF500542), *Lates calcarifer* (91% identity; HQ646109) and *Seriola quinqueradiata* (91% identity; AB436470). In general results of homologous comparisons, all the fish individuals used for analysis showed at least 80% identity with ChHSP70. A phylogenetic tree of ChHSP70 with other HSP70 was reconstructed by the neighbour-joining method as shown in Figure 4.1.

Table 4.1: Sequence similarities (%) between ChHSP70 and other fishes HSP70

Species name	GenBank accession no.	a.a length	Identity%	Similarity%	Gap
Siniperca chuatsi	KF500542	639	91	99	0
Lates calcarifer	HQ646109	639	91	99	0
Seriola quinqueradiata	AB436470	639	91	99	0
Monopterus albus	KC455493	640	90	99	2
Sciaenops ocellatus	GU244375	639	90	99	0
Lutjanus sanguineus	HQ331120	639	89	99	0
Neolamprologus brichardi	XM006805275	640	89	99	0
Oreochromis aureus	FJ358426	640	89	99	0
Oreochromis niloticus	FJ213839	640	89	99	0
Oreochromis mossambicus	AJ001312	639	89	99	0
Acanthopagrus schlegelii	AY762970	638	89	99	9
Xiphophorus maculatus	AB062114	639	87	99	4
Oncorhynchus mykiss	NM_001124228	644	84	99	10
Cyprinus carpio	JN544930	643	83	99	2
Haplochromis burtoni	XM_005947278	649	83	99	17
F.rubripes	Y08577	526	85	99	6
Latimeria chalumnae	XM_006000954	641	82	94	10

- a.a: amino acid

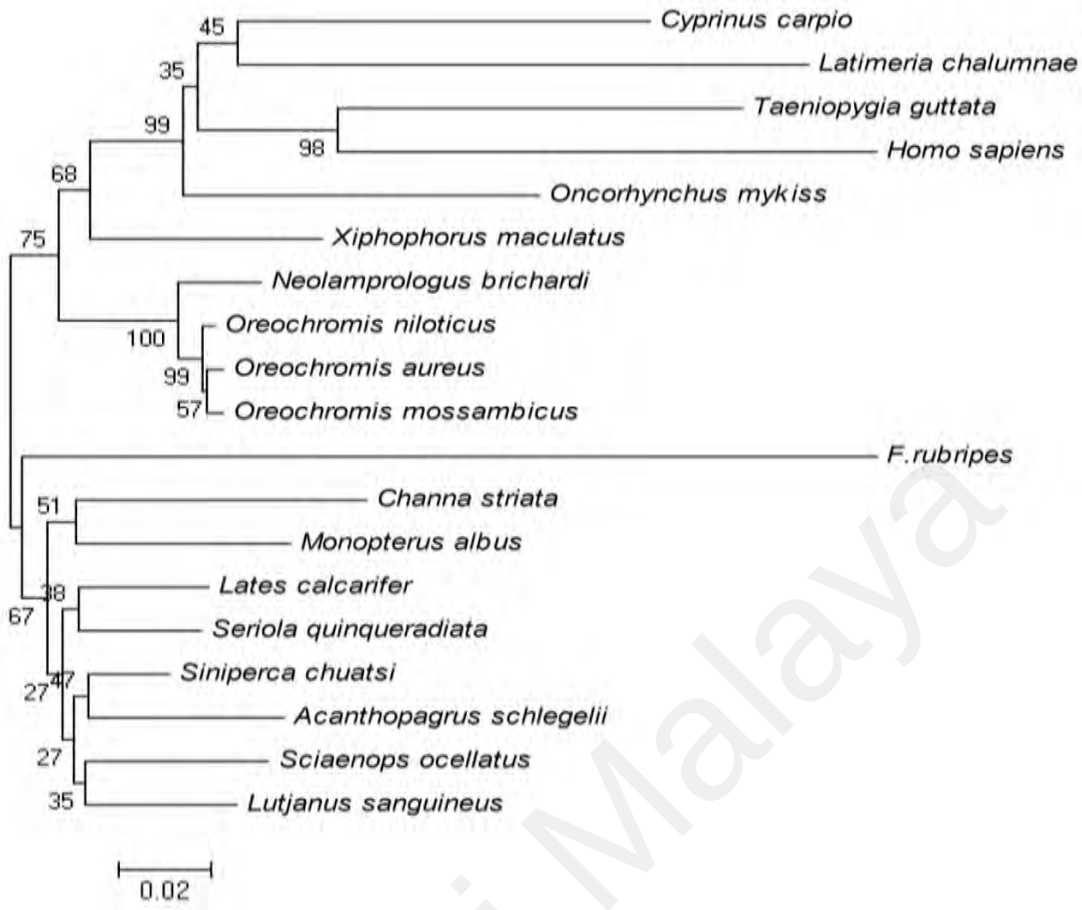

Figure 4.1: A phylogenetic tree of ChHSP70 with other fishes and vertebrates HSP70 was reconstructed by the neighbour-joining method. The numbers shown at the branches denote the bootstrap majority consensus values of 1000 replicates. The gene and GenBank accession number and details are given in Table 4.1.

4.1.2 Variations of HSP70 in vertebrates

To understand the molecular diversity of HSP70 genes, to prove that this gene is conserved as well as to identify in which group of vertebrates the gene is more conserved1528bp of the gene sequence in 54 vertebrate individuals which include fish, amphibian, reptiles, bird and mammals were aligned using the ClustalO program available online (Figure 4.2). The conserved sites for all used sequences were 734 bp and the variable sites were 776 bp. The mRNA sequence of HSP70 belonging to birds (Bir) showed highly conserved (1368bp), while that of amphibians (Amp) showed the lowest conserved sites (1002bp).

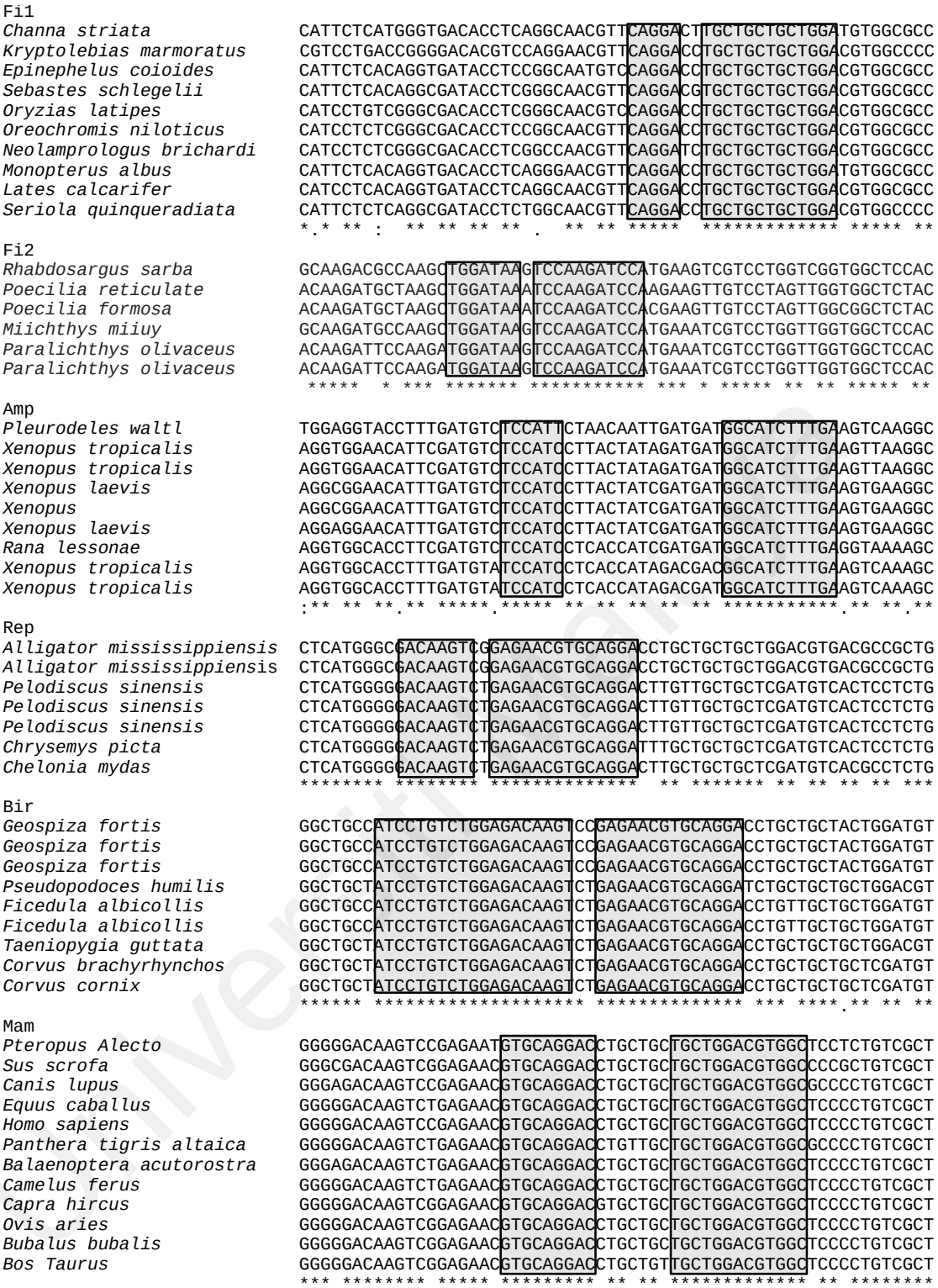

Figure 4.2: Alignment of 54 HSP70 sequences from different vertebrate classes produced by ClustalO. The highlighted box (grey) are the conserved region of HSP70 among the group. The asterisk at the bottom of the sequences is the similar nucleotide among the HSP70 gene

The phylogenetic tree of the studied sequences (Figure 4.3) was clearly divided into six main clusters: fish (Fis 1 and Fis 2), amphibians (Amp), reptiles (Rep), birds (Bir) and mammals (Mam). Among all the genomes examined, the fish group was the largest and the most diverse, thus consisted to be composed of two genetically distinct groups namely Fis 1 and Fis 2.

In these two later groups (Fis 1 and Fis 2), polymorphic sites and number of base substitutions were specified. Results for these two variables were 1081 bp & 193 bp and 383 & 383 for Fis 1 and Fis 2 respectively. In addition, gene diversity for them was 1.000±0.0447 and 1.000±0.0962 correspondingly. Likewise, the AMOVA test between these two groups revealed that 30.39% of the variation was attributed to difference between the sequences of the two groups while 69.61 were within the sequences of the same group.

Furthermore, FST pairwise comparisons among classes were statistically significant ($p<.0001$), with the minimum significant genetic difference in different classes being between Fis 1 and Amp (0.3453), and the maximum difference was about 0.8031 in between Bir and Mam (Table 4.2).

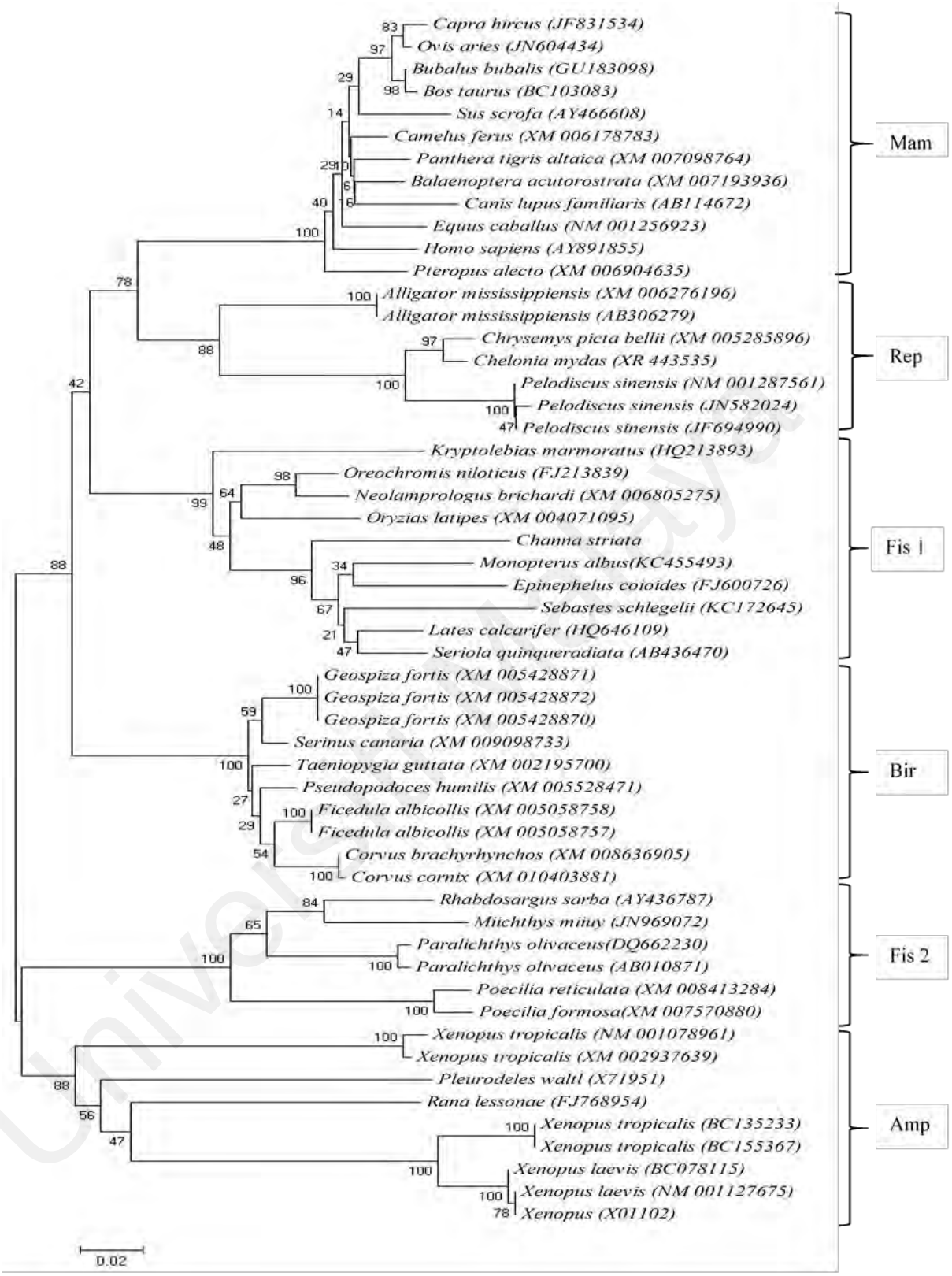

Figure 4.3: Phylogenetic analysis of the HSP70 gene of vertebrates. Neighbor-joining pairwise-based tree including Fis1, Fis2, Amp, Rep, Bir and Mam. Bootstrap analysis (1000 replications) values are shown at each node. Sequences of the HSP70 genes were obtained from the NCBI GenBank database. Sequence names and accession numbers are indicated in the tree

Table 4.2: Pairwise FST comparison of six vertebrate groups based on HSP70 gene.

	Fis 1	**Fis 2**	**Amp**	**Rep**	**Bir**	**Mam**
Fis 1	0.00000					
Fis 2	0.30395	0.00000				
Amp	0.34535	0.40982	0.00000			
Rep	0.43447	0.59715	0.55060	0.00000		
Bir	0.51749	0.68966	0.60419	0.73288	0.00000	
Mam	0.50403	0.67802	0.63176	0.69181	0.80312	0.0000

Besides, AMOVA from the analysis of HSP70 gene sequences of the vertebrate classes revealed significant ($p < .0001$) differences between them, with ~ 55% of the variations attributable to differences between classes and ~ 44% within classes. The subdivisions between classes (FST = 0.5684) meant that there was significant evolution of the gene in the studied sequences.

4.1.3 Tissue distribution of ChHSP70 mRNA expression induced by temperature challenge

The exposure of *Channa striata* to 32°C resulted in the death of half of the fish within one hour. The highest ChHSP70 mRNA expression was observed in the stomach and fin (90-fold), while the lowest was in the kidney (8-fold) in comparison with ChHSP70 mRNA expression in liver. Difference level of expressions of ChHSP70 mRNA on other organs were very substantial; 8-fold expression for kidney, 10-fold expression for muscle and 80-fold expression for gills when compared with liver (Figure 4.4A). In contrast, when *Channa striata* was exposed to 16°C, the results were rather different.

By far the highest level of ChHSP70 mRNA expression was in the kidney which is 130-fold higher than ChHSP70 mRNA expression in liver. The differences in level of expression between the stomach, fin and muscle were 50-fold, 75-fold and 10-fold, respectively. (Figure 4.4B).

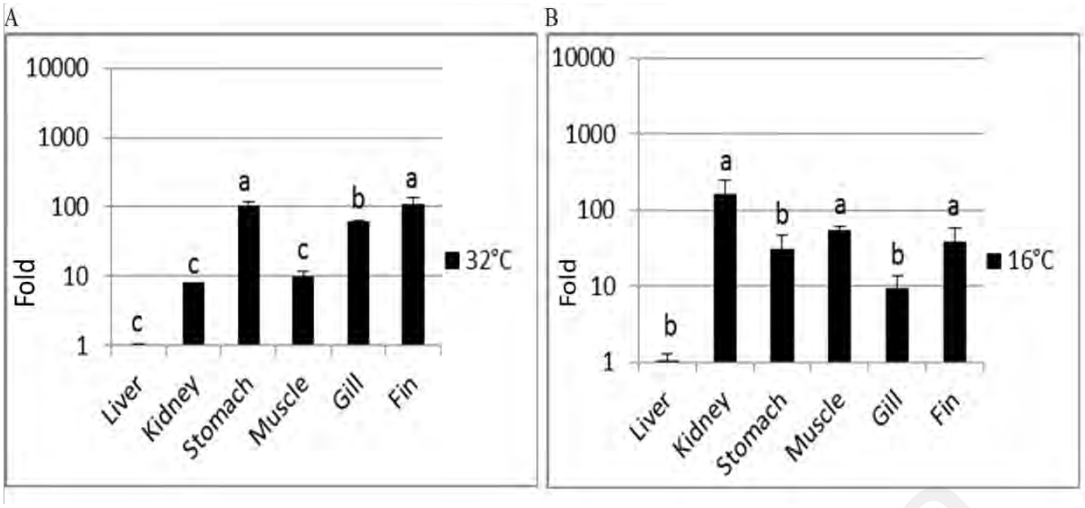

Figure 4.4: Tissue distribution of ChHSP70 expression in different organs of *C. striata* by qRT-PCR. Y axis represents number of expression folds. Data are expressed as a ratio to ChHSP70 mRNA expression in liver. The different alphabets are statistically significant at the P< 0.05 level by MANOVA and Duncan's Multiple Range Test. A and B exposed to 32°C and 16°C respectively

Overall, the expression of the ChHSP70 mRNA in the kidney, muscle and stomach when *C. striata* was exposed to a high temperature was significantly different (p<.05) from the level of expression induced on the same organs by exposure to a low temperature (Table 4.3).

Table 4.3: MANOVA analysis for gene expression of different organs under heat challenge

Dependent variable	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Muscle	Model	1	34.60527354	34.60527354	394.06	<.0001
	Error	4	0.35127157	0.08781789		
Stomach	Model	1	41.64818652	41.64818652	2569.52	<.0001
	Error	4	0.06483430	0.01620858		
Kidney	Model	1	36.82383880	36.82383880	20.74	0.0104
	Error	4	7.10306703	1.77576676		
Liver	Model	1	0.01228804	0.01228804	0.07	0.8123
	Error	3	0.54905917	0.18301972		
Gills	Model	1	13.20630288	13.20630288	6.61	0.0619
	Error	4	7.98724689	1.99681172		
Fins	Model	1	5.61079571	5.61079571	2.32	0.2023
	Error	4	9.67148448	2.41787112		

MANOVA of mRNA expression in different organs treated with exposure to16°C showed significant differences among different organs ($F = 6.48$, $df = 5$, $p < .05$), with Duncan's comparisons showing that the muscle, kidney and fins had substantially higher expression levels than the liver, stomach and gills (Table 4.4).

Table 4.4: Duncan multiple range test of mRNA expression in different organs treated with exposure to 16°C

Pr > F	Duncan Grouping	Organ
0.0039	A	Muscle
	A	Kidney
	A	Fins
	B	Gills
	B	Stomach
	B	Liver

* Means with the same letter are not significantly different

Also, when *C. striata* was exposed to 32°C, there were significant differences between the organs ($F = 93.51$, $df = 5$, $p < .0001$), but Duncan's multiple range comparisons demonstrated no significant differences in the mRNA levels between the liver, muscle and kidney (Table 4.5). The level of ChHSP70 mRNA expression in *Channa striata* for both extreme temperatures (32°C and 16° C) was very low in liver.

Table 4.5: Duncan multiple range test of mRNA expression in different organs treated with exposure to 32°C

Pr > F	Duncan Grouping	Organ
<.0001	A	Fins
	A	Stomach
	B	Gills
	C	Liver
	C	Muscle
	C	kidney

* Means with the same letter are not significantly different

4.2 Results of determining the infectious bacteria

4.2.1 Molecular identification using 16S rRNA gene sequence

Six isolates of bacteria were purified. However, only one of them (*Enterobacter soli* strain LF7a) gave typical symptoms of the disease observed in the fish farm. The identification of the bacterial isolate (Appendices F & G) was based on the sequence of 16S rRNA gene (1160bp). A similarity-based search of 16S rRNA nucleotide sequences through the NCBI server showed that *Enterobacter soli* strain LF7a had the highest similarity (99.31%) with the bacterium isolated from the fish in this study. Whereas the similarity to *Enterobacter asburiae* strain JCM 6051 and *Enterobacter cloacae* strain LMG 2683 was 98.36% & 98.18%, respectively (Table 4.6 and Figure 4.5)

Table 4.6: Similarity of isolated bacteria with other species based on 16S rRNA gene sequences

Species strain	Strain	Accession no.	Similarity (%)	Completeness (%)
Enterobacter soli	LF7a	NR_117547	99.31	100
Enterobacter asburiae	JCM 6051	NR_024640	98.36	97.26
Enterobacter cloacae	LMG 2683	NR_044978.	98.18	100
Enterobacter cloacae	ATCC 13047	NR_102794.	98.1	100
Enterobacter aerogenes	JCM1235	NR_024643	98.01	100
Enterobacter ludwigi	EN-119	NR_042349	97.93	100
Enterobacter kobei	JCM8580	NR_113321	97.84	99.32
Enterobacter cancerogenus	LMG 2693	NR_044977	97.83	100

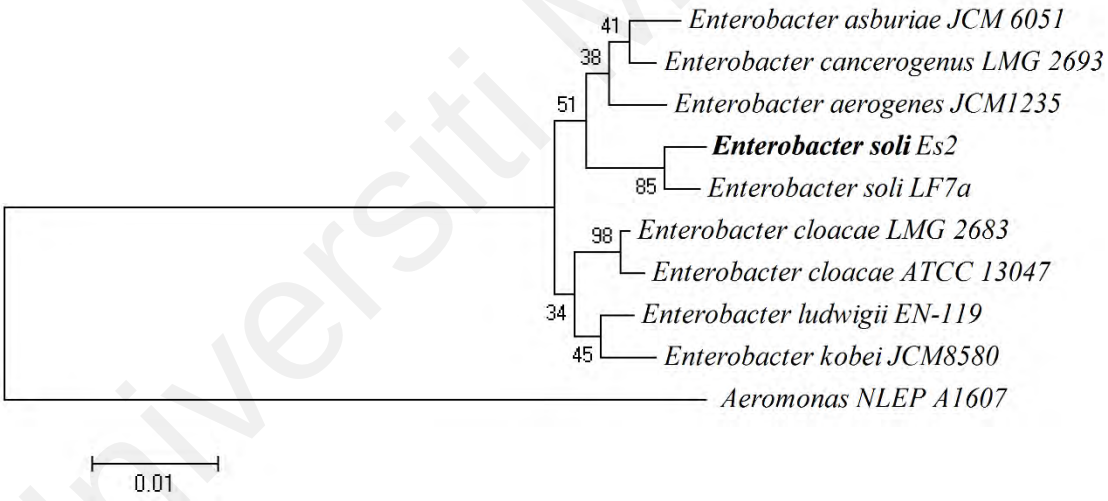

Figure 4.5: Phylogenetic tree deduced from the 16S rRNA gene sequences of the isolated bacterium with other *Enterobacter* spp. reconstructed by the neighbor-joining method. The numbers shown at the branches denote the bootstrap majority consensus values of 1000 replicates

4.2.2 Experimental infection of fish

4.2.2.1 Clinical signs

Clinical signs of the disease or abnormal behavior were not observed within the control group of fish (PBS-injected) throughout the experimental period but only observed in fishes injected with *Enterobacter soli* Es2. Three days after the injection, the main clinical signs observed in fish injected with the bacteria included scale loss and white skin lesions (Figure 4.6). Seven days' post-injection, skin lesions further developed into ulcerations. On the 10th day post injection, abnormal swimming behavior such as tethering movement and unusual backward and forward activity were detected. On the 14th day post injection moribund fish showed weakness with loss of appetite, and remained sunk at the surface of the water tank before dying. Although half of the experimentally infected fish died, mortality rate was not identified in the farm in which infected fishes were first observed in the early beginning.

Figure 4.6: Clinical signs of *C. striata* infected with isolated *Enterobacter soli* Es2. The main clinical signs observed in fish injected with the bacteria included scale loss and white skin lesions. Photo was taken 3 days post infection while the fish was alive inside the water

4.2.2.2 Histopathological changes

On histopathological examination of the liver of the injected fish, blood congestion in the blood vessel was observed in addition to hemorrhage and necrosis beside rupture of central vein lining. The liver also showed dilation and congestion of central vein. The pathological findings in the muscle of challenged fish included degeneration in muscle bundles and aggregations of inflammatory cells among them (Figure 4.7).

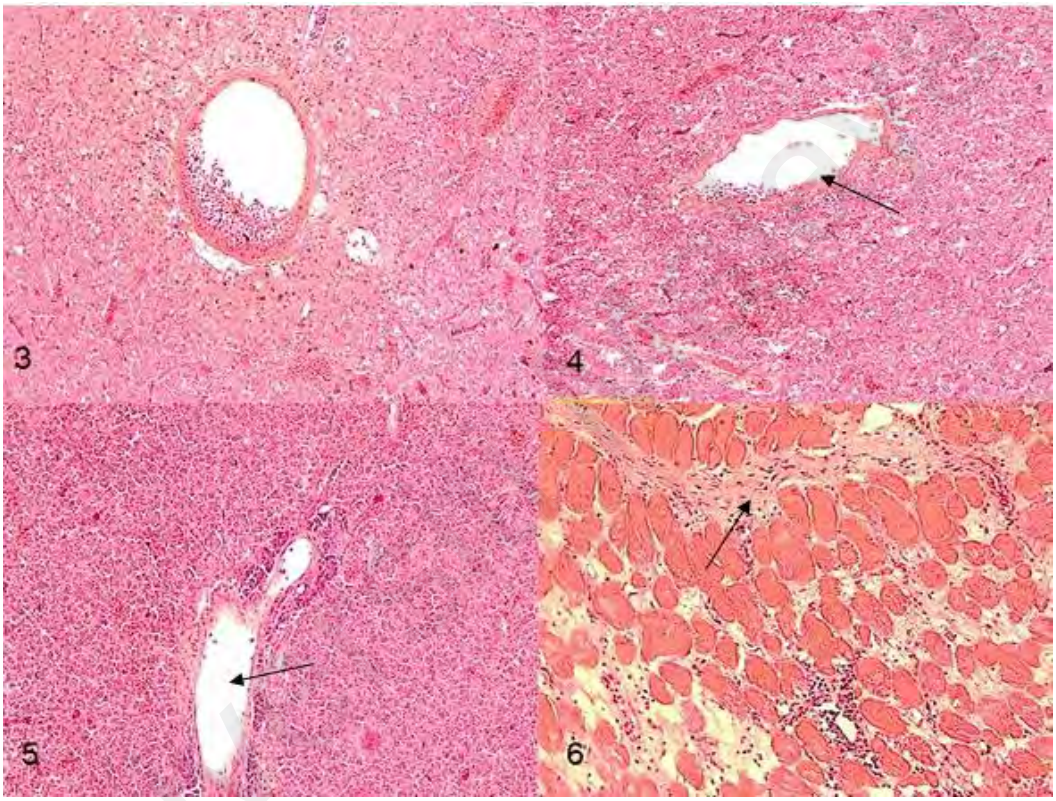

Figure 4.7: *C. striata* liver and muscle infected with *Enterobacter soli* Es2 strain bacteria. (3) early inflammatory cells infiltration in blood vessels, hemorrhage and necrotic area. (4) rupture of central vein lining (arrow) with hemorrhage and blood congestion. (5) marked dilation (arrow) and congestion of central vein. (6) *Channa striata* muscle with acute inflammation and neutrophilic infiltrate (arrow) had led to necrosis and loss of muscle fiber structure. (H&E staining)

4.3 Results of gene expression of challenged *Channa striata*

Generally, both tissue type (Table 4.7 & Table 4.8) and time elapsed (Table 4.9 & Table 4.10) had affected the expression significantly (P<.05). Excluding CsGlut expression results, 1-day elapse was not enough to show significant difference in all organs. Actually, even three days elapse was not enough to exhibit significance difference in CsCyto expression. CsCyto expression was significant for only 7 days and 10 days, that means it had the least difference in expression level. Results of expression for 3 days and 10 days were significant for both glutathione and CsCalp genes.

The expression of the four genes differed in almost all tested tissues according to the time elapsed from the injection by bacteria. Precisely, the expression of CsCyto and CsGlut was significant in all tissues (P<.05), that of CsGale was insignificant in spleen only, while CsCalp expression was significant only in intestine, spleen and gills.

Table 4.7: Analyses of variance (ANOVA) examining variation in gene expression of CsCyto and CsCalp genes. Significance levels are according to change of tissue type

		CsCyto			CsCalp			
Time		df	F	Sig.	Between groups	df	F	Sig.
Control	Between groups	5	0.258	0.927	Within groups	6	1.025	0.449
	Within groups	12			Total	14		
	Total	17			Between groups	20		
1 day	Between groups	5	1.841	0.179	Within groups	6	0.985	0.471
	Within groups	12			Total	14		
	Total	17			Between groups	20		
3 days	Between groups	5	3.729	0.029	Within groups	6	5.736	0.003
	Within groups	12			Total	14		
	Total	17			Between groups	20		
7 days	Between groups	5	10.095	0.001	Within groups	6	8.324	0.001
	Within groups	12			Total	14		
	Total	17			Between groups	20		
10 days	Between groups	5	19.934	0.001	Within groups	5	29.716	0.001
	Within groups	12			Total	12		
	Total	17			Between groups	17		
14 days	Between groups	5	2.238	0.117	Within groups	6	12.428	0.001
	Within groups	12			Total	13		
	Total	17			Between groups	19		

Table 4.8: Analyses of variance (ANOVA) examining variation in gene expression of CsGlut and CsGale genes. Significance levels are according to change of tissue type

		CsGlut				CsGale		
Time		df	F	Sig.		df	F	Sig.
Control	Between groups	5	0.012	1.000	Between groups	6	1.519	0.243
	Within groups	12			Within groups	14		
	Total	17			Total	20		
1 day	Between groups	5	18.08	0.001	Between groups	6	2.22	0.103
	Within groups	12			Within groups	14		
	Total	17			Total	20		
3 days	Between groups	5	5.408	0.008	Between groups	6	14.045	0.001
	Within groups	12			Within groups	14		
	Total	17			Total	20		
7 days	Between groups	5	14.824	0.001	Between groups	6	3.259	0.032
	Within groups	11			Within groups	14		
	Total	16			Total	20		
10 days	Between groups	5	6.532	0.004	Between groups	6	4.605	0.009
	Within groups	12			Within groups	14		
	Total	17			Total	20		
14 days	Between groups	5	3.343	0.040	Between groups	6	4.469	0.01
	Within groups	12			Within groups	14		
	Total	17			Total	20		

Table 4.9: Analyses of variance (ANOVA) examining variation in gene expression of CsGlut and CsGale genes. Significance levels are according to change in timing point

		CsGlut			CsGale			
Organ Tissue		Df	F	Sig.		df	F	Sig.
Intestine	Between groups	8	11.208	.000	Between groups	8	2.993	.027
	Within groups	18			Within groups	17		
	Total	26			Total	25		
Fin	Between groups	8	4.548	.004	Between groups	8	8.992	.000
	Within groups	18			Within groups	18		
	Total	26			Total	26		
Gill	Between groups	8	13.095	.000	Between groups	8	30.029	.000
	Within groups	18			Within groups	18		
	Total	26			Total	26		
Liver	Between groups	8	15.172	.000	Between groups	8	5.751	.001
	Within groups	17			Within groups	18		
	Total	25			Total	26		
Spleen	Between groups	NA	NA	NA	Between groups	8	1.953	.114
	Within groups	NA			Within groups	18		
	Total	NA			Total	26		
Stomach	Between groups	8	14.944	.000	Between groups	8	57.113	.000
	Within groups	18			Within groups	18		
	Total	26			Total	26		
Muscle	Between groups	8	2.629	.042	Between groups	8	4.532	.004
	Within groups	18			Within groups	18		
	Total	26			Total	26		

Data for spleen CsGlut is not available (NA).

Table 4.10: Analyses of variance (ANOVA) examining variation in gene expression of CsGlut and CsGale genes. Significance levels are according to change in timing points

		CsCalp				CsCyto		
Organ Tissue		Df	F	Sig.		df	F	Sig.
Intestine	Between groups	8	13.535	0	Between groups	8	19.547	.000
	Within groups	18			Within groups	18		
	Total	26			Total	26		
Fin	Between groups	8	2.284	0.069	Between groups	8	54.171	.000
	Within groups	18			Within groups	18		
	Total	26			Total	26		
Gill	Between groups	8	7.178	0	Between groups	NA	NA	NA
	Within groups	18			Within groups	NA		
	Total	26			Total	NA		
Liver	Between groups	8	2.613	0.043	Between groups	8	14.62	.000
	Within groups	18			Within groups	18		
	Total	26			Total	26		
Spleen	Between groups	8	15.795	0	Between groups	8	5.417	0.001
	Within groups	18			Within groups	18		
	Total	26			Total	26		
Stomach	Between groups	8	1.436	0.248	Between groups	8	8.97	.000
	Within groups	18			Within groups	18		
	Total	26			Total	26		
Muscle	Between groups	NA	NA	NA	Between groups	8	14.932	.000
	Within groups	NA			Within groups	18		
	Total	NA			Total	26		

Data for muscle CsCalp and gills CsCyto are not available (NA)

The four tested genes showed different patterns of expression in different tissue types (Figure 4.8). CsGale showed its minimum expression in spleen and gills compared to other genes whereas it showed its maximum expression in muscle at all tested points (except one-day post injection) compared with other genes.

CsCyto showed its minimum expression in intestine, spleen and muscle in almost all the tested points compared to the rest of tested genes. That means in muscle galectin is best expressed while cytochrome is poorly expressed. CsCalp showed its minimum expression in the liver and stomach (except for 3 days post injection) compared to other genes and its maximum expression in intestine in all tested points with exception of 3days point at which only CsGlut exceeds it. CsGlut showed its maximum expression in liver compared with other genes except at 1-day post injection point.

Regarding the expression pattern of fishes brought from the farm with clear signs of disease, and sampled on the second and third days of arrival at the laboratory, the expression of all genes and all tissue organs (except in gills) of fish sampled on the third day was higher than those sampled on the second day.

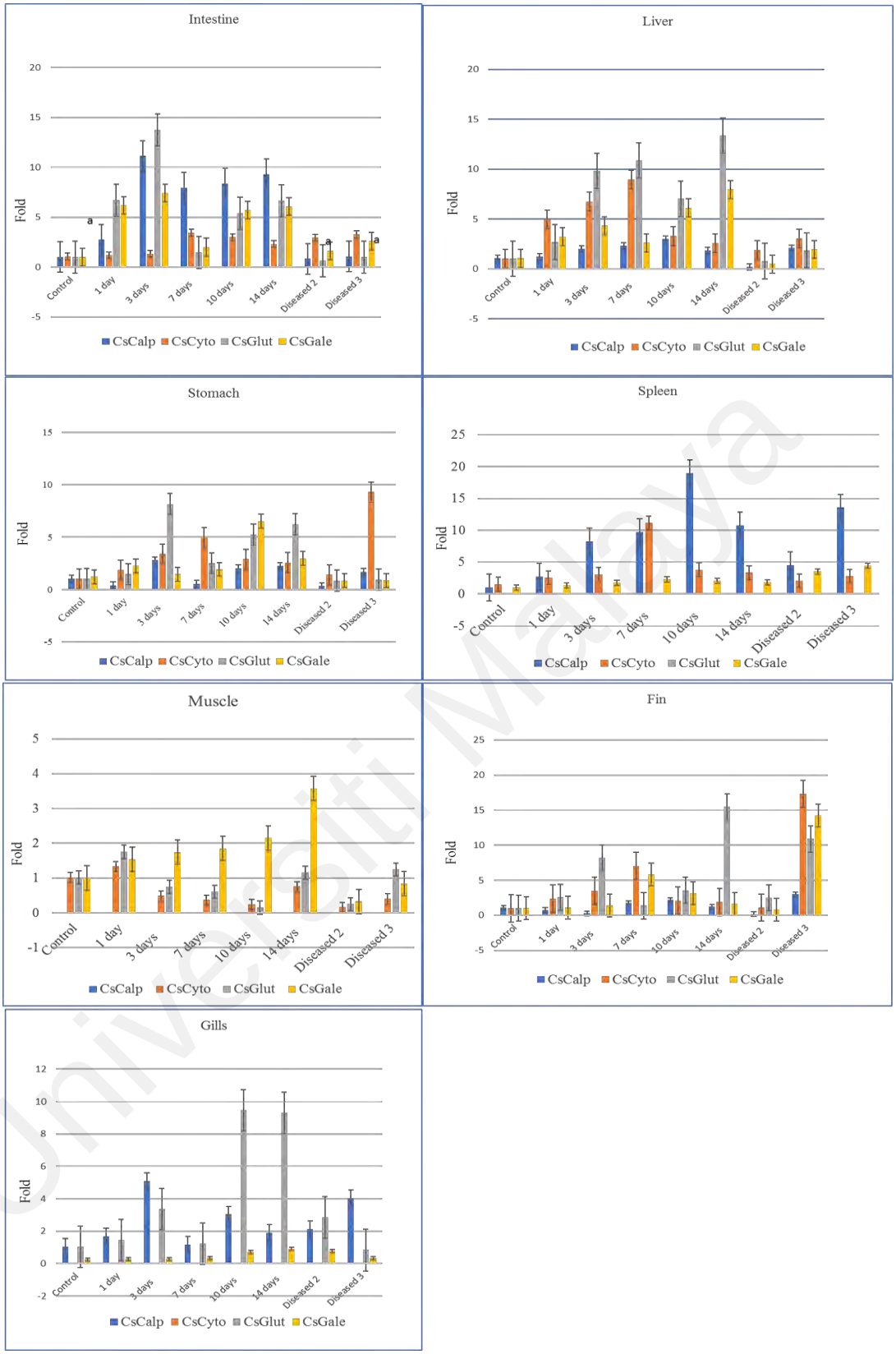

Figure 4.8: mRNA expression levels of the four tested genes analyzed by qrtPCR in different organs of *C. striata* at different time intervals after *Enterobacter soli* Es2 challenge. Y axis represents number of expression folds. Data for muscle CsCalp, gill CsCyto and spleen CsGlut are not available

CHAPTER 5: DISCUSSION

In this study, a partial length of ChHSP70 cDNA sequence had exhibited high sequence similarity with known HSP70 genes from other species of fish. The results showed that the genes encoding HSP70 are highly conserved between these species. Moreover, a phylogenetic tree of the sequences of the gene studied was constructed for six main clusters. From there HSP70 sequences in to the same class were remained conserved by having high sequence similarity except for fish in general. The classes for fish were revealed to be more diversified and divided into two groups. This relationship suggests that HSP70 may have proceeded through gene duplication and sequence divergence (Waters & Rioflorido, 2007). One possible reason for this could be the gradual increase in the temperature of the environment (Safdar et al., 2012). The presences of two HSP70 isoforms of the heat-inducible genes belonging to fish is also reported (Yamashita et al., 2004). Comparing the two groups of fish according to their polymorphic sites, polymorphism was found to be higher in Fis 1 while the substitutional sites are less than that of Fis 2 this means there is a clear bias in mutation rates in the two sets, which may result in new speciation in FIS-1 in the future. The clear differences in the evolutionary patterns of HSP70 between different fish groups suggest that there may be so far undiscovered functional differences between these subgroups. Genes that are phylogenetically stable have preserved their core functions, while genes that display a much more varied or unstable phylogeny have much more varied functions (Thomas, 2007). This diversity in fish species may be due to the environmental adaptation of this gene to deal with environmental stresses that are more pronounced for aquatic organisms than higher, land-based vertebrates (Das et al., 2015). The phylogenetic tree, together with the population pairwise FST, reveals that Fis 2 are closely related to Amp in this respect-proof of the fact that fishes and amphibians share many similar features. Similarly, Picone et al. (2014) found that the predicted olfactory receptors which able to

detect water-soluble odorants were found in teleost fish and amphibians, but not in reptiles, birds or mammals, consistent with their respective habitats. Further studies of HSP70 sequence diversity within and between closely related species should provide crucial information and help to elucidate additional differences in structure and function of this gene.

Members of the HSP 70 families have been shown to be stimulated by a wide variety of physiological and environmental stresses, including thermal shock, heavy metals, free radicals and microbial infection (Simoncelli et al., 2010; Zhang et al., 2011; Bakiu et al., 2014). HSP gene responses can vary according to tissue, distinct HSP families, and stressors. The sensitivity of HSP gene expression can also vary according to species, developmental stage and season (Kayhan & Duman, 2010). Higher expression levels of HSP70 indicate better thermal stress tolerance in cells (Li et al., 1992). Interestingly, the expression of the gene under 32°C in the stomach was higher than that of the gills although the latter are more exposed to external temperature change than the former in a poikilothermic animal like fish. Together with the stomach, the highest expression level of ChHSP70 under thermal stress was observed in the fin, in line with a previous study in *S. torgalensis* (Jesus et al., 2013). This may be because fins are directly exposed to water and hence are more susceptible to environmental changes. Under cold stress, in contract, ChHSP70 expression was highest in the kidney and relatively low in the fin, this suggests that ChHSP70 may adopt a similar strategy to LrHSP70 (Giri et al., 2014) to protect cells against thermal or cold stress.

Currently, the bacterial family *Enterobacteriaceae* has 53 genera and over 170 named species (Public Health England, 2015) indicating its importance. Hitherto a small number of these species, if any, were isolated from warm-blooded vertebrates (Gordon & Frances, 1999). Its main genera are *Escherichia, Proteus, Klebsiella, Citrobacter* and

Enterobacter (Cabral, 2010). The aim of the present study is to investigate the causative agents elaborated in observed selective mortalities of the *Channa striata* fish in aquaculture premises in Malaysia. Results revealed that *Enterobacter soli* Es2 was the causative agent for these mortalities. *Enterobacter soli* strain LF7a was first isolated from soil by Manter et al. (2011). As *C. striata* fish are found in freshwater bodies such as rivers and ponds, contamination by bacteria through run-off water to these water bodies, which is the ultimate source of water for tropical rivers, could not be excluded. Recently, Hassan et al. (2012) isolated 37 *Enterobacteriaceae* strains from 40 *Oreochromis niloticus* fish samples. However, as a disease-causing agent *Enterobacter cloaca* had earlier been found to have possible involvement with fish infection (Sekar et al., 2008). Thus, the current study is the first to report *Enterobacter soli* as a disease causative agent in fish.

In this study, the main clinical signs in experimentally diseased fish were skin ulcer, dullness, appetite loss and abnormal swimming behavior which may be pretty similar to those noted by Hassan et al. (2012). Histology is the study of tissues, including their role in the body, their anatomy, their interaction with body systems and the ways they are affected by the disease. In this study, we observed hepatic lesions including blood congestion that may be due to increased blood influx to the liver. This finding is in line with Mekkawy et al. (2012) in *Orechromis niloticus* and Darwish et al. (2000) in channel catfish infected with *Edwardsiella tarda*. Sinusoids and small blood vessels congestion were observed that makes blood movement from the hepatic portal vein and hepatic artery into the central vein quite hard. These difficulties in blood flow cause dilation of sinusoids that may reach the extent of rupture of blood vessels; similar findings were also observed by Ikpesupp & Ezemonye, (2014). Other histological alterations observed microscopically in the liver of challenged fish were cellular degeneration and necrosis which were also observed by Alaa, (2012). Hassan et al. (2012) elucidated that

inflammatory and necrotic changes in rainbow trout fry and cyprinids indicate typical acute bacterial septicemia caused by *Citrobacter freundii*. A notable gathering of inflammatory cells infiltration next to some blood vessels could be attributed to the presence of necrotic cells that act as irritant substances that bring these inflammatory cells. This is fairly consistent with Mekkawy et al. (2012). In general, the accumulation of these cells is an actual response of body tissue facing injury or bacterial infection (El-Banhawy, 1993).

Histological changes in the muscle include inflammatory cells infiltration due to the bacterial infection. Therefore, this result is in line with the findings of El-Banhawy (1993) who studied the pollution of *Solea vulgaris* and *Tilapia zillii* fish from a lake that receives large amounts of contaminated drainage water.

Immune responses are key mechanisms that defend against exotic pathogens. As the security force of the host organism, hypersensitivity reactions and autoimmunity may occur when immune responses are too intense or too lengthy. Thus, a large number of negative regulatory mechanisms are involved in the regulation of immune-related signaling and maintenance of immune system homeostasis (Zhu et al., 2013). This study discussed complex changes in the expression of some candidate genes associated with infection with *Enterobacter soli* Es2 bacteria.

The qRT-PCR results revealed that CsGale mRNA was constitutively expressed in all the detected tissues. These results were also consistent with other researchers (Kong et al., 2012; Inagawa et al., 2001). Inagawa et al., (2001) reported that galectin from rainbow trout was widely expressed in various tissues such as spleen, head, kidney, thymus, peritoneal exudate cells, gills and heart. This result suggests that CsGale is an inducible protein that may play an important role during pathogen invasion. It has been shown by various researchers that CsGlue gene expression is influenced by bacteria (Hu et al., 2012;

Wang et al., 2013) and virus (Duan et al., 2013). In the present study challenges with pathogenic bacteria promoted upregulation of CsGlue gene expression. This finding is consistent with other researchers work on crustacean (Hu et al., 2012; Arockiaraj et al., 2014; Li et al., 2015) supporting that Glutathione S-transferase plays a role in the immune roles of aquatic organisms.

Although CsCyto showed minimum expression in intestine, spleen and muscle, but the differences in various other tissues according to timing point is significant. it plays a major role in immune system of a wide range of living organisms from microbes to human being. CsCyto acts during various inflammations and injuries in different cells (Menoret et al., 2012). They also act simultaneously with bacterial and fungal infections (Karlsson, 2005).

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

The freshwater snakehead fish *Channa striata* (Bloch, 1793) is extensively distributed across tropical Africa and Asia. This fish becomes an important commodity in the aquaculture business that has been industrialized very fast over the last 10 years

In this research the effect of temperature and bacterial stresses were studied. Temperature tolerances (including lethal limits) and the associated rates of thermal acclimation of fish are critical data when predicting fish responses to global climate changes. Consequently, a partial sequence of one of the heat shock proteins, namely HSP70 cDNA of *Channa striata* was isolated and characterized. The sequence was 918 bp in length and contained an open-reading frame of 854 bp (encodes 290 amino acids). The evolutionary process that led to the diversity of HSP70 specific to vertebrates was analyzed. Results revealed that the gene is highly homologous to HSP70 genes in other fish families, with lesser similarity to that in other vertebrate classes. This may indicate that accumulated mutations due to climate change play important roles in the evolution and diversity of the gene. To examine the extent to which different temperature influence the expression patterns of HSP70 transcripts, we exposed *Channa striata* to high temperature (32°C) and low temperature (16°C) shocks. The expression of the gene is higher at 32°C than 16°C (higher and lower extremes) in most of the organs. Indicating that the HSP70 gene is inducible with different temperature levels. *Channa striata* is more tolerant to lower temperature levels than higher ones. More investigations are needed in other fish species for more elaborations on the effect of heat stress.

Diseases were reported to cause economic loss to *Channa* aquaculture that is estimated to be in billions. Although wild *Channa striata* is known to be highly resistant to diseases, but the case of being under captivity is different. For instance, an occurrence of selective mortality was found in a farm of *Channa striata* in Selangor, Malaysia. The etiology of

the infection was attributed to bacteria that belong to *Enterobacteriaceae* family. This family is known to be widely distributed in nature and was previously found to be involved in the mortality of different fish species. So, 16S rRNA gene sequencing was used to identify bacteria isolated from skin and liver of moribund fish; the present research confirmed that the causative disease agent had been *Enterobacter soli* Es2. To our best knowledge, our work is the first to report this bacteria species as a disease causative agent in fish. Furthermore, clinical and histopathological changes of healthy fish samples were conclusive according to the Koch's postulates.

C. striata is known for its capability of surviving in harsh environmental condition and also under limited water araciality. Moreover, identification and characterization of the important immune genes in *C. striata* is vital and can be a platform for further research in understanding the immune function of this fish. Hence, the expression of galectin 8 (CsGale), glutathione S-transferase (CsGlut), h2 Calponin (CsCalp) and cytochrome b5 (CsCyto) genes, which are well known as immune related genes, were tested after challenging the fish with *Enterobacter soli* Es2. The four tested genes showed different Patterns of expression in different tissue types. CsGale showed its minimum expression in spleen and gills compared to other genes while showing its maximum expression in muscle at all tested points (except one-day post injection) compared with other genes. CsCyto showed its minimum expression in intestine, spleen and muscle in almost all the tested points compared to the rest of tested genes. That means in muscle galectin is best expressed while cytochrome is poorly expressed. CsCalp showed its minimum expression in the liver and stomach (except for 3 days post injection) compared to other genes and its maximum expression in intestine in all tested points with exception of 3days point at which only CsGlut exceeds it. CsGlut showed its maximum expression in liver compared with other genes except at 1-day post injection point.

Regarding the expression pattern of fishes brought from the farm with clear signs of disease, and sampled on the second and third days of arrival at the laboratory, the expression of all genes and all tissue types (except in gills) of fish sampled on the third day was higher than those sampled on the second day.

For gene expression experiments, if the cost become reasonable, it is better to increase the samples size so as to get more accurate results. Overall, based on the present results, further studies are possible on over-expressing and purifying the recombinant proteins of the genes to study their biological activities at proteomic level.

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