THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Aquaculture)

DEGREE

Aquaculture	Aquaculture
FIELD	**DEPARTMENT**

TITLE: Characterization of Two Constitutive Heat Shock Protein 70 Genes in Bighead Catfish *Clarias macrocephalus* (Günther, 1864): cDNA Cloning and Their Response to Heat Stress and Bacterial Challenge

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THESIS

CHARACTERIZATION OF TWO CONSTITUTIVE HEAT SHOCK PROTEIN 70 GENES IN BIGHEAD CATFISH *Clarias macrocephalus* (Günther, 1864): cDNA CLONING AND THEIR RESPONSE TO HEAT STRESS AND BACTERIAL CHALLENGE

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Aquaculture)
Graduate School, Kasetsart University
2013

Pornpun Poompoung 2013: Characterization of Two Constitutive Heat Shock Protein 70 Genes in Bighead Catfish *Clarias macrocephalus* (Günther, 1864): cDNA Cloning and Their Response to Heat Stress and Bacterial Challenge. Doctor of Philosophy (Aquaculture), Major Field: Aquaculture, Department of Aquaculture. Thesis Advisor: Associate Professor Supawadee Poompuang, Ph.D. 137 pages.

Heat shock proteins are ubiquitous, highly-conserved proteins that play a central role in controlling cellular homeostasis in response to a wide range of stresses including heat stress, oxidative stress, heavy metals and viral infection. Two heat shock cognate protein 70 genes were isolated from liver of bighead catfish. The length of complete cDNA sequences for bighead catfish *HSC70-1* and *HSC70-2* were identical with 2,278 bp, with the open reading frame of 1,950 bp encoding a 649 amino acid protein. Amino acid sequences of both proteins shared 94% similarity with 38 substitutions. The two genes were different in genomic DNA length, with 3,178 and 2,909 bp, respectively for *HSC70-1* and *HSC70-2*. Seven introns and eight exons were found in the genomic structure of both *HSC70s*. Bacterial infection induced *HSC70-2* expression in liver and muscle, but not in gills and brain tissues. The increased transcription of *HSC70-2* may indicate their important roles as molecular chaperones under oxidative stress.

Reproductive cycle of female bighead catfish is normally interrupted by the presence of a resting phase which precludes a year-round fry production, posing problem to aquaculture of bighead catfish and their hybrid. This study demonstrated the possibility of using temperature manipulation to stimulate ovarian activities during the resting period for bighead catfish. Ovarian development and monthly change in GSI of females held under pond conditions was monitored over a 1-year period from April 2008 to March 2009. In October, thirteen-month old female fish were collected from earthen pond and exposed to elevated temperatures at 30°C and 35°C under hatchery conditions for six weeks. Ovarian growth was determined at a 2-week interval. Significant variation of mean GSI values ($P<0.05$) was observed among months with the highest value (13.91±3.63%) in July. Histological examination of ovaries revealed that in females held under 30°C and 35°C, the number of vitellogenic oocytes progressively increased from 0.5 and 4.7% at week 2 to 4.6 and 19.7% at week 6, whereas in the earthen pond, ovaries remained in resting stage. Exposure to warm temperatures resulted in significant elevations of plasma cortisol but not glucose concentrations. The progression of ovarian development was likely due to the stimulatory effect of increased plasma cortisol on synthesis of yolk protein as inferred from the increased numbers of vitellogenic oocytes. Although fully matured females could not be obtained, the present study suggested that temperature manipulation was probably the practical way to increase the number of maturing females at the end of the reproductive season for bighead catfish.

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ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude and deep appreciation to my advisor, Associate Professor Dr. Supawadee Poompuang, for her providing great opportunities, encouragements, invaluable suggestions and comments which enable me to carry out my Ph.D. study and research successfully. I am very grateful to my advisory committee, Professor Dr. Uthairat Na-Nakorn and Dr. Wongpathom Kamonrat, for their precious guidance and commentaries for the completion of my thesis. I am appreciate greatly for acknowledge comments from the external examiner, Dr. Somsri Ngamwongchon and the chairperson of my final exam, Dr. Prapansak Srisapoome.

This thesis was supported by the Agricultural Research Development Agency (ARDA).

Finally, I wish to express my deepest gratitude to my parents for giving birth and all members in my family for their love, understanding, patience, support and encouragement until I accomplish my study.

Pornpun Poompoung

January 2013

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LIST OF ABBREVATIONS

ANOVA	=	Analysis of variance
bp	=	Base pairs
BW	=	Body weight
°C	=	Degree Celsius
cm	=	Centimeter
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxydinucleotide triphosphates
g	=	Gram
HCl	=	Hydrochloric acid
M	=	Molar
mg	=	Milligram (s)
$MgCl_2$	=	Magnesium chloride
ml	=	Milliliter (s)
mM	=	Millimolar
mm.	=	Millimeter
NaCl	=	Sodium chloride
ng	=	Nanogram (s) (10^{-9})
PCR	=	Polymerase Chain Reaction
pH	=	Logarithm of reciprocal of hydrogen (H) ion
pmol	=	Picomole (s) (10^{-12})

CHARACTERIZATION OF TWO CONSTITUTIVE HEAT SHOCK PROTEIN 70 GENES IN BIGHEAD CATFISH *Clarias macrocephalus* (Günther, 1864): cDNA CLONING AND THEIR RESPONSE TO HEAT STRESS AND BACTERIAL CHALLENGE

INTRODUCTION

Stress in fish has been shown to impact their growth, reproduction, immune system and viability both in the wild and in captivity. Stress is unavoidable and more importantly, it is a major concern in aquaculture due to its negative impact on production. Sources of stress include water quality conditions, exposure to crowding, diseases, handling and transportation. Fish have the ability to respond to stress depending on the severity and duration of the stressor. In general, response of fish to stress can be measured at three levels of organization, including behavior response, physiological response, and cellular response (Iwama *et al.* 2004). Behavior response is the immediate sign of fish under stress conditions, in which fish display changes in normal activities such as predator avoidance, prey capture and habitat selection. Response at physiological level involves increase of stress hormones, mainly cortisol, and glucose in the circulation. At the cellular level, stress response is determined by the expression of heat shock protein (*HSP*) families in key tissues of fish (Iwama *et al.* 1999). Heat shock proteins play an important role in protecting organism from damage after exposure to a wide variety of stressors.

As in other vertebrates, physiological stress response in fish can be categorized into the primary, secondary, and tertiary responses. A primary response involves releasing of a cascade of hormones, also known as the pituitary-interrenal axis (Sumpter, 1997). The secretion of pituitary hormone, adrenocorticotropic hormone (ACTH) in response to stress induces the release of catecholamines and cortisol into the circulation. Catecholamines are synthesized in the chromaffin tissue in the head kidney, whereas cortisol is produced in the interrenal tissue. In a secondary response,

cortisol activates a number of metabolic pathways including glucose production in the circulation to provide support for the increased energy demand of the animals (Iwama *et al.*, 1999). Glucose is produced mainly in liver tissue through glycogenolysis and/or gluconeogenesis. The stress hormones, adrenaline and cortisol play an important role in increasing glucose production in fish resulting in increased plasma glucose concentration. The tertiary response to stress can be described as changes in whole animal and community level. Whole animal changes may occur when fish is unable to acclimate or adapt to the stressor. As a result, fish will experience decreased reproductive capacity and growth. Under stress conditions, energy demand in fish increases and energy substrates are diverted from growth and reproduction processes of the animals to cope with stress. This can have negative impacts on their growth and reproduction. Moreover, decreased reproductive capacity of the animals can lead to change in population size (Barton *et al.*, 2002).

The cellular stress response in fish involves the synthesis of a highly conserved family of heat shock proteins (*HSPs*). These proteins play a central role in controlling cellular homeostasis in response to a wide range of stresses, including heat stress, oxidative stress, heavy metal exposure and viral infection (Feder and Hofmann, 1999). Exposure to stress induces an accumulation of denatured and mis-folded proteins within the cell; *HSPs* act as molecular chaperones to mediate the assembly, correct folding and localization of nascent proteins as well as degradation of denatured proteins (Krone *et al.*, 1997). Heat shock proteins are present in a wide range of organisms from *E. coli*, *Drosophila* to humans. *HSPs* are characterized by their molecular weights with the predominant classes being *HSP90* (85-90 kDa), *HSP70* (68-73 kDa), *HSP60* (16-24 kDa) and the small *HSP* (MW< 40 kDa) (Feder and Hofmann, 1999). Due to their significance functions in various aspects of fish physiology, studies of *HSP70s* in fish and shellfish continue to increase. Most studies have focused on molecular characterization and expression analysis of these proteins. *HSP70* is not expressed in most tissues, but is highly inducible under stress conditions. Some members of the *HSP* families are constitutively expressed in cells under normal conditions, such as the 70 kDa heat shock cognate protein (*HSC70*) (Iwama *et al*., 1998). *HSC70s* have received attention in fish and shellfish due to increasing evidence

of the involvement of *HSC70* in the immune system (Basu *et al*., 2002; Robert, 2003). For example, *HSC70* and *HSP70* levels in liver and hepatopancreas tissues were modulated during bacterial and viral infections (Leung and Hightower 1997; Deane and Woo, 2004; Chuang *et al*., 2007; Ming *et al*., 2010; Rungrussamee *et al*., 2010; Yue *et al*., 2011). It has been suggested that the role of *HSC70* during infection is associated with various pathways of protein translocation, whereas *HSP70* is able to stimulate innate immune response through the nuclear factor (NF)κb signaling pathway (Basu *et al*., 2002; Robert, 2003; Deane and Woo, 2004). *HSC70* genes have been cloned from several fish species, including platyfish (Yamashita *et al*., 2004), wuchang bream (Ming *et al*., 2010), and grass carp (Zhang *et al*., 2011). In addition, two *HSC70* genes were identified in zebrafish *Danio rerio* (Graser *et al*., 1996; Santacruz *et al*., 1997), common carp *Cyprinus carpio* (Ali *et al*., 2003) and yellowtail *Seriola quinqueradiata* (Yabu *et al*. 2010).

Bighead catfish (*Clarias macrocephalus*) is a high priced food fish because of its good taste and the beautiful meat color. Aquaculture of this species was established in Thailand for more than 50 years. At present, however, farming of bighead catfish nearly disappears due to the existence of problems including slow growth rate and low disease resistance. Hybrid catfish (*C. macrocephalus* × *C. gariepinus*) has replaced the culture of bighead catfish because they grow rapidly and are more resistant to diseases. The production of hybrid catfish in 2009 were 130,100 ton valued 235,912,000 Thai baht, respectively (DOF statistics, 2010). Demand for bighead catfish today is mostly for use as broodstock to produce hybrid larvae. High quality female bighead catfish, therefore, is required to support growing market of hybrid catfish. Under pond conditions, bighead catfish reach maturity at the age of approximately 9-10 months. Bighead catfish is asynchronous spawner, which can spawn several times in one season. A female broodfish can be used to produce larvae approximately 2-3 times in a year. In general, the onset of reproduction normally occurs at the beginning of rainy season (May) and continues through October. The availability of ripe females is low at the end of spawning season during December to February, when the temperature is 5-10°C below average (28°C). To overcome the shortage of female broodfish in the

country, hybrid catfish breeders acquired wild broodfish from neighboring countries, such as Cambodia and Viet Nam.

Reproductive cycle of *C. macrocephalus* resembles that of African catfish (*C. gariepinus*) and can be divided into three different phases; a pre-spawning phase (recrudescence of the ovary), a spawning phase, and a resting phase (Rodriguez *et al*., 1995). Under pond conditions, bighead catfish generally exhibit regression of the ovary at the end of spawning season during December to February, during which, induced spawning at this period is difficult. Several techniques have been used to induce ovarian development outside breeding seasons, including hormone injection, photoperiod and temperature manipulation. It is suggested that in temperate zone fishes, the reproductive cycle is strongly affected by seasonal changes in water temperature and photoperiod (Amita and Bhavna. 2011). These environmental factors may have direct or indirect effects on initiation of oogenesis, production of vitellogenin, or secretion of pituitary and reproductive hormones (Rottmann *et al.*, 1991). Although temperature is considered less important in tropical species, a number of studies have documented the influences of temperature on reproductive maturation. For instance, in African catfish, ovarian development was enhanced and the resting period was shortened from 4-7 months to 2 months when the fish were kept at a constant 25°C water temperature compared to those kept in outdoor ponds with ambient temperature (15 to 30°C) (Richter *et al*., 1987). In snakehead, a combination of high temperature (30°C) and long photoperiod stimulated reproduction and extended the spawning period (Andrew *et al.*, 2011). Ovarian development of bighead catfish has been well studied but less is known about the effects of heat stress on their reproduction.

Disease outbreak is a major problem that catfish farmers facing when cold weather moves in, especially at the end of breeding season. The common disease is bacterial septicemia caused by *Aeromonas hydrophila* (Wei and Klesius, 2011). During a breeding season, the outbreak can results in severe loss of broodfish. *A. hydrophila* infections occur most during environmental changes, change in the temperature, in contaminated environments, and when an organism is already infected

with a virus or another bacterium. Prevention and cure of the hemorrhagic septicemia are relied on the conventional approaches such as antibiotic treatment (Jongjareanjai *et al.,* 2009). The repeated use of such treatment has led to increased bacterial resistance to drug. Alternative treatment strategies have been developed such as development of novel vaccination approaches and the search for new immune stimulating treatments. Recently, the potential of heat shock protein 70 as a new prevention method was demonstrated in platyfish (Yamashita *et al*., 2004). The study reported that the fish received an injection with recombinant bacterial *HSP70* purified from *E. coli* had reduced rate of mortality when challenged with *Yersinia ruckeri* compared to the control (Kartik *et al*., 2010).

With the discovery of heat shock protein genes in *C. macrocephalus*, research on cellular stress response is now possible. Three major families of heat shock proteins, *HSC90, HSC70*, and low molecular *HSP25*, have been identified in the liver cDNA library of female *C. macrocephalus* (Panprommin *et al*., 2007). EST sequences of these heat shock protein genes have been deposited in GenBank. Molecular characterization, including cDNA and genomic cloning as well as expression analysis of heat shock proteins will provide basic knowledge of their role and function in physiological stress response in walking catfish. The partial cDNA sequence information can be used to obtain complete cDNA sequences of heat shock protein genes by rapid amplification of cDNA ends (RACE) techniques.

The goal of this research is to determine the molecular characteristics of *HSC70* genes and their response to heat stress and bacterial infection. From background and problems of bighead catfish aquaculture, it is interesting that despite the negative impacts on health and growth, elevated temperature and heat stress can also exert stimulating effects on reproduction of the fish. In this study, I used water temperature (30 and 35°C) to induce ovarian growth of bighead catfish for six weeks and measured the response to heat stress. Because heat shock protein research in *C. macrocephalus* is in its early stage compared with those in other teleosts, this study provides information on molecular biology of *HSC70* genes and their relationship

between the expression levels and the temperature stress response of this species. Furthermore, it contributes to better understanding of heat shock protein effects on reproductive biology of female broodfish. Finally, information of differential tissue expression of *HSC70* genes against bacterial infection is useful for further studies to develop new measures for prevention of bacterial diseases in bighead catfish.

OBJECTIVES

1. To clone complete cDNA sequences of two heat shock cognate (*HSC70-1* and *HSC70-2*) genes from *C. macrocephalus* by rapid amplification of cDNA ends (RACE).

2. To examine the seasonal expressions of heat shock cognate protein 70 (*HSC70*) and heat shock cognate protein 90 (*HSC90*) in tissues of *C. macrocephalus* cultured under natural pond conditions.

3. To determine the effects of elevated temperatures on ovarian development of female *C. macrocephalus*.

4. To investigate the response of *HSC70* and *HSC90* to heat stress in female *C. macrocephalus*.

5. To determine the expression of *HSC70-1* and *HSC70-2* genes in response to bacterial infection in key tissues of *C. macrocephalus*.

LITERATURE REVIEW

1. Fish stress and response

Stress has been recognized to cause detrimental effects on health, growth as well as reproduction of fish under either natural or aquaculture conditions. Stress can be defined as threat or disturbance of homeostasis produced by environmental or other factors (Barton, 1997). For intensive aquaculture, sources of stress include water quality conditions, diseases, fish density, nutrition, handling and transportation. Fish respond to stress according to the severity and duration of the stressors, i.e., acute or chronic. The effects of stress can be examined from the physiological responses to one or more of these stressors, by measuring the levels of hormones or other related metabolic enzymes. Stress responses are considered adaptive that promote the best chance of survival of the animal in the threatening situation. In response to short-term or acute stressors, energy resources are diverted from immediately non-essential processes, e.g., growth and reproduction to increase probability of survival of the animals. The detrimental effects of stress are more prominent for continuous or chronic stressors when the adaptive response is compromised (Wendelaar, 1997).

Stress responses in fish can be divided into three levels of organization including, behavior response, physiological stress response, and cellular stress response (Iwama *et al*., 2004). Behavior response is the immediate sign of fish stress as indicated by changes of several activities that are important to survival of the animals, such as food acquisition, predator avoidance, migration and habitat preference (Schreck *et al*., 1997). These behaviors are closely correlated with physiological responses and tend to return to normal in the shortest time to increase chances of survival for that individual and the population. In general, behavioral response to stressors helps lessening the energy demand on the physiological systems to response to such stress (Schreck *et al*., 1997). The physiological mechanisms, on the other hand, are responsible for initiating, and maintaining the behavioral reaction (Iwama *et al*., 2004).

The physiological response of fish can be categorized into the primary, secondary and tertiary responses (Iwama *et al*., 1997) (Fig. 1). Primary response or generalized stress response comprises the production of neuroendrocrine hormones. Stress hormones, catecholamines and cortisol are released into the circulation in response to several pituitary hormones, mainly adrenocorticotrophic hormone (ACTH). Catecholamines are released from the chromaffin tissue situated in the head kidney of teleosts whereas cortisol is released from the interrenal tissue of the head kidney..The measure of cortisol is generally used as an indicator for primary response. Cortisol also is important for carbohydrate metabolism which stimulates gluconeogenesis in liver to increase the protein disintegration for energy (Carl and Alena, 2011). Animals lacking cortisol cannot use carbohydrate and are not tolerance to stress.

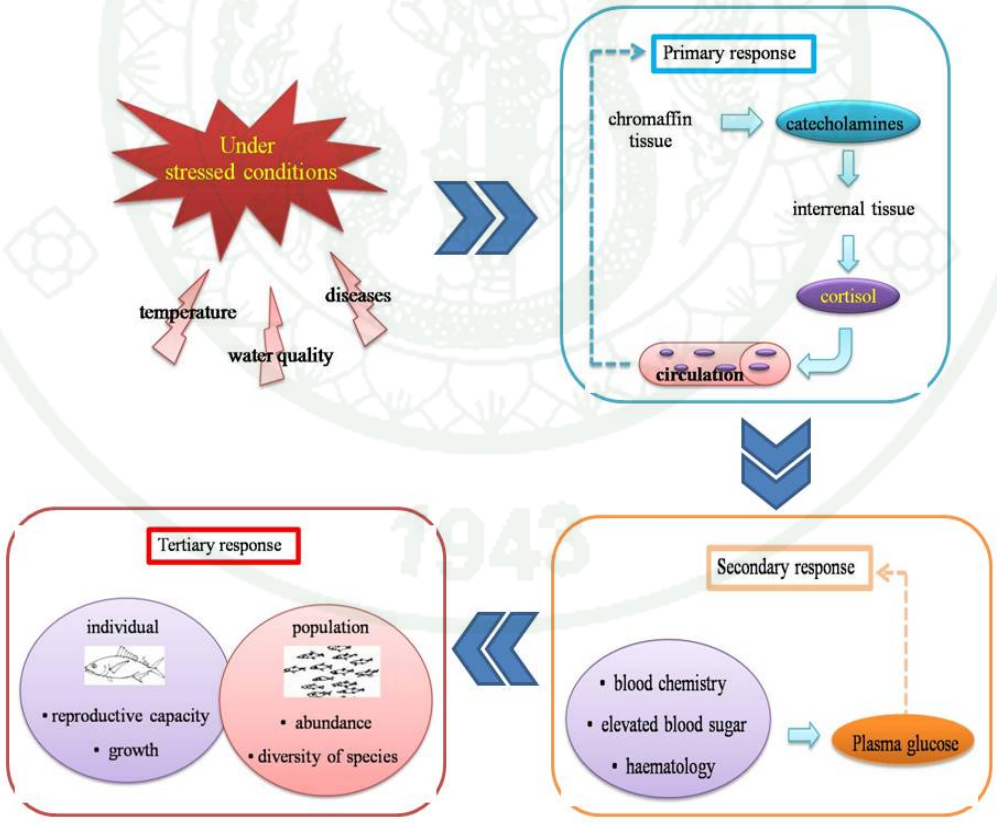

Figure 1 Three stages of physiological stress response in fish; primary, secondary and tertiary.

Secondary response is largely mediated by cortisol, which activates a number of metabolic pathways causing changes in blood chemistry and haematology (Iwama *et al.*, 2004). In fish, adaptive secondary response includes increased gill perfusion, elevated blood sugar (hyperglycemia), and reduced blood-clotting time (Barton *et al*., 2002). Plasma glucose concentration has been used as an indicator of secondary (metabolic) response to stressors in fish (Iwama *et al.*, 1997). Glucose is produced primarily in liver of the animal under stress to provide energy substrates to other tissues such as brain, gills and muscles, in order to cope with the increased energy demand. It is well understood that cortisol is important for glucose production in fish and plays an important role in the stress associated increase in plasma glucose concentration (Iwama *et al.,* 1999). In addition, cortisol helps maintaining plasma glucose levels in post-stress fish. Other common indicators for the secondary response are haematocrit, erythrocyte (RBC) numbers, and plasma chloride and sodium concentration (Iwama *et al.,* 1997).

The tertiary response to stress can be described as changes in whole animal and population level. Decreased reproductive capacity, growth and disease resistance are used as the indicators for tertiary stress response (Barton *et al.*, 1997). Whole animal changes may occur when fish is exposed to long-term exposure to a stressor and is unable to acclimate or adapt. When fish is under stress, its energy demand increases and energy substrates are diverted from vital processes such as growth and reproduction. Consequently, decreased reproductive success and growth of the animals can lead to change in population size and diversity (Barton *et al.*, 1997).

2. Cellular stress response: heat shock proteins (*HSPs*)

The cellular stress response is described by a synthesis and the expression of a family of proteins referred to as the heat shock proteins (*HSPs*). Heat shock proteins are ubiquitous, highly-conserved proteins that play a central role in controlling cellular homeostasis in response to a wide range of stresses including heat stress, oxidative stress, heavy metals and viral infection (Iwama *et al*., 1998). Stress conditions which induce the synthesis of heat shock proteins results in an accumulation of denatured and

aberrantly folded proteins within the cell. *HSPs* act as molecular chaperones to assist the correct assembly and localization of intracellular and secreted polypeptides and oligomeric protein structures (Krone *et al*., 1997). Several heat shock proteins are constitutively expressed in normal and stressed cells indicating their importance role in the protein folding pathway.

2.1 Characterization of heat shock proteins in fish and shellfish

Heat shock proteins are classified into three major families based on molecular weight: *HSP90* (85-90 kDa), *HSP70* (68-73 kDa), and low molecular weight *HSP* (MW< 40 kDa) (Iwama *et al*., 1998). *Hsp70* is known to assist the folding of newly synthesized polypeptide chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured proteins (Fig 2). *HSP90* is active in supporting various components of cell signaling, including the cytoskeleton, enzymes, and steroid hormone receptors. The low molecular weight *HSPs* have diverse functions that are species specific and unlike other *HSPs*, these proteins have no known constitutive function and seem to only be induced during stress (Iwama *et al*., 2004).

Studies of heat shock proteins in fish indicate that *HSP70* and *HSP90* exist in constitutive and inducible forms (Feder and Hofmann, 1999). Under stressed conditions, these protein isoforms are differentially expressed in various tissues. For example, *HSC70* is the constitutively expressed form and only slightly induced during stress conditions while *HSP70* is highly inducible upon exposure to stress. In addition, two isoforms of *HSP90* have been characterized in common carp *Cyprinus carpio*, *HSP90α* and *HSP90β*. The *HSP90β* was constitutively expressed in brain, liver and kidney and was slightly inducible by high temperature (Hermesz *et al*., 2001). In contrast *HSP90α* was highly inducible in brain tissue following heat stress.

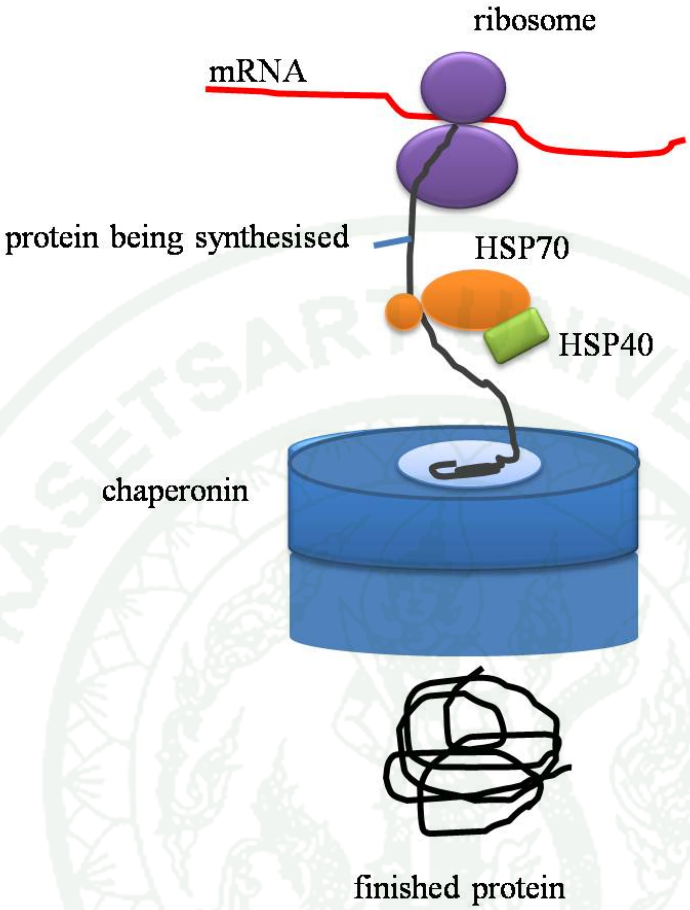

Figure 2 Role of heat shock proteins as molecular chaperone in protein folding.

Three heat shock protein families, *HSC90*, *HSC70* and *HSP25* were identified in liver tissues of *C. macrocephalus* (Panprommim *et al*., 2007) by expressed sequence tag (EST) techniques. Among them, heat shock cognate protein 70 (*HSC70*) was the most abundant with the highest number of transcripts. Partial cDNA sequences of the three heat shock protein genes have been deposited in EST database in GenBank.

Most studies revealed that the expression of heat shock proteins in teleost fish were different across tissues, developmental stages and species level. Palmisano *et al*. (2000) reported that the 5-hour exposure of chinook salmon *Oncorhynchus tshawytscha* to elevated temperature (mean 21.6°C ± 10.6°C over ambient) induced a significant increase in *HSP90* mRNA accumulation in heart, brain, gill, muscle, liver,

kidney, and tail fin tissues. An increase of approximately 35-fold was detected in heart tissue whereas heat shock induced no increase in plasma cortisol. However, there were no increases of *HSP90* mRNA level or plasma cortisol after the overnight exposure to seawater. In other study, exposure of juvenile Atlantic salmon *Salmo salar* to hatchery stressors, other than heat stress, failed to up-regulate expression of *HSP90*, *HSP70* and *HSP30* (Zarate and Bradley, 2003). The results indicated that all three isoforms of *HSPs* present in the larvae were least sensitive to stress. In contrast, the rapid response of *HSP90* and *HSP70* was detected in brain tissue of channel catfish *Ictalurus punctatus* exposed to low temperature when temperature was reduced from 24 to 12°C (Ju *et al*., 2002). By using cDNA microarray, they found that gene expression occurred within 2 hours of the temperature shift. Genes encoding proteins involved in signal transductions and chaperones such as *HSP70* and *HSP90* were highly expressed in brain. Several genes, primarily ribosomal protein genes, were down-regulated, indicating reduced metabolic activities after extended incubation at the low temperature. Results supported the significant role of heat shock proteins for adaptation and tolerance of catfish to low environmental temperature.

A study in tiger shrimp *Peneaus monodon* suggested that *HSP70* might be an indicator of acclimation temperature stress in this species (Doungpunta *et al*., 2000). Differential expression of *HSP60* and *HSP70* were detected at 35°C whereas both protein families were expressed at 30°C. On the other hand, there was no significant difference of glucose levels in haemolymph at water temperature of 30°C and 35°C.

In other studies, the potential for using *HSP70* as a biomarker has been demonstrated in black bullhead (*Ameiurus melas*) and bluegill sunfish (*Lepomis macrochirus*) (Yoo and Janz, 2003). The fish were exposed chronically to sub-lethal concentrations of cadmium and zinc. The fish were collected from two creeks during spring (pre-spawning season and average water temperature of 26.5 ± 0.95°C) and winter (water temperature ranged from 4.8 to -0.80 °C). The *HSP70* expression was examined in head kidney, liver, gill, including ovarian tissues to determine the reproductive physiological responses in female fishes. Significant seasonal differences

were observed in expression of *HSP70* in gill tissue of both species, in ovarian and liver tissue of bluegill sunfish and in head kidney of black bullhead.

The generalized stress response at the cellular level of *HSP70* in whole fish and cell lines has been extensively reviewed by Iwama *et al*. (1998). A number of studies have focused on the relation between *HSP70* and stress hormones adrenaline and cortisol. Evidence from cultures of rainbow trout hepatocytes revealed that adrenaline caused an increase in *HSP70*. In contrast, cortisol did not directly affect *HSP70* levels in fish tissues. The relationship between stress hormones and *HSP70* induction may be modulated by several factors *in vivo*. For instance, Mazur (1996) has shown physical handling of rainbow trout (45 s dip-net stress) which caused an increase in plasma cortisol concentration significantly reduced the heat shock-related increase of *HSP70* in the gill tissue. These findings were contrast with the results of Vijayan *et al*. (1994), which show that physical handling, which caused an increase in circulating cortisol levels, did not cause an increase in liver *HSP70* levels. Most studies suggested that further investigations are required to understand the relationship between the cellular *HSP* response and the physiological stress response in fish.

2.2 Structure of heat shock protein 70

HSP70/HSC70 have two structural domains, an N-terminal nucleotide binding domain (NBD) and a C-terminal protein substrate binding domain (SBD). Both domains are connected by a conserved linker that mediates communication between the two domains (Babak *et al*., 2007). The NBD which has ATPase activity binds ATP and hydrolyzes it to ADP (Fig. 3). The exchange of ATP drives conformational changes in the substrate binding domain that contains a groove with an affinity for neutral, hydrophobic amino acid residues. The groove can interact with peptides up to seven residues in length. The C-terminus has alpha helical structure and is also referred to as the crystalline domain (Basha *et al*., 2006).

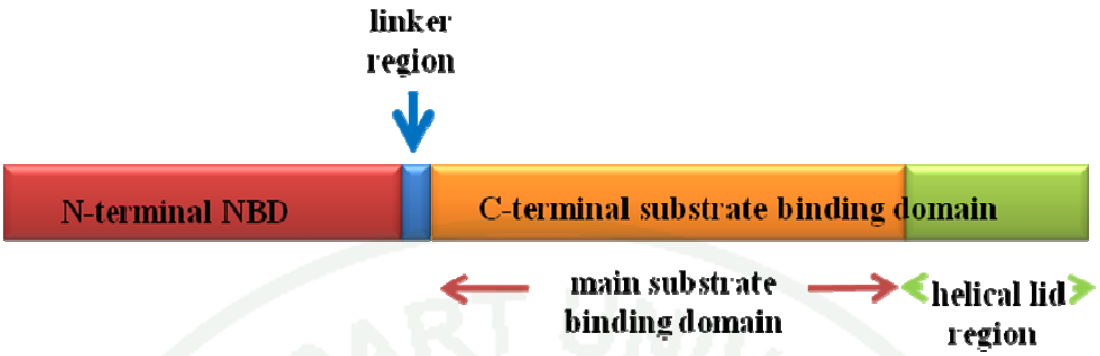

Figure 3 Schematic of the domain structures of *E. coli HSP70* including two major domains: N-terminal NBD (red) and C-terminal substrate binding domain (orange and green). This region comprises the main substrate binding domain (orange; 18 kDa) and helical lid region (green; 10kDa). Both molecules have a linker region (blue) that is thought to mediate communication between the major sub domains.

Source: Babak *et al.* (2007).

The amino acid sequences are highly conserved at the N-terminal domain. In addition, glycine residues are highly conserved in the middle domain of the protein. The sequence conservation in the middle domain signifies its importance in efficient interaction between HSP70 and substrate (Desai *et al.*, 2010). In contrast the amino acid sequences are very more variable at the C-terminal domain. The beta sheets dominate the HSP 70 homologue structures compared to the helical structures (Desai *et al.*, 2010). The beta sheet structure plays a major role in providing stability to the proteins and maintaining homeostasis in the cell during stress condition.

2.3 Molecular mechanisms of heat shock protein expression

A review of molecular processes involved in heat shock protein expression is provided by Iwama *et al.* (1998). The expression of heat shock proteins is mediated by heat shock transcription factor (HSF). Under stress conditions, HSF is activated and binds to heat shock element (HSE) located upstream of the heat shock

genes. A heat shock factor contains DNA-binding domain located about 100 amino acids at the amino-terminus and three leucine zippers (trimerization domain) located nearby the DNA-binding domain (Fig. 4). Yeasts and *Drosophila* are known to contain only one HSF, while mice and humans have multiple distinct HSFs (HSF1, HSF2, and HSF3) with different functions. HSF1 is the major isoform and important in regulating the cellular response to thermal and oxidative stress. In response to stress, HSF1 undergoes the transition from an inactive monomeric form to a homotrimer form and binds to the promoter region to activate transcription.

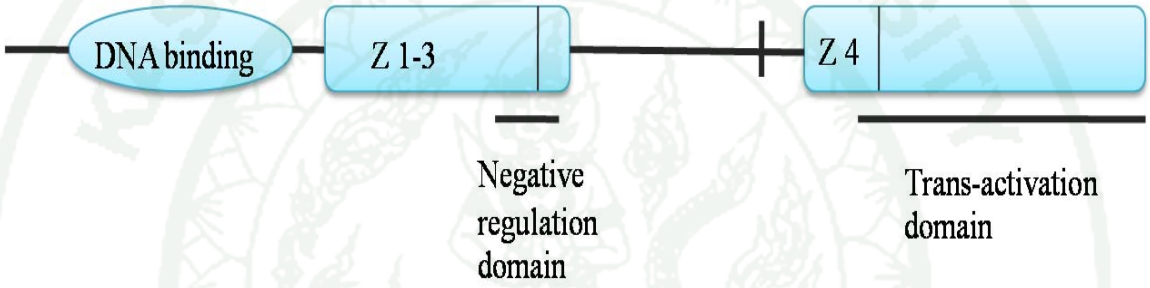

Figure 4 Diagram of heat shock factor (HSF1) structure showing DNA binding (carboxyl-terminus), leucine zippers (Z) 1-3, and leucine zipper 4 structures. Thick bars indicate areas of constitutive phosphorylation.

Source: Iwama *et al.* (1998).

The vertebrate HSFs have highly conserved amino acid sequences at the DNA-binding and leucine zipper 1-3 domains. For example, there is 85-95% amino acid sequence identity between human, mouse and chicken HSF1 (Morimoto *et al.*, 1994). In *Drosophila* and higher eukaryotes, HSF1 contains an additional leucine zipper 4 at the carboxyl-terminus. The quantity of HSF1 in a cell does not vary with stress. On the other hand, stress induces conversion of the inactive form of HSF1 to an active protein which subsequently regulates the expression of *HSP70* (Rabindran *et al.*, 1991). However, the mechanism of HSF1 activation and the nature of the sensor of the stressor in the cell are still not known.

To understand the regulation of heat shock protein expression, a model based on proteotoxicity was proposed (Morimoto *et al.*, 1996; Voellmy, 1996). In this model, stressors would cause damage to native proteins inside the cell resulting in *HSP* production (Fig. 5). In the cytosol of normal cells, HSF1 is found as a monomer or forms a complex with *HSP70* to facilitate releasing from DNA. In stressed cells, the damaged proteins sequester *HSP70* and other chaperone proteins such as *HSC70*, and *HSP40*, to assist their repair or destruction.

The initiation of *HSP* expression occurs with dissociation of *HSP70* from the monomeric form of HSF1 in the cytosol (Morimoto *et al*., 1996). As a result, HSF1 moves to the nucleus and changes to a trimeric form which can bind to the promoter regions of the *HSP* genes. The trimeric HSF1 remains inactive until stress-induced phosphorylation of the HSF1 takes place (Cotto *et al.*, 1996; Morimoto *et al.*, 1996). The production of *HSP70* occurs through the normal transcription and translation processes. When the translation of *HSP70* is complete, free *HSP70* binds to HSF1 to release HSF1 from heat shock element (HSE) of the DNA. In addition, the binding of free *HSP70* to HSF1 aids in the dissociation of the trimeric form back to the inactive monomers and the relocation of monomeric HSF1 back into the cytoplasm. During recovery from stress, the increased amount of *HSP70* in the cell is from the newly synthesized proteins and those that bound by denatured proteins which are released after repair process.

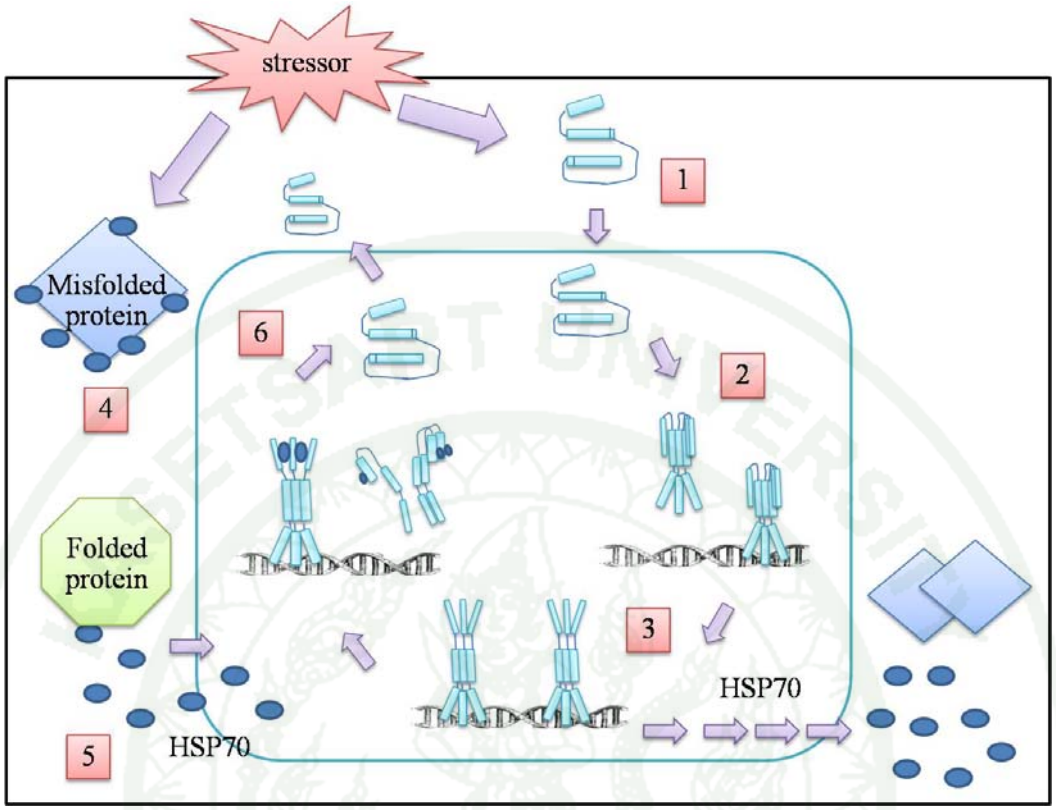

Figure 5 The model of the role of heat shock factor (HSF1) in the regulation of heat shock protein expression. (1) Latent monomeric form of HSF1 in cytoplasm or nucleus. (2) Activation by stressor leads to trimerization of HSF1, which facilitates binding to heat shock element of the *HSP70* gene promoter. (3) Phosphorylation and activation of *HSP70* transcription results in *HSP70* expression. (4) Increase in cytosolic *HSP70* repairs misfolded proteins, along with other *HSPs*. (5) Subsequent repair of damaged proteins results in higher levels of free *HSP70* in cytosol. (6) Higher concentration of *HSP70* results in binding to HSF1, facilitating release from DNA, and dissociation of HSF1 back to the monomeric form.

Source: Morimoto *et al*. (1996) and Iwama *et al*. (1998).

The regulation of *HSP* production involves signal transduction mechanisms for sensors of the stressors such as redox changes in the cell (Voellmy, 1996). For example, reducing agents are found to prevent the activation of HSF in HeLa cells exposed to stressors such as mild heat shock, hypoxia, hydrogen peroxide or dinitrophenol (Huang *et al*. 1994). In addition, it is suggested that the mitogen-activated protein (MAP) kinase cascades in the cell may involves in the *HSP* production (Bensaude *et al.*, 1996). The intracellular signal transduction cascades are triggered by an increase in calcium concentration and the stimulation of enzymes such as phospholipases C and A2 caused by stressors and may affect the phophorylation of the HSF1.

2.4 Functions of heat shock cognate protein 70 (*HSC70*)

A comprehensive review on functions of the human *HSC70* has been provided by Liu *et al* (2012). Members of the heat shock protein 70 family, *HSP70* and *HSC70* share high levels of amino acid similarity, have similar functions and interact with each other in an ATP-dependent manner. Significant differences between the two forms were found in the carboxyl-terminal domain which mediates substrate specificity and particular biological functions.

HSC70 acts as molecular chaperone in facilitating folding of newly synthesized proteins and stabilizing their normal structures under stress conditions (Fig 6). In addition, *HSC70* is suggested to involve in translocation of protein across endoplasmic reticulum and antigen presentation. Details of the cellular functions of *HSC70* have been described as follows (Liu *et al*., 2012). (1) *HSC70* functions as an ATPase to dissociate coat proteins from clathrin coated vesicles (CCVs) to facilitate endocytosis (Chang *et al*., 2002). (2) *HSC70* facilitates transportation of receptors between nucleus and cytoplasm (Kose *et al*., 2005). (3) *HSC70* maintains protein homeostasis (protein folding, translocation, assembly, disassembly, and degradation) in both normal and stress conditions. (4) *HSC70* suppresses protein aggregation and reactivates heat denatured proteins. (5) *HSC70* is involved in facilitating maturation of newly synthesized proteins and interacts with nascent polypeptides in the process of

translocation into organelles. (6) *HSC70* can regulate the translocation of proteins into different cellular organelles such as endoplasmic reticulum and mitochondria. (7) *HSC70* can recognize misfolded proteins and delivers them to lysosomes for degradation. (8) *HSC70*, together with other molecular chaperones, is involved in regulating cellular signaling and functions. (9) *HSC70* is needed for the activation of heat shock factor 1 (HSF1) in mammalian cells. (10). *HSC70* can regulate apoptosis and is required for prevention of apoptosis induced by white spot syndrome virus (WSSV) infection (Yan *et al*., 2010).

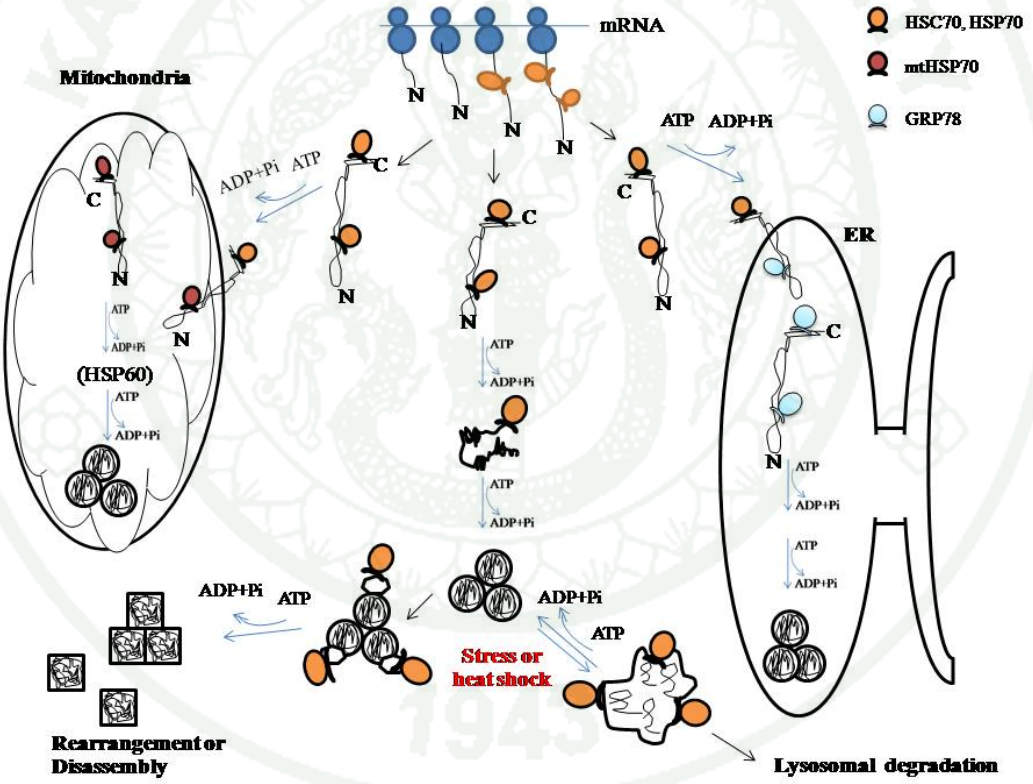

Figure 6 Model of molecular chaperon functions of the HSP70 family. HSP70/HSC70 function as intracellular chaperones for other proteins and mediate protein folding, translocation and degradation.

Source: Yamashita *et al*. (2004).

3. Reproductive development of tropical fish

Ovarian development in most teleost fish has been characterized as synchronous or asynchronous, according to stages of oocyte development during reproductive cycle. In synchronous spawners, there are two strategies for the egg production. In the first strategy, egg production occurs only once in the fish's life as in Pacific salmon and the fish die after spawning. A second strategy is called group synchronous with several cycles of oocyte development and spawning as in brown trout and striped bass. In asynchronous spawners, oocyte development is continuous and spawning occurs several times throughout the breeding season. Bighead catfish is identified as asynchronous spawners and contain oocytes of different stages of development.

Oocyte development for bighead catfish can be broadly classified into four stages according to Lehri (1968): immature oocyte, maturing oocyte, mature oocyte and atretic oocyte. The immature oocytes stain dark blue cytoplasm and light pink germinal vesicle with Hematoxylin and Eosin with large sized nucleus and germinal vesicle located in the center of the cell. Oocytes in the maturing stage start to produce yolk vesicle and accumulate yolk granule in cytoplasm. In mature oocytes, vitellogenesis stops and the germinal vesicle migrates to the animal pole. Atretic or degenerate oocytes can be characterized by the presence of enlarged granular cell wall with no nuclei.

Stress can have a profound effect on gamete development and quality, as well as on egg and larval development and viability in fish. Stress affects circulating levels or change seasonal patterns of reproductive hormones (Pankhurst and Van Der Kraak, 1997). Stress is known to effect changes of plasma gonadotropins which can result in reduction of plasma levels of testosterone and/or estrogen. In female fish, the reduction of plasma estrogen due to stress is found to be associated with a higher incidence of ovarian atresia. In some cases, the effects of stress resulting in ovary degeneration were irreversible during the reproductive season. Further, stress can

result in delayed ovulation and reduced egg size in females and lowered sperm counts in males. Progeny from stressed fish have lowered survival rate.

The release of cortisol into the circulation during the primary stress response is believed to affect reproductive capacity of fish. Reduced ovary size and lower levels of plasma estradiol and vitellogenin were observed in mature females treated with exogenous cortisol. The lower levels of estradiol can have direct effect on the synthesis of vitellogenin in the hepatocytes which results in reduced egg size (Pacoli *et al*., 1990).

4. Important diseases in bighead catfish culture

During the culture period, bighead catfish have been found to suffer from a number of bacterial diseases, in particular, *Aeromonas hydrophila*. Bacterial septicemia caused by the Gram-negative intracellular bacterium *A. hydrophila* also commonly affects other freshwater teleosts (Areerat, 1987). *A. hydrophila* is a rod shaped bacterium, mainly found in areas with a warm climate. This bacterium can also be found in fresh, salt, marine, estuarine, chlorinated, and un-chlorinated water. *A. hydrophila* can survive in aerobic and anaerobic environments. *A. hydrophila* was isolated from humans and animals in the 1950s (Jongjareanjai *et al*., 2009). It is also highly resistant to multiple medications, chlorine, and cold temperatures.

Because of *A. hydrophila*'s structure, it is very toxic to many organisms. When it enters the body of fish, it travels through the bloodstream to the first available organ. It produces Aerolysin Cytotoxic Enterotoxin (ACT), a toxin that can cause tissue damage (Rodriguez *et al.*, 1993). And it is considered to be opportunistic pathogens, meaning it rarely infect healthy individuals. *A. hydrophila* is widely considered a major fish and amphibian pathogen, and its pathogenicity in humans has been recognized for decades (Angka *et al.*, 1995)

The pathogenicity of *Aeromonas* spp. is mediated by a number of extracellular proteins such as aerolysin, lipase, chitinase, amylase, gelatinase, hemolysins and enterotoxins. However the pathogenic mechanisms of *Aeromonas* spp. are unknown. The recently proposed type III secretion system (TTSS) has been linked to *Aeromonas* pathogenesis. TTSS is specialized protein-secretion machinery that exports virulence factors directly to host cells (Yu *et al.*, 2005). These factors subvert normal host cell functions to the benefit of invading bacteria. In contrast to the general secretory pathway, type III secretion system is triggered when a pathogen comes in contact with host cells. ADP-ribosylation toxin is one of the effector molecules secreted by several pathogenic bacteria and translocated through TTSS and delivered into the host cytoplasm leads to interruption of NF-κB pathway (Hideaki *et al.*, 2008), cytoskeletal damage and apoptosis which can cause disease both in human and fish.

A. hydrophila infections occur most during environmental changes, stressors, change in the temperature, in contaminated environments, and when an organism is already infected with a virus or another bacterium (Eissa *et al.*, 1994). *A. hydrophila* is associated with diseases mainly found in fish and amphibians, because these organisms live in aquatic environments. When infected with *A. hydrophila*, fish develop ulcers, tail rot, fin rot, and hemorrhagic septicemia. Hemorrhagic septicemia causes lesions that lead to scale shedding, hemorrhages in the gills and anal area, ulcers, exophthalmia, and abdominal swelling (Gado, 1998).

MATERIALS AND METHODS

Part I

Molecular characterization of *HSC70* genes

1. Clone *HSC70* cDNAs and *HSC70* genes

1.1 Cloning of two *HSC70* cDNAs

1.1.1 Total RNA extraction and purification of mRNA

Total RNA was extracted from liver tissue of female bighead catfish (152 g) which stocked in 800 m^3 earthen ponds using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instruction. The extracted RNA was purified using a QuickPrep™ Micro mRNA Purification kit (Amersham Biosciences, Buckinghamshire, UK). The quantity of mRNA was determine by measuring OD at 260 nm and electrophoresed on 1% formaldehyde-agarose gel.

1.1.2 Rapid amplification of cDNA ends (RACE)

The 371 bp *HSC70* EST clone (EB360505) containing partial open reading frame and the 3′ untranslated region (UTR) was obtained from the previous work (Panprommin *et al*., 2007), therefore, only 5′ RACE was performed. The 5′ RACE-technique was utilized to recover the 5′ information of target cDNA using a BD Smart™ RACE cDNA Amplification kit (BD Biosciences ClonTech, San Jose, CA, USA) according to the manufacturer's instruction. Approximately 1 µg of mRNA was used for first strand cDNA synthesis. For the 5′-RACE-Ready cDNA, 1 µg of mRNA, 1 µl of BD Smart II A-oligo (5′-AAGCAGTGGTATCAACGCAGAG TACGCGGG-3′) and 1 µl of 5′-CDS primer (5′-$(T)_{25}$VN-3′, N = A, C, G or T; V = A,

G or C) were used to generate the nucleotide sequences at the 5′ end. Sterile water was added in each reaction to a final volume of 5 µl and the reaction was spun briefly in a microcentrifuge. The tube was incubated at 70°C for 2 min and cooled on ice for 2 min. The tube was spun briefly to collect the contents at the bottom. Two µl of 5x first-strand buffer, 1 µl of 20 mM of dithiothreitol, 1 µl of 10 mM of dNTP Mix and 1 µl of BD PowerScript Reverse Transcriptase were added to each reaction tube. The contents was mixed gently and spun briefly to collect the contents at the bottom. The tube was incubated at 42°C for 1.5 hrs in a hot lid thermal cycle. The first-strand reaction product was diluted with 250 µl of Tricine EDTA buffer and incubated at 72°C for 7 min.

After first strand cDNA synthesis, the 5′-RACE-Ready cDNA was used as templates to generate the nucleotide sequences at the 5′ ends. For the RACE PCR reaction, a 50 µl PCR reaction mixture consisted of 2.5 µl of 5′-RACE-Ready cDNA, 5 µl of 10x BD Advantage 2 PCR buffer, 1 µl of 10 mM dNTP mix (each at 2.5 mM), 1 µl of 50x BD Advantage 2 polymerase mix, 5 µl of 10x Universal Primer Mix (UPM: Long (0.4 µM), 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ and Short (2 µM), 5′-CTAATACGACTCACTATAGGGC-3′) and 1 µl of 10 µM gene specific primer (Table 1). The 5′ RACE was carried out using reverse primers in the PCR for 25 cycles as follows: 94°C for 3 min, 94°C for 30 sec, 60°C for 30 sec and 72°C for 3 min. The positions of primer are shown in Figure 7 and Figure 8.

Table 1 Nucleotide sequences and positions of primers used in 5′ RACE cloning of cDNA *HSC70-1* and *HSC70-2* genes.

Name	Primer sequence (5′ to 3′)	Position	
		HSC70-1	*HSC70-2*
HSC70-1R1	CCAATGCCTGGTTTGCCTTGAA	2210-2231	
HSC70-1R2	GTCTCTGCTGTTGACAAGAGC	1495-1515	
HSC70-1R3	CCAGTATTGAGATCGACTCCC	872-892	
HSC70-1R4	GCTCTGAGAGAAATGTCCTTATTTTCGA	602-629	
HSC70-2R1	CCAATGCCTGGTTTGCCTTGAA		2210-2232
HSC70-2R2	GGAGTTCCTCAAATCGAAGTG		1441-1461
HSC70-2R3	CTACAGCAGGAGACACTCACC		695-715
HSC70-2R4	CTCAGCGTCAAGCCACAAAAGATGCTGG		491-518

[a] Nucleotide positions are based on *C. macrocephalus* sequence submitted to GenBank (Accession number JX112294 of cDNA for *HSC70-1* and accession number JX12295 of cDNA for *HSP70-2*).

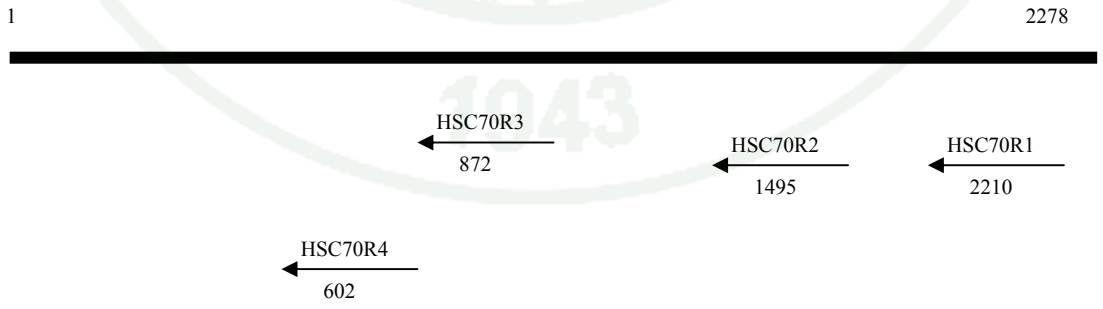

Figure 7 Positions of primers for amplification of *C. macrocephalus HSC70-1* cDNA (thick black line). The orientations of amplification are indicated by arrows with the primer's name on the top and the first position for amplification is represented by the number under the arrow.

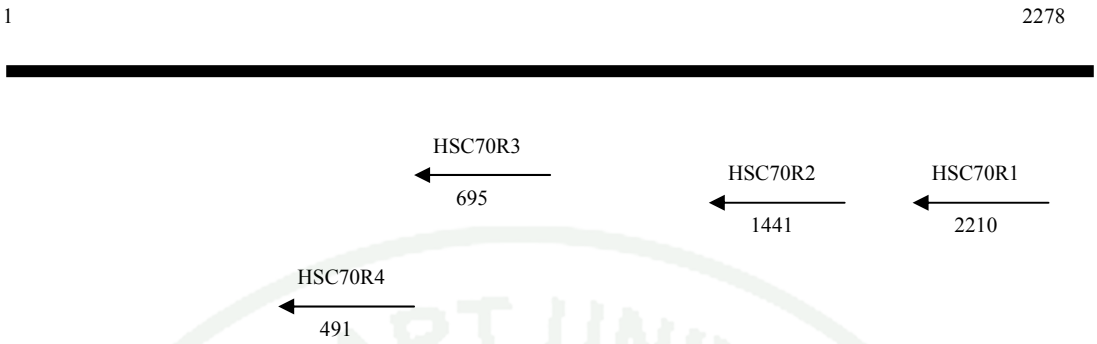

Figure 8 Positions of primers for amplification of *C. macrocephalus HSC70-2* cDNA (thick black line). The orientations of amplification are indicated by arrows with the primer's name on the top and the first position for amplification is represented by the number under the arrow.

1.2. Genomic cloning of two *HSC70* DNAs

Two heat shock cognate cDNAs obtained in this study were called bighead catfish *HSC70-1* and *HSC70-2*. Primers for genomic cloning of bighead catfish *HSC70-1* and *HSC70-2* were designed based on the full length cDNAs (Fig. 9 and Fig. 10). Genomic DNA was extracted from liver tissue (20-30 mg) of female fish using Genomic DNA purification kit (Fermentas, Germany). The extracted DNA was subjected to PCR amplification using primers (Table 2 and 3). A 25-µl PCR mixture consisted of 1µl of genomic DNA, 2.5 µl of 10x *Taq* buffer, 0.75 µl of $MgCl_2$, 1.5 µl each of dNTPs, 1 µl each of forward and reverse primers, 0.2 µl of 1 U *Taq* DNA polymerase, and 17.05 µl of sterile water under the following conditions for 25 cycles: 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min 30 s. PCR products were cloned and sequenced. The genomic nucleotide sequences were compared to cDNA sequences to determine the exon-intron boundaries of the genes.

Table 2 Nucleotide sequences and positions of primers used in genomic cloning and 5′ RACE of *HSC70-1* gene.

Name	Primer sequence (5′ to 3′)	sequence	
		Position	bp
HSC70F1	CAGATTGAGGTCACATTTGAC	2085-2105	1046
HSC70R1	CCAATGCCTGGTTTGCCTTGAA	3111-3132	
HSC70F2	GCTATTGCCTATGGGTTG	800-817	1351
HSC70-1R2	GTCTCTGCTGTTGACAAGAGC	2130-2150	
HSC70F3	CCAGCTGTTGGCATTGATCTG	46-66	1165
HSC70-1R3	CCAGTATTGAGATCGACTCCC	1190-1210	
HSC70-1R4 (5′ RACE)	GCTCTGAGAGAAATGTCCTTATTTTCGA	602-629	

Table 3 Nucleotide sequences and positions of primers used in genomic cloning and 5′ RACE of *HSC70-2* gene.

Name	Primer sequence (5′ to 3′)	sequence	
		Position	bp
HSC70F1	CAACCTGCTCGGGAAGTTTGA	1824-1844	1110
HSC70R1	CCAATGCCTGGTTTGCCTTGAA	2911-2933	
HSC70F2	GCTATTGCCTATGGGTTG	800-817	1094
HSC70-2R2	GGAGTTCCTCAAATCGAAGTG	1873-1893	
HSC70F3	CCAGCTGTTGGCATTGATCTG	46-66	921
HSC70-2R3	CTACAGCAGGAGACACTCACC	946-966	
HSC70-2R4 (5′ RACE)	CTCAGCGTCAAGCCACAAAAGATGCTGG	491-518	

[a] Nucleotide positions are based on *C. macrocephalus* genomic sequence submitted to GenBank (Accession number JX273642 for *HSC70-1* and Accession number JX273643 for *HSP70-2*).

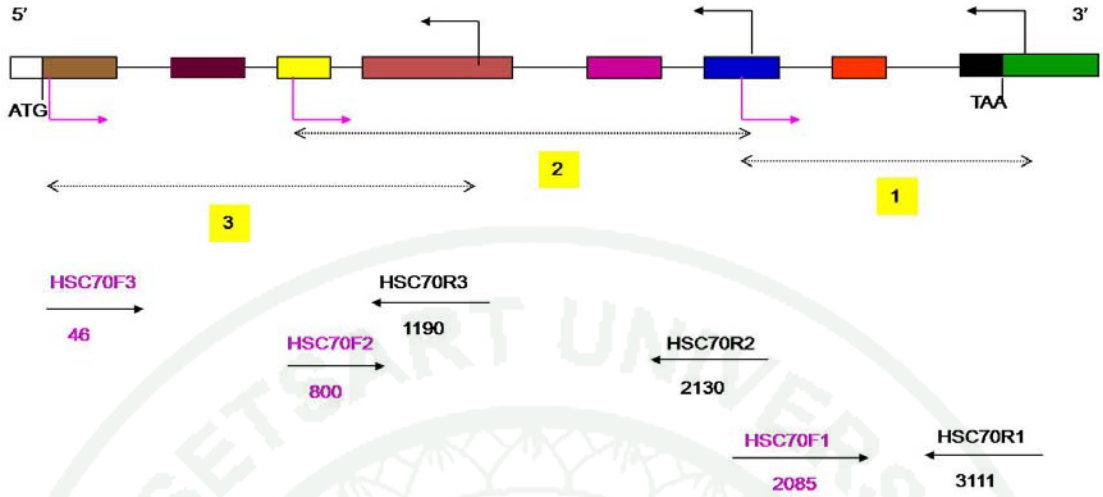

Figure 9 Positions of primers for amplification of *C. macrocephalus HSC70-1* genomic DNA (thick black line). The orientations of amplification are indicated by arrows with the primer's name on the top and the first position for amplification is represented by the number under the arrow.

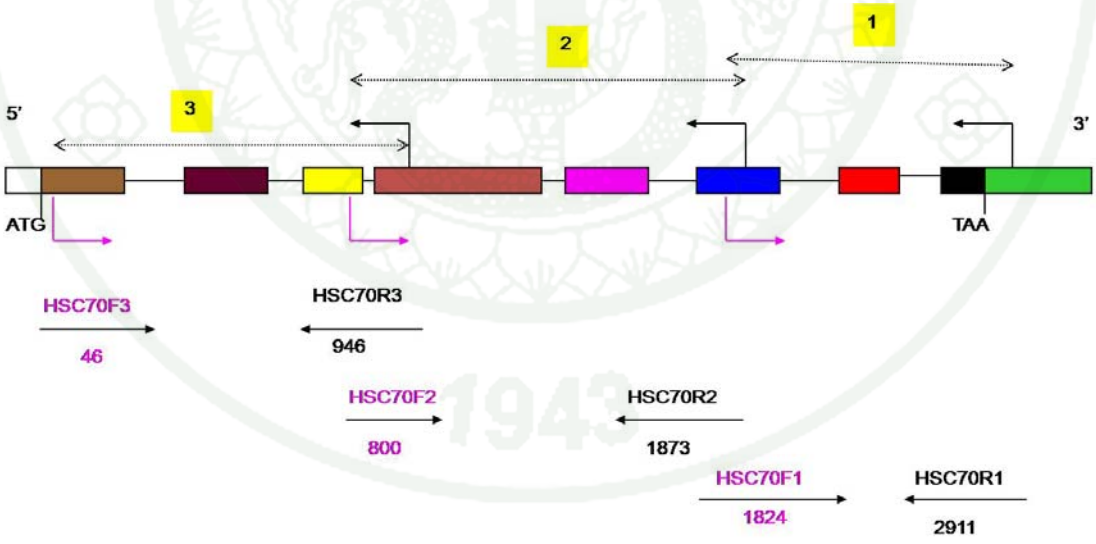

Figure 10 Positions of primers for amplification of *C. macrocephalus HSC70-2* genomic DNA (thick black line). The orientations of amplification are indicated by arrows with the primer's name on the top and the first position for amplification is represented by the number under the arrow.

2. Cloning and sequencing

2.1 Gel purification

The RACE product and PCR products of genomic DNA were gel purified using theWizard®SV Gel and PCR Clean Up System kit (Promega, USA) and GeneJET™ Plasmid Miniprep kit (Fermentas, Germany) according to the manufacturer's instruction. The total product was loaded and run on 1% agarose gel with 1x TBE buffer. The run gel was stained with ethidium bromide and visualized under UV light. The DNA fragment of interest was excised from gel slice and transferred to a 1.5 ml microcentrifuge tube. The membrane binding solution was added at a ratio of 10 µl per 10 mg of gel slice. The mixture was vortexed and incubated at 65°C for 10 min. Dissolved gel slice was transferred to SV Minicolumn and incubated for 1 min at room temperature. The column was centrifuged at 14,000 rpm for 1 min and washed by adding 700 µl of membrane wash solution and centrifuge for 1 min. The column was washed with 500 µl of membrane wash solution and centrifuge for 5 min. The SV Minicolumn was transferred to a clean 1.5 ml microcentrifuge tube. The DNA fragment was eluted by adding 50 µl of Nuclease free water directly to the center of the column. The column and tube was incubated at room temperature for 1 min and centrifuged for 1 min.

2.2 cDNA cloning

The DNA fragment was ligated into vector using pGEM®-T Easy Vector (Promega, USA). The 10 µl ligation reaction consisted of 5 µl of 2x Rapid Ligation Buffer, 1 µl of pGEM®-T Easy Vector, 3 µl of PCR product and 1 µl of T4 DNA ligase. The reaction was mixed by pipetting and incubated overnight at 4°C. The ligation reaction was transformed into *E. coli* strain JM109 high efficiency competent cells. The tube containing the ligation reaction was centrifuged to collect contents at the bottom of the tube. Ten µl of ligation reaction was transformed to 100 µl of competent cells. The mixture was mixed and placed on ice for 20 min. Cells were heat shock for 45-50 sec in water bath at exactly 42°C and immediately returned to ice for

2 min. Nine hundred µl of SOC medium with ligation reaction was added to the tube containing transformed cells and incubated for 1.5 hrs at 37°C with shaking. The mixture was centrifuged at 1,000 x g for 10 min, resuspended in 100 µl of SOC medium, plated on LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37°C. White colonies which contained recombinant plasmids was picked up to the fresh LB ampicillin agar plates and incubated overnight at 37°C.

2.3 Determination of positive clones by colony PCR

Insert size of white colonies was determined by colony PCR. The 20 µl PCR reactions contained 1 U of Taq DNA polymerase, 1x Taq buffer, 0.2 mM of each dNTP, 0.5 µM of each primer and 1.25 mM of $MgCl_2$. White colonies were scraped by the micropipette tip and mixed in the mixture reaction. The colony PCR was performed for 30 cycles: 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min and 30 sec. the PCR products were electrophoresed on 1.5% agarose gel in 1x TBE buffer at 100 volts. A DNA ladder 100 bp was used as a DNA marker. The run gel was stained with ethidium bromide and visualized with UV light.

2.4 Preparation of plasmid DNA

Positive clones were selected for extraction of the plasmid using GeneJET™ Plasmid Miniprep kit (Fermentas, Germany) according to the manufacturer's instruction. Colonies were cultured in 3 ml of LB ampicillin broth and incubated overnight at 37°C with shaking. The plasmid containing bacterial cells were centrifuged at 12,000 x g for 1 min at room temperature. The cell pellets were re-suspended in 250 µl of re-suspension solution. Two hundred and fifty µl of lysis solution were added and the reaction was mixed by inverting the tube 6-8 times. The solution was mixed with 350 µl of neutralization solution by inverting the tube 6-8 times. The neutralized lysate was centrifuges for 5 min. The supernatant was removed to the plasmid mini column by pipetting. The column was centrifuged for 1 min and the flow through was discarded. The column was washed by adding 750 µl of wash solution and centrifuged for 1 min and the flow through was discarded. The column

was centrifuged for 1 additional min to remove residual wash solution. The plasmid mini column was transferred to a 1.5 ml microcentrifuge tube. Fifty µl of elution solution were added to the column to elute plasmid. The column was let stand for 1 min and centrifuged for 1 min.

2.5 Restriction enzyme digestion

After plasmid preparation, restriction enzyme digestion was used to confirm the size of DNA inserts. The *Eco*RI enzyme was used to cut the inserts cDNA. The reaction was performed in 10 µl reaction containing 2 µl of DNA plasmid, 0.7 µl of EcoRI enzyme, 0.7 µl of BSA and 1 µl of *Eco*RI buffer and incubated overnight at 37°C. Four µl of 6x loading dye solution were added to the reaction and loaded in 1.5% agarose gel and run in 1x TBE buffer at 100 volts. The gel was stained with ethidium bromide and visualized under ultraviolet light. A DNA ladder 100 bp was used as a DNA marker.

3. Sequencing data analysis

Three µl of the extracted plasmid DNA was sequenced using Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The M13 forward and reverse primers were used for sequencing the 5′ and 3′ ends of the cDNA. After sequencing, the vector sequences were removed using the computer program Genetyx version 7.0. The nucleotide and deduced amino acid sequences were searched for homology using the BLAST programs (NCBI, http://www.ncbi.nlm.nih.gov) (Altschul *et al*., 1997). BLASTn program was used to search for nucleotide sequences homology and BLASTx program was used to search for translated amino acids homology.

4. Phylogenetic Analysis

The 5′ and 3′ UTR sequences of the full length of *HSC70-1* and *HSC70-2* cDNA were removed using Genetyx version 7.0. Only the open reading frames of genes were analyzed. Computer program Clustal W (Thompson *et al*., 1994) was used for multiple sequence alignment to determine the longest possible consensus sequence, protein alignment and alignment consensus sequence. Sequence alignment was carried out on *HSC70* from other fishes and vertebrates. The *HSC70* rooted phylogenetic tree was constructed for the amino acid sequences by neighbor joining method (Saitou and Nei, 1987) and bootstrapped for 1000 replicates using computer program MEGA version 3.1 (Kumar *et al*., 2004).

Part II

Seasonal expression of *HSC70* and *HSC90* genes and gonad development under pond culture

1. Experimental fish

1.1 Tissue collection

Fish used in this study were produced by hormone injection and artificial fertilization of 120 females (weight 210±26 g) and 30 males (weight 202±12 g) in August 2007 and were obtained from the hatchery of the Pathumthani Inland Fisheries Research and Development Center, Pathumthani Province. Larvae were reared in concrete tanks (2×3×1 m) at stocking density of 3,000/m^2 for two months (length = 5-7±1.6 cm). Thereafter, juveniles were stocked in 800 m^3 earthen ponds and reared until six month of age. They were fed commercial catfish pellet with 25% protein (2% body weight) twice daily at 0700 and 1600. Water quality was measured every month throughout the experiment. Fifteen females were collected from the earthen pond monthly from April 2008 (age = 8 months) to March 2009, measured and weighed. Total length and body weight of each fish were measured. Ovary tissues were measured for calculating the gonadosomatic index, GSI (total gonadal weight x 100/ total fish weight). Gills, liver, brain and muscle tissues were placed immediately in TRIzol reagent (Molecular Research Center) for extraction of total RNA.

Ovarian tissues were fixed in Bouin's solution for 24 hr, washed in 79% ethanol, and dehydrated using the automated tissue processor. The tissues were embedded in paraffin, sectioned to 5-10 μm, mounted on slides and stained with Haematoxylin and eosin. At least three hundred oocytes from the anterior, middle and posterior parts of the ovary of a female were examined using light microscope. Oocytes were classified into six stages of maturation according to Groman (1982) as follows: stage I: oocytes are undifferentiated and occur in nests with large nuclei and acidophilic cytoplasm; stage II: oocytes increase in size, each contains a large nucleus

with basophilic chromatin; stage III: oocytes show a well defined follicular epithelium and basophilic cytoplasm; stage IV: oocytes are characterized by the appearance of euvitellin nucleoli along the nuclear membrane, the presence of yolk granules in the cytoplasm, and a more distinct zona radiata in the cell membrane; stage V: oocytes are larger with abundant yolk vesicles, showing fewer euvitellin nucleoli in the nucleus and a degenerating nuclear membrane; stage VI: oocytes are fully grown, containing large amount of acidophilic yolk granules in the cytoplasm.

1.2 Data analysis

Data of GSI were presented as means ± S.E. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests for comparisons of means by month. For all statistical tests, significance was set at $P < 0.05$.

2. Tissue expression by RT-PCR

2.1 Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from gill, liver, brain and muscle tissues of five bighead catfish (150±5.3 g in weight) using TRIzol reagent (Molecular Research Center) according to the manufacturer's instructions. The quantity and quality of total RNA was calculated using the absorbance at 260 nm and electrophoresed on 1% formaldehyde-agarose gel. Approximately 1 μg of total RNA was used for first strand cDNA synthesis using iScript™ Select cDNA Synthesis kit (BIO-RAD) according to the manufacturer's instructions.

2.2 Design of primers

The gene specific primers were designed from the EST partial sequences of *HSC70* and *HSC90* (Panprommin *et al.,* 2007) (Table 4).

Table 4 Nucleotide sequences and positions of primers used in semi-quantitative RT-PCR for *HSC70* and *HSC90* expression.

Name	Primer sequence (5′ to 3′)	*HSC70*, *HSC90*	
		Position	bp
RTHSC70F	CTGTAGAGGATGAGAAGCTA	1688-1707	281
RTHSC70R	GATCTTCTGGTCCCACCATC	1949-1968	
RTHSC90F	CACCATGGGTTACATGATGG		567
RTHSC90R	AGGCTGGTGTATGTTCAGTG		
Ipβ-actin-F	AGAGAGAAATTGTCCGTGACATC		414
Ipβ-actin-R	CTCCGATCCAGACAGAGTATTTG		

[a] Nucleotide positions are based on *C.macrocephalus* sequence submitted to GenBank (Accession number JX112294 for *HSC70-1* and accession number JX12295 for *HSP70-2*). [b] Primers for β-actin are based on GenBank sequence (Accession number AY555575)

2.3 RT-PCR

First strand cDNA from each tissue was used as template. PCR amplifications were performed using primers listed in Table 3. Primers for the β-actin gene was based on information obtained from channel catfish *Ictalurus punctatus* (AY555575) (Kocabas *et al*., 2002). PCR was carried out in a 25 μl reaction mixture containing 1x *Taq* buffer, 0.75 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each forward and reverse primer, 1 U *Taq* DNA polymerase (Fermentas) and 0.5 μl of first strand cDNA. PCR amplification was carried out under the following conditions: pre-denaturation at 96°C for 3 min, 26 cycles consisted of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min. The last extension at 72°C was extended for 5 min. The level of expression of β-actin was used as an internal control. Ten μl of each PCR product was subjected to electrophoresed on

1.5% agarose gel in 1x TBE buffer at 100 volts. A DNA ladder 100 bp was used as a DNA marker. The gel was stained with ethidium bromide (0.5 mg/ml).

3. Quantification of *HSC70* and *HSC90* mRNA in gills, liver and brain tissue by semi-quantitative RT-PCR

3.1 Semi-quantitative RT-PCR analysis

Total RNA was extracted from muscle, gill, liver and brain tissue by using the TRIzol reagent (Molecular Research Center) according to the manufacturer's instructions. First strand cDNA synthesis was carried out using RevertAid™ First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. Approximately 1μg of total RNA was incubated with 1 μl of oligo$(dT)_{18}$ at 70°C for 5 min. Four μl of 5x reaction buffer, 1 μl of RiboLock™ Ribonuclease inhibitor and 2 μl of 10 mM dNTP mix was added and the reaction was incubated at 37°C for 5 min. One micro-liter of RevertAid™ M-MuLV Reverse Transcriptase was added and the mixture was incubated at 42°C for 60 min followed by 70°C for 10 min.

PCR reactions were performed using different sets of gene-specific primers as shown in Table 1. Primers were designed from EST sequences of these genes obtained from the liver cDNA library of *C. macrocephalus* (Panprommin *et al.*, 2007). Primers for *β*-actin gene was obtained from channel catfish (*Ictalurus punctatus*). PCR amplification was carried out in a 25 μl reaction volume containing 0.5 μl of cDNA first strand template, 0.2 mM of each dNTPs, 1x *Taq* buffer, 1 U of *Taq* DNA polymerase, 1 μM of forward primer, and 1 μM of reverse primer. PCR profiles were as follows: denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. The optimal cycle numbers were determined by performing PCR reactions in triplicates between 22 and 35 cycles to generate a product within the exponential range.

The PCR products were separated and visualized on 1.5% agarose gel containing ethidium bromide. The density of bands was measured by FluorChem™ 8000 Advanced Fluorescence, Chemiluminescence and Visible Light Imaging (Alpha Innotech Corporation) which corresponds to pixel density values (Integrated Density Value, or IDV). The pixel density value of each product was used to calculate ratio of the selected gene expression relative to β-actin expression. The expression of β-actin was normalized to 100. Differential expression of the selected genes between different sample groups was evaluated according to the pixel density values.

3.2 Data analysis

Data of ratio of the target gene: β-actin was presented as means ± S.E. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS 11.0, followed by Duncan's multiple range tests for comparisons of means by stage of development. For all statistical tests, significance was set at $P < 0.05$.

Part III

Oocyte development and expression of *HSC70* and *HSP 90* genes in response to elevated temperature

1. Experimental fish and experimental design

Three hundred female fish (age = 13 months; weight = 108±12.7 g) were collected from the earthen pond during October 2008 and transferred to 5x10x1m concrete tanks seven days prior to the experiment. One hundred and eighty fish were stocked randomly over fifteen 50-l aquaria (length, 90 cm; width, 45 cm; depth, 45 cm.) at a density of twelve fish per aquarium. The fish were divided into three groups. Fish in the first group were maintained at an ambient temperature of 25°C and served as a control. Fish in the second and third groups were raised in the aquaria containing aerated water heated by immersion heater to 30°C and 35°C respectively. The experimental fish were fed commercial pellets containing 25% protein at 2% body weight twice daily and maintained for six weeks. Dissolved oxygen and pH were measured daily. Blood samples and ovaries were collected from fifteen individuals each of control and test groups after two, four and six weeks of rearing.

Based on information from the preliminary study, the range of temperature and duration of exposure were non-lethal for this species under hatchery conditions. Oocyte development and GSI was determined as described in experiment 2.

2. Plasma analyses: cortisol and glucose

2.1 Blood samples

Before tissue and blood collection, fish were anesthetized with 2-phenoxyethanol (0.75 mg/l). Blood samples were taken from the caudal vein using 1-ml syringes coated with Na_2-EDTA (Titriplex III, Merck, Darmstadt, Germany), placed into 1.5 ml microcentrifuge tubes, and centrifuged at 1,500 rpm at 4°C for 10

min. Plasma was stored at -20°C until use. Cortisol levels were quantified by radioimmunoassay (RIA) using a commercial kit (Cortisol Bridge kit, TKCO1, L&R Enterprise Co. Ltd, Thailand). Plasma glucose was measured by colorimetric test using a commercial kit (no. RA 122-10: End point, BIOTECH, Bangkok, Thailand).

2.2 Data analysis

Data for plasma cortisol and glucose were presented as means ± S.E. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests for comparisons of means by month. For all statistical tests, significance was set at $P < 0.05$.

3. Semi-quantitative RT-PCR

As described in experiment 2.

Part IV

Expression of two *HSC70* genes in response to *Aeromonas hydrophila* infection

1. Bacterial infection

One hundred four-month old healthy fish (body weight = 29.7±6.4 g) were collected from the earthen pond at the Department of Aquaculture, Kasetsart University, and transferred to a 6×4 m^2 concrete tanks seven days prior to the experiment. The fish were stocked randomly over two 250 L fiber glass tank 26-28 °C at fifty individuals each. Fish in the first group were injected intraperitoneally with 0.1 ml of 0.85% NaCl and served as a control. Fish in the second group were injected with 0.1 ml of 10^9 CFU/ml of *A. hydrophila* by the same route as control. The experimental fish were fed commercial pellets containing 25% protein at 5% body weight twice daily. Tissue samples, including brain, gills, liver and skeletal muscle, were randomly collected from injected and control fish at 6, 12, 24 and 48 h post-injection, for total RNA extraction.

2. Tissue analysis quantification of *HSC70s* mRNA by semi-quantitative RT-PCR

Total RNA was extracted from muscle, gills, liver and brain tissues using the TRIzol reagent (Molecular Research Center) according to the manufacturer's instructions. First strand cDNA synthesis was carried out using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. Approximately 1μg of total RNA was incubated with 1 μl of oligo$(dT)_{18}$ at 70°C for 5 min. Four μl of 5x reaction buffer, 1 μl of RiboLock™ Ribonuclease inhibitor and 2 μl of 10 mM dNTP mix was added and the reaction was incubated at 37°C for 5 min. One micro-liter of RevertAid™ M-MuLV Reverse Transcriptase was added and the mixture was incubated at 42°C for 60 min followed by 70°C for 10 min.

Primers were designed from full-length cDNA of *HSC70-1* and *HSC70-2* obtained in part I. The *β*-actin gene was used as internal control.

Table 5 Nucleotide sequences and positions of primers used in semi-quantitative RT-PCR for *HSC70-1* and *HSC70-2* expression.

Name	Primer sequence (5′ to 3′)	*HSC70*	
		Position	bp
RTHSC70-1F	GCTATTGCCTATGGGTTGGAC	571-591	945
RTHSC70-1R	GTCTCTGCTGTTGACAAGAGC	1495-1515	
RTHSC70-2F	GCTATTGCCTATGGGTTGGAC	571-591	522
RTHSC70-2R	CAGAAACTTCTGCAGGACTTC	1072-1092	
Ipβ-actin-F	AGAGAGAAATTGTCCGTGACATC		414
Ipβ-actin-R	CTCCGATCCAGACAGAGTATTTG		

[a] Nucleotide positions are based on *C. macrocephalus* sequence submitted to GenBank (Accession number JX112294 for *HSC70-1* and accession number JX12295 for *HSP70-2*). [b] Primers for β-actin are based on GenBank sequence (Accession number AY555575)

PCR reactions were performed using different sets of gene-specific primers as shown in Table 5. PCR amplification was carried out in a 25-μl reaction volume containing 0.5 μl of cDNA first-strand template, 0.2 mM of each dNTPs, 1x *Taq* buffer, 1 U of *Taq* DNA polymerase, 1 μM of forward primer, and 1 μM of reverse primer. PCR profiles were as follows: denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min. PCR profiles for 26 cycles were as follows: denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min. The level of expression of β-actin was used as an internal control.

The PCR products were separated and visualized on a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml). The density of bands was measured by FluorChem™ 8000 Advanced Fluorescence, Chemiluminescence and Visible Light Imaging (Alpha Innotech Corporation) which corresponded to pixel density values (Integrated Density Value, or IDV). Expression of the *HSC70-1* and *HSC70-2* genes was measured as pixel density values. The pixel density value of each product was used to calculate the ratio of the selected gene's expression relative to *β*-actin expression. The expression of *β*-actin was normalized to 100. Expression of the *HSC70s* genes was measured as pixel density values.

3. Statistical analysis

Data on relative expression levels of the two *HSC70* genes at four time points were presented as means ± S.E. for each tissue, where $n = 20$ fish. Statistical differences in gene expression levels between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS 11.0, followed by Duncan's multiple range tests for comparisons of means in the same gene and tissue across four different time points. A student's *t*-test was used to compare means between groups of *HSC70* genes at the same time point. For all statistical tests, significance was set at $P < 0.05$.

RESULTS

Part I

Molecular Characterization of *HSC70* gene

1. Cloning of two full-length *HSC70* cDNAs

1.1 *HSC70-1*

The full-length cDNA of *C. macrocephalus HSC70-1* was shown in Figure 11 and 12 together with the deduced amino acid sequence. The 2,278 bp sequence contained 33 bases of 5′-untranslated region, the open reading frame of 1,950 bp encoding a 649 amino acid protein, 295 bases of 3′-untranslated region and a poly(A) tail of 33 nucleotides. The 3′-untranslated region included the termination signal (TAA) and a conserved polyadenylation signal (AATAAA) located 19 bases before the poly(A) tail (Fig. 12). Molecular mass determined from the deduced amino acid sequence was approximately 71.24 kDa and was submitted in GenBank database with accession number of JX112294.

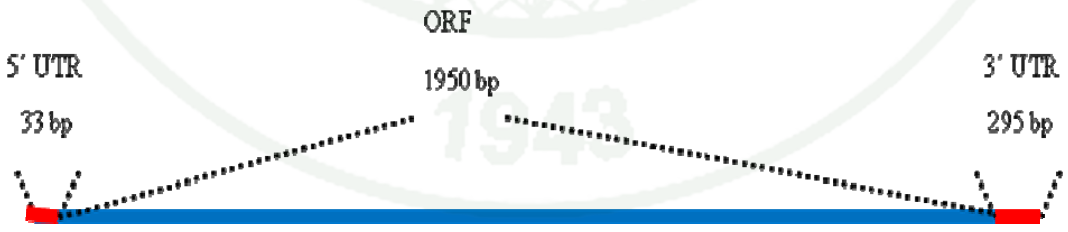

Figure 11 Schematic structure of cDNA of *C. macrocephalus HSC70-1* gene.

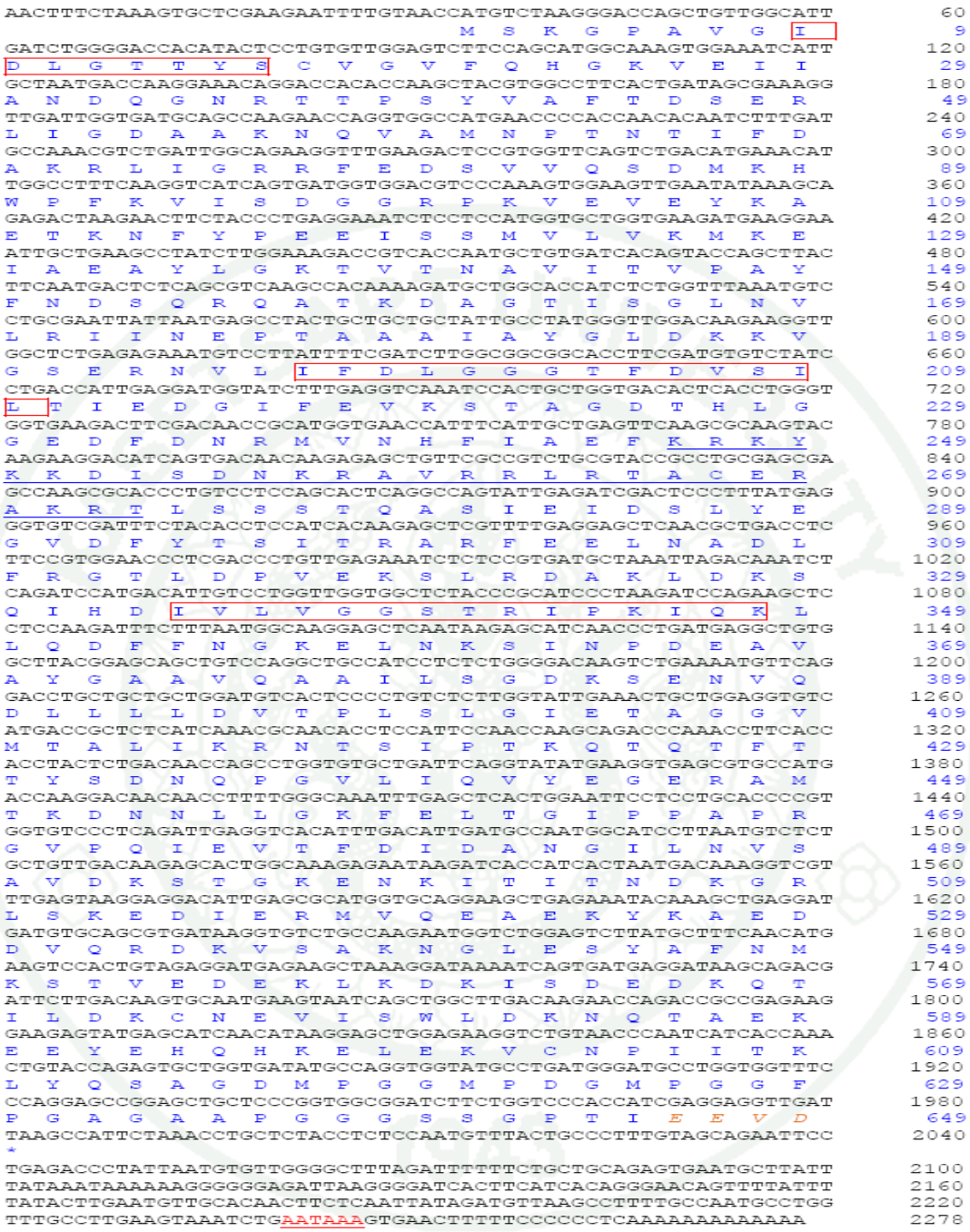

Figure 12 Nucleotide and deduced amino acid sequence of *C. macrocephalus HSC70-1*. Three signatures of *HSP70* family are shown in boxes, a putative nuclear localization signal is underlined, and consensus sequence EEVD at the C-terminus is indicated in italics. The numbers on the right are positions of the nucleotides and amino acid. The polyadenylation signal (AATAAA) is underlined.

1.2 *HSC70-2*

The 2,278 bp of complete cDNA sequence for bighead catfish *HSC70-2* contained 33 bases of 5′-untranslated region, the open reading frame of 1,950 bp, 295 bases of 3′-untranslated region (Fig. 13) and a poly(A) tail of 13 nucleotides. The 3′-untranslated region included the termination signal (TAA) and a conserved polyadenylation signal (AATAAA) located 19 bases before the poly(A) tail. The cDNA sequence is shown in Figure 14, together with the deduced sequence of 649 amino acids with a predicted molecular mass of 71.27 kDa and was submitted in GenBank database with accession number of JX112295.

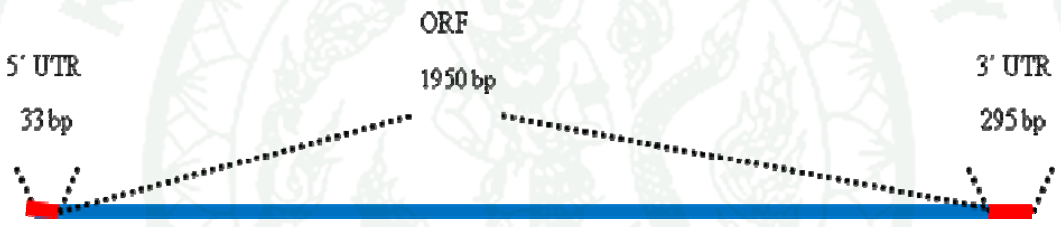

Figure 13 Schematic structure of cDNA of *C. macrocephalus HSC70-2* gene.

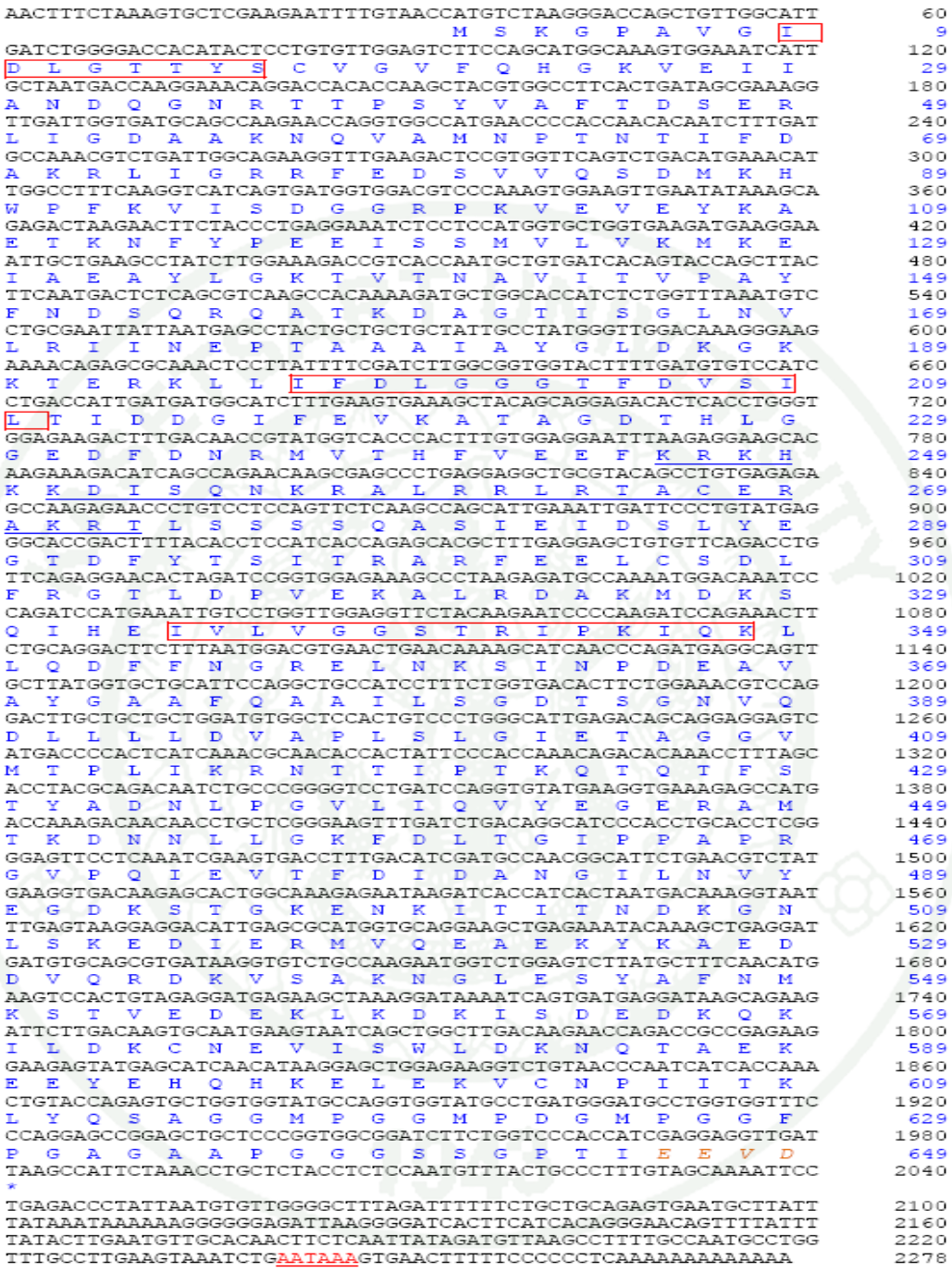

Figure 14 Nucleotide and deduced amino acid sequence of *C. macrocephalus HSC70-2*. Three signatures of *HSP70* family are shown in boxes, a putative nuclear localization signal is underlined and consensus sequence EEVD at the C-terminus is indicated in italics. The numbers on the right are positions of the nucleotides and amino acid. The polyadenylation signal (AATAAA) is underlined.

1.3 Analysis of amino acid sequences

Amino acid sequence analysis identified three signature sequences of the *HSP70* family: IDLGTTYS in the N-terminal domain, IFDLGGGTFDVSIL, and IVLVGGSTRIPKIQK in the middle domain, and the EEVD cytoplasmic motif at the C-terminus for both *HSC70-1* and *HSC70-2*. One repeat of the tetrapeptide motif (GGMP) was present in the C-terminal region of *HSC70-1*, while *HSC70-2* contained two GGMP motif repeats. *HSC70-1* and *HSC70-2* amino acid sequences were 96% similar (Table 6) and 94% identical (Table 7), with 38 substitutions between the two predicted proteins (Fig. 15).

Figure 15 Alignment of amino acid sequences of *C. macrocephalus HSC70-1* and *HSC70-2*. Identical amino acids are marked with asterisk (*), highly related amino acids with a colon (:), and related amino acids with single dot (.). Numbers refer to the amino acid sequence.

2. Genomic DNA structure of *HSC70-1* and *HSC70-2*

2.1 *HSC70-1*

Band doubles at 1,000 bp were observed on the agarose gel, therefore, two bands were cloned separately. Initially, primers (*HSC70F1-R1*) were designed based on information of a 371 bp EST clone (EB360505) of *C. macrocephalus* which contained the 3′ UTR. These primers were used to clone a fragment of 1,048 bp. Subsequently, primers *HSC70F2-1R2*, and *HSP70F3-1R3* were designed and were used to clone a 1,351 bp and a 1,165 bp fragment respectively. Finally, the 5′end was cloned using a reverse primer *HSC70-1R4*. After removal of overlapping nucleotide sequences, a genomic sequence of *HSC70-1* (3,179 bp) was obtained. The coding region consisted of seven introns (901 bp) which including 144, 85, 89, 198, 119, 122, and 144 bp respectively, and eight exons (1,950 bp) which including 205, 206, 153, 556, 203, 199, 233, and 195 bp respectively. The genomic sequence of walking catfish *HSC70-1* gene was deposited in GenBank under accession number JX273642 (Fig. 16).

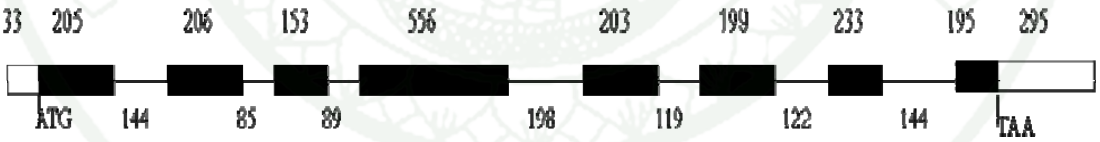

Figure 16 Schematic structure of full-length genomic DNA of *C. macrocephalus* *HSC70-1* gene. Black boxes represent exons, white boxes are untranslated regions, and lines represent introns. The sizes (base pairs) of each exon and intron are indicated. The start codon (ATG) and stop codon (TAA) are also indicated.

2.2 *HSC70-2*

The same set of primers (*HSC70F1-R1, HSC70F2-2R2*, and *HSC70F3-2R3*) was used to clone three genomic fragments (921 bp, 1,094 bp, and 1,110 bp respectively) of *HSC70-2*. Finally, the 5′end was cloned using a reverse primer *HSC70-2R4*. After removal of overlapping nucleotide sequences, a genomic sequence of *HSC70-2* (2,980 bp) was obtained. The coding region consisted of seven introns (702 bp) which including 144, 85, 22, 64, 117, 126, and 144 bp and eight exons (1,950 bp) including 205, 206, 172, 539, 201, 213, 219, and 195 bp respectively. The genomic sequence of bighead catfish *HSC70-2* gene was deposited in GenBank under accession number JX273643 (Fig. 17).

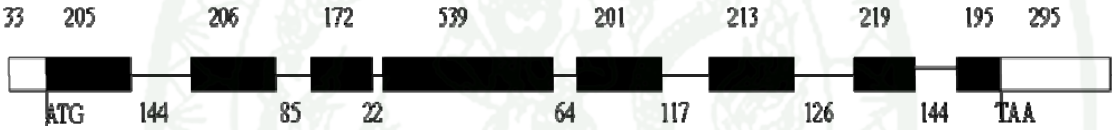

Figure 17 Schematic structure full-length of genomic DNA of *C. macrocephalus HSC70-2* gene. Black boxes represent exons, white boxes are untranslated regions, and lines represent introns. The sizes (base pairs) of each exon and intron are indicated. The start codon (ATG) and stop codon (TAA) are also indicated.

The comparisons genomic structure revealed that the exon and intron sizes of the two genes were different for exons 3, 4, 5, 6 and 7 and for introns 3, 4, 5 and 6 (Fig. 18).

Figure 18 Alignment genomic sequences of walking *C. macrocephalus HSC70-1* and *HSP70-2*. Identical sequences are marked with asterisk (*), highly related sequences with a colon (:), and related sequences with single dot (.). Numbers refer to the nucleotide sequence. The 5′ and 3′ UTRs are shown in boxes, the start codon (ATG) and stop codon (TAA) are underlined and the highlighted sequences are exons.

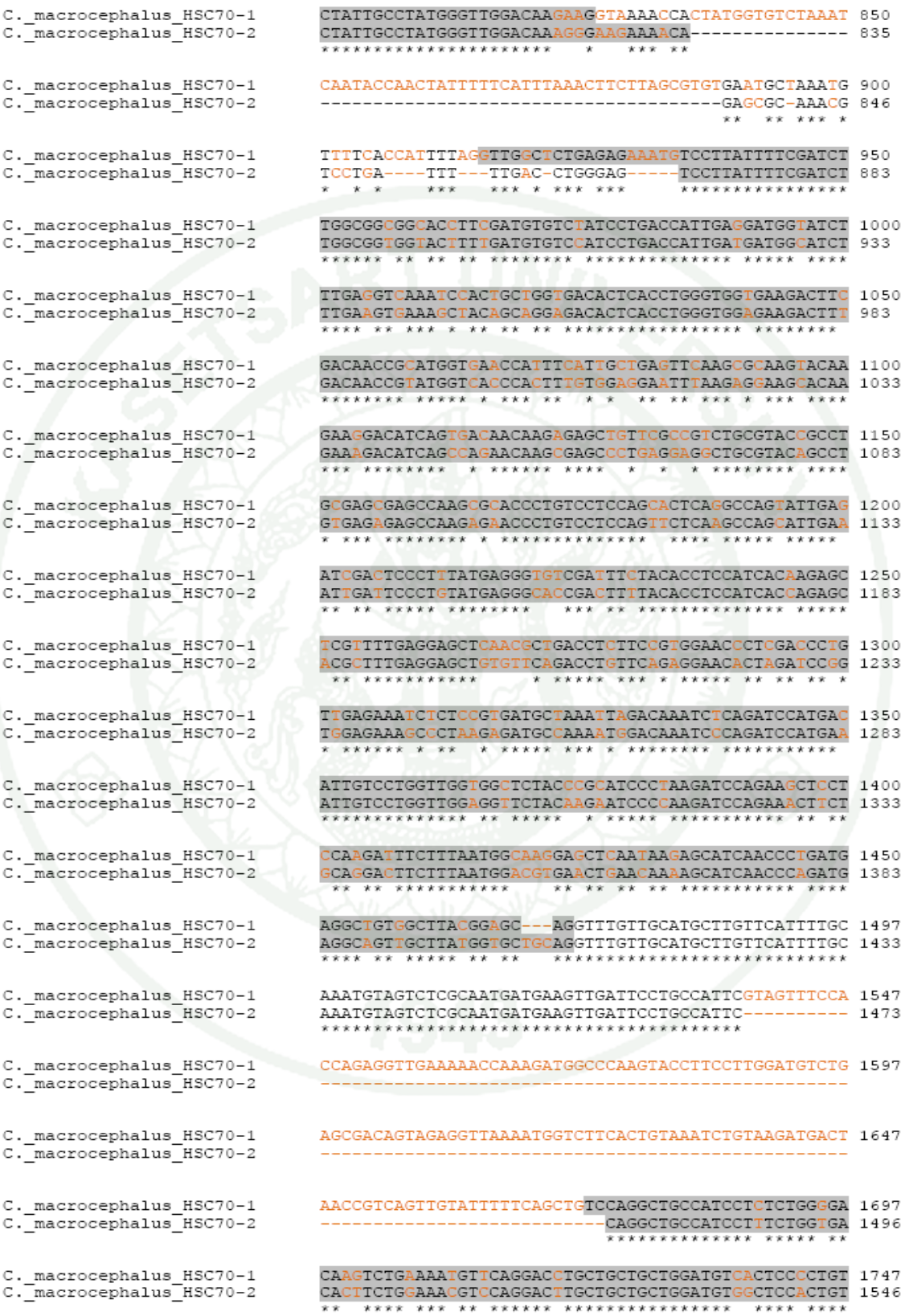

Figure 18 (Continued)

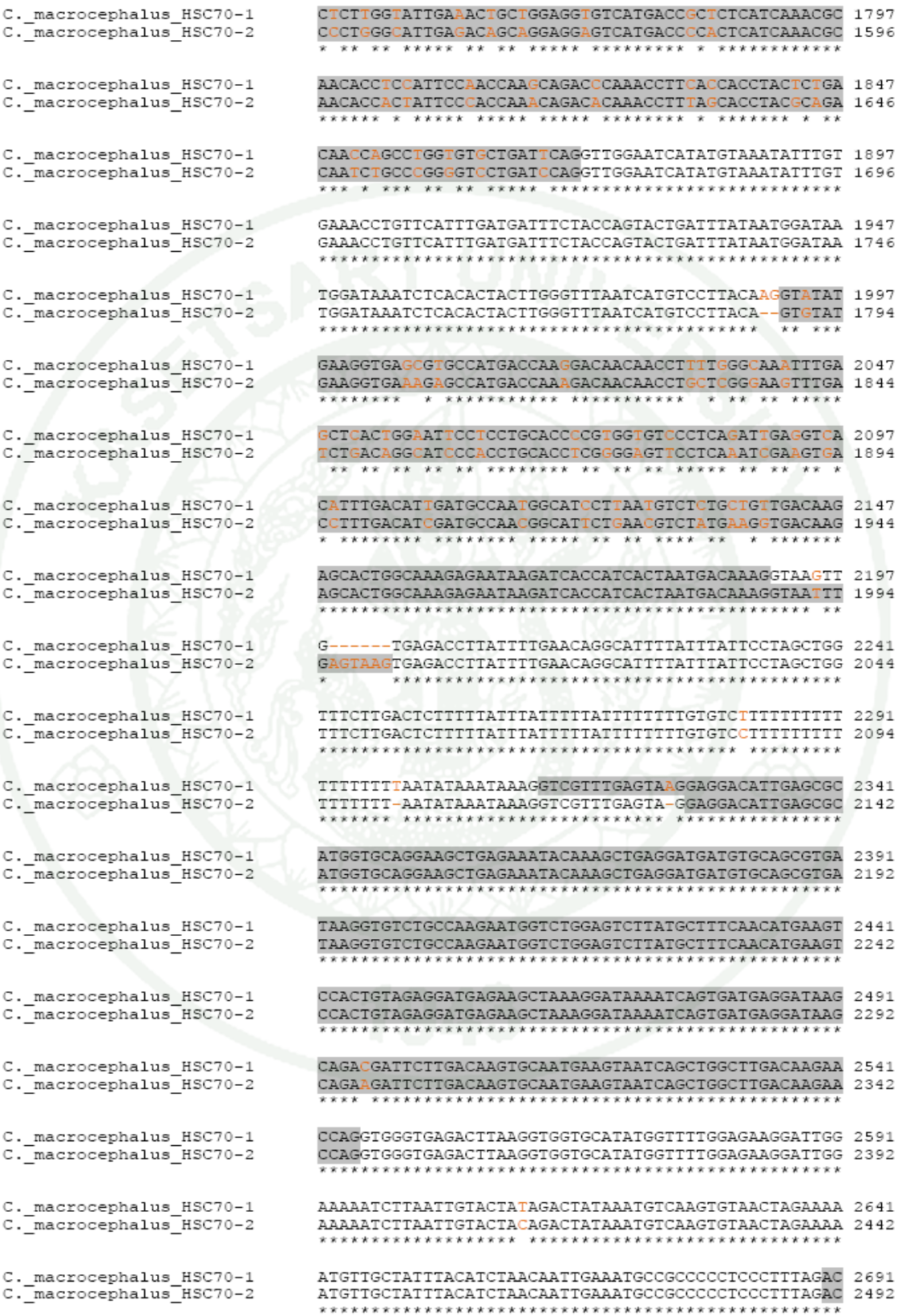

Figure 18 (Continued)

Figure 18 (Continued)

3. Phylogenetic analysis

Alignment of the amino acid sequences of *C. macrocephalus HSC70-1* and *HSC70-2* with those of *HSC70*, *HSC71* and *HSP70* from other species (Fig. 19) revealed that the three signature sequences of heat shock protein 70 family and the EEVD motif were highly conserved. The two *HSC70* proteins of bighead catfish contained 649 amino acids, similar to those of zebra fish *HSC70*, channel catfish *HSC71* and Wuchang bream *HSC70*. The *HSC70* amino acid sequences of all fish

species varied from 612 to 650 residues, whereas, clawed frog, chicken and human *HSC70* contained 646 amino acids. The amino acid sequences of bighead catfish *HSC70-1* and *HSC70-2* shared 82-95% and 82-91% identity of amino acids with other teleosts, chicken and human. (Table 7)

The organism name and GenBank accession number are as follows: *HSP70*_Salmo, *Salmo salar*, ACI34374.1; *HSP70*_Acanthopagrus, *Acanthopagrus schlegelii*, AAX07834.1; *HSP70*_Megalobrama, *Megalobrama amblycephala*, ACG63706.; *HSP70*_Carassius, *Carassius auratus*, BAC67184.2; *HSP70*_Fundulus, *Fundulus heteroclitus*, ABB17041.1; *HSP70*_Paralichthys, *Paralichthys olivaceus*, ABG56390.1; *HSC70-1*_Clarias, *Clarias macrocephalus,* HM044876; *HSC70-2*_Clarias, *Clarias macrocephalus,* JN258960; *HSC71*_ Ictalurus, *Ictalurus punctatus*, P47773.1; *HSC70*_Fundulus, *Fundulus heteroclitus*, DQ202278; *HSC70*_Acanthopagrus, *Acanthopagrus schlegelii*, AAX07833.1; *HSC71*_Paralichthys, *Paralichthys olivaceus*, AB006814.1; *HSC70*_Pimephales, *Pimephales promelas*, AAS46619.1; *HSC70*_Megalobrama, *Megalobrama amblycephala*, EU623471.2; *HSC70*_Danio, *Danio rerio*, CAA72216.1; *HSC70*_Cyprinus, *Cyprinus carpio*, AAP51388.1; *HSC70*_Carassius, *Carassius gibelio*, AAO43731.1; *HSC70*_Gallus, *Gallus gallus*, NP_990334.1; *HSC70*_Homo, *Homo sapiens*, NP_006588.1; *HSC70*_Xenopus, *Xenopus laevis*, AAH41201.1; *HSP70*_Xenopus, *Xenopus laevis*, NP_001079632.1; *HSC70*_Salmo, *Salmo salar*, BT059361.1; *HSP70*_Gallus, *Gallus gallus*, AAN18282.1; *HSP70*_Homo, *Homo sapiens*, NP_005518.3

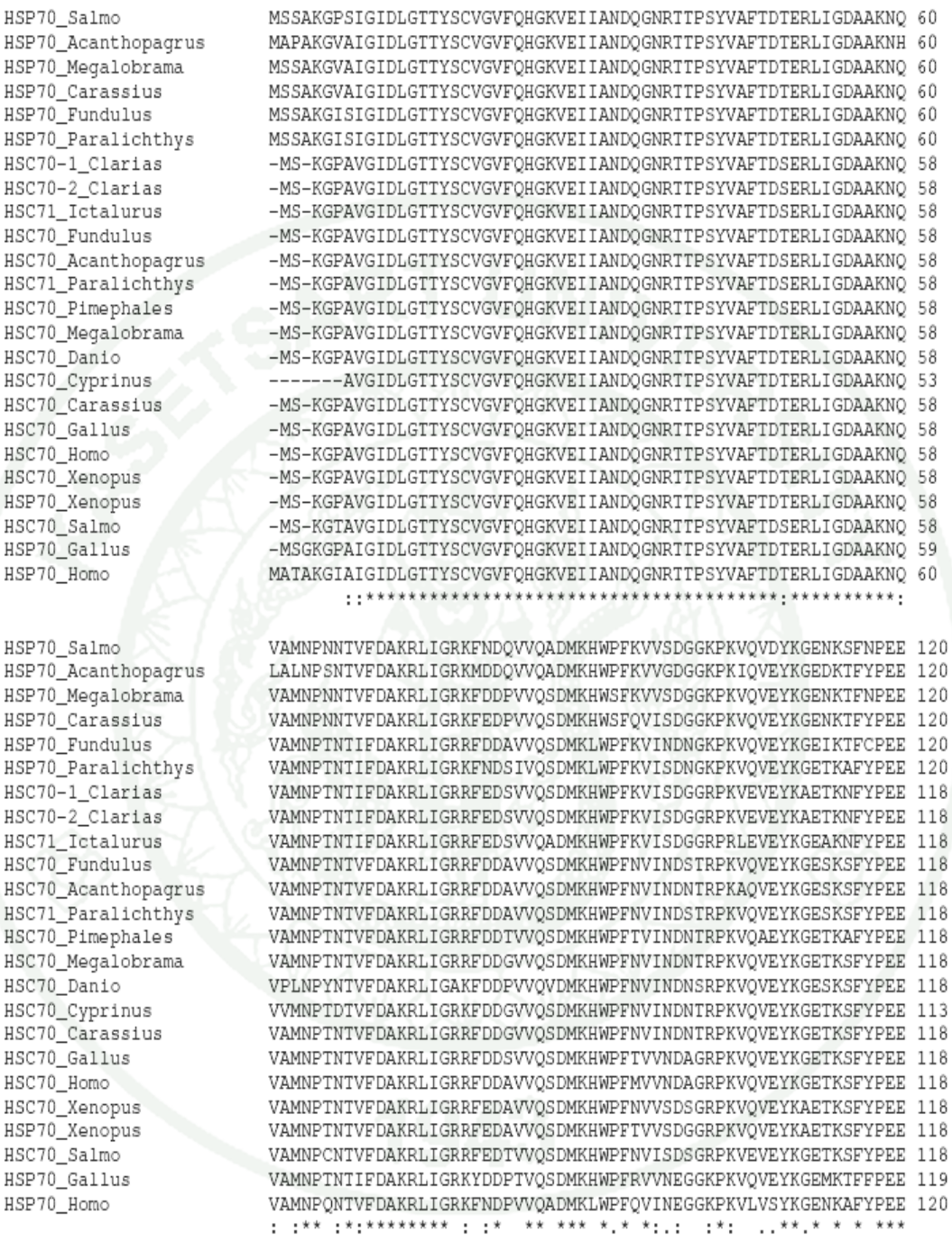

Figure 19 Alignment of amino acid sequences of *C. macrocephalus HSC70-1* and *HSP70-2* and other fishes and vertebrates. Identical amino acids are marked with asterisk (*), highly related amino acids with a colon (:), and related amino acids with single dot (.). Numbers refer to the amino acid sequence.

```
HSP70_Salmo             ISSMVLVKMREIAEAYLGQKVSNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 180
HSP70_Acanthopagrus     ISSMVLVKMKEIAEAYLGQQVSNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 180
HSP70_Megalobrama       ISSMVLVKMKEIAEAYLGQKVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 180
HSP70_Carassius         ISSMVLVKMKEIAEAYLGQKVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 180
HSP70_Fundulus          ISSMVLVKMREIAEAFLGQSVSNAVITVPAYFNDSQRQATKDAGVISGINVLRIINEPTA 180
HSP70_Paralichthys      ISSMVLVKMKEIAEAYLGQKVSNAVITVPAYFNDSQRQATKDAGVISGLNILRIINEPTA 180
HSC70-1_Clarias         ISSMVLVKMKEIAEAYLGKTVTNAVITVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSC70-2_Clarias         ISSMVLVKMKEIAEAYLGKTVTNAVITVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSC71_Ictalurus         ISSMVLVKMKEIAEAYLGKSINNAVITVPAYFNDSQRQRTKDAGTISGLNVLRIINEPTA 178
HSC70_Fundulus          ISSMVLTKMKEIAEAYLGKTVNNAVITVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSC70_Acanthopagrus     VSSTVLTKMKEIAEAYLGKTVNNAVITVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSC71_Paralichthys      ISSMVLTKMKEIAEAYLGKTVNNAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSC70_Pimephales        ISSMVLTKMKEIAEAYLGKTITNAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSC70_Megalobrama       ISSMVLTKMKEIAEAYLGKTVSNAVITVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSC70_Danio             ISSMVLTKMKEIAEAYLGKTVSNAVITVPAYFNDSQRQATKDAGVLAGLNVLRIINEPTA 178
HSC70_Cyprinus          ISSMVLTKMKEIAEAYLGKTVSNAVVTVPAYFNDSQRQATKDAGTISGLNVLGIINEPTA 173
HSC70_Carassius         ISSMVLTKMKEIAEAYLGKTVSNAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSC70_Gallus            ISSMVLTKMKEIAEAYLGKTVTNAVVTVPAYFNDSQRQGTKDAGTIAGLNVLRIINEPTA 178
HSC70_Homo              VSSMVLTKMKEIAEAYLGKTVTNAVVTVPAYFNDSQRQATKDAGTIAGLNVLRIINEPTA 178
HSC70_Xenopus           ISSMVLTKMKEIAEAYLGKTVTNAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSP70_Xenopus           VSSMVLTKMKEIAEAYLGKTVTNAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSC70_Salmo             ISSMVLVKMKEIAEAYLGKTVSNAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA 178
HSP70_Gallus            ISSMVLTKMKEIAEAYLGKKVQNAVITVPAYFNDSQRQATKDAGTITGLNVMRIINEPTA 179
HSP70_Homo              ISSMVLTKLKETAEAFLGHPVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 180
                        :** **.*::* ***:**: : ***:************ *****.::*:*:: *******

HSP70_Salmo             AAIAYGMDKG--MSRERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNR 238
HSP70_Acanthopagrus     AAIAYGLDKG--KTGERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNR 238
HSP70_Megalobrama       AAIAYGLDKG--KAAERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNR 238
HSP70_Carassius         AAIAYGLDKG--KASERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNR 238
HSP70_Fundulus          AAIAYGLDKG--KRGERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNR 238
HSP70_Paralichthys      AAIAYGLDKG--KRGERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNR 238
HSC70-1_Clarias         AAIAYGLDKK--VGSERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSC70-2_Clarias         AAIAYGLDKG--KKTERKLLIFDLGGGTFDVSILTIDDGIFEVKATAGDTHLGGEDFDNR 236
HSC71_Ictalurus         AAIAYGLDKK--VGSERNVLIFDLGGGTFDVSILTIEDGIFDLKSTAGDTHLGGEDFDNR 236
HSC70_Fundulus          AAIAYGLDKK--VGSERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSC70_Acanthopagrus     AAIAYGLDKK--VGSERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSC71_Paralichthys      AAIAYGLDKK--VGSERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSC70_Pimephales        AAIAYGLDKK--VGSERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSC70_Megalobrama       AAIAYGLDKK--VGAERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSC70_Danio             AAIAYGLDKK--VGAERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSC70_Cyprinus          AAIAYGLDKK--VGAERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 231
HSC70_Carassius         AAIAYGLDKK--VGAERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSC70_Gallus            AAIAYGLDKK--VGAERNLLIFDLGGGTFDVSILTIENGIFEVKSTAGDTHLGGEDFDNR 236
HSC70_Homo              AAIAYGLDKK--VGAERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSC70_Xenopus           AAIAYGLDKK--VGAERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSP70_Xenopus           AAIAYGLDKK--VGVERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSC70_Salmo             AAIAYGLDKK--VGAERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 236
HSP70_Gallus            AAIAYGLDKKGTRAGEKNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR 239
HSP70_Homo              AAIAYGLDKG--GQGERHVLIFDLGGGTFDVSILTIDDGIFEVKATAGDTHLGGEDFDNR 238
                        ******:**      *:::*******************:***::*:***************

HSP70_Salmo             LVSHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASIEIDSLFEGIDFYTS 298
HSP70_Acanthopagrus     MVNHFVEEFKRKHKKDISHNKRALRRLRTACERAKRTLSSSSQASIEIDSLFEGVDFYTS 298
HSP70_Megalobrama       MVNHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASLEIDSLYEGIDFYTS 298
HSP70_Carassius         MVNHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASIEIDSLYEGIDFYTS 298
HSP70_Fundulus          MVSHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQASIEIDSLFEGIDFYTS 298
HSP70_Paralichthys      MVSHFLEEFKRKYKKDISQNKRAVRRLRTACERAKRTLSSSTQASIEIDSLFEGIDFYTS 298
HSC70-1_Clarias         MVNHFIAEFKRKYKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGVDFYTS 296
HSC70-2_Clarias         MVTHFVEEFKRKHKKDISQNKRALRRLRTACERAKRTLSSSSQASIEIDSLYEGTDFYTS 296
HSC71_Ictalurus         MVNHFIAEFKRKHKKDISDNKRAVRRLATACERAKRTLSSSTQASIEIDSLYEGVDFYTS 296
HSC70_Fundulus          MVNHFIAEFKRKYKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGVDFYTS 296
HSC70_Acanthopagrus     MVNHFIAEFKRKYKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGVDFYTS 296
HSC71_Paralichthys      MVNHFIAEFKRKYKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGVDFYTS 296
HSC70_Pimephales        MVNHFISEFKRKYKKDISDNERAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS 296
HSC70_Megalobrama       MVNHFITEFKRKHKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS 296
HSC70_Danio             MVDHFITEFR-KHKKDISDNKRAVRRLRTACERAKPTLSSSTQASIEIDSLYEGIDFYTS 295
HSC70_Cyprinus          MVNHFITEFKRKHKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS 291
HSC70_Carassius         MVNHFITEFKRKHKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS 296
HSC70_Gallus            LVNHFIAEFKRKHKKDISENKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS 296
HSC70_Homo              MVNHFIAEFKRKHKKDISENKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS 296
HSC70_Xenopus           MVNHFVAEFKRKHKKDIIDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS 296
HSP70_Xenopus           MVNHFVAEFKRKHKKDIIDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS 296
HSC70_Salmo             MVNHFIAEFKRKYKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTS 296
HSP70_Gallus            MVNHFVEEFKRKHKRDIAGNKRAVRRLRTACERAKRTLSSSTQASIEIDSLFEGIDFYTS 299
HSP70_Homo              LVSHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQANLEIDSLYEGIDFYTS 298
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Figure 19 (Continued)

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HSP70_Salmo             ITRARFEEMCSDLFRGTLEPVEKALGDAKMDKAQIHDVVLVGGSTRIPKVQKLLQ-DFFN 357
HSP70_Acanthopagrus     ITRARFEELCSDLFRGTLEPVEKALRDAKMDKAQIHDIVLVGGSTRIPKIQKLLQ-DFFN 357
HSP70_Megalobrama       ITRARFEEMCSDLFRGTLEPVEKALRDAKMDKSQIHDIVLVGGSTRIPKIQKLLQ-DFFN 357
HSP70_Carassius         ITRARFEEMCSDLFRGTLEPVEKALRDAQMDKSQINDVVLVGGSTRIPKIQKLLQ-DFFN 357
HSP70_Fundulus          ITRARFEELNSDLFRGTLEPVEKALQDAKLDKSKIHEIVLVGGSTRIPKIQKLLQ-DFFN 357
HSP70_Paralichthys      ITRARFEELNSDLFRGTLEPVEKALQDSKMDKSKIHEIVLVGGSTRIPKIQKFLQ-DFFN 357
HSC70-1_Clarias         ITRARFEELNADLFRGTLDPVEKSLRDAKLDKSQIHDIVLVGGSTRIPKIQKLLQ-DFFN 355
HSC70-2_Clarias         ITRARFEELCSDLFRGTLDPVEKALRDAKMDKSQIHEIVLVGGSTRIPKIQKLLQ-DFFN 355
HSC71_Ictalurus         ITRARFEELNADLFRGTLDPVEKALRDAKMDKAQVHDIVLVGGSTRIPKMEKLLQ-DFFN 355
HSC70_Fundulus          ITRARFEELNADLFRGTLDPVEKSLRDAKMDKGQIHDIVLVGGSTRIPKIQKLLQ-DFFN 355
HSC70_Acanthopagrus     ITRARFEELNADLFRGTLDPVEKSLRDAKMDKGQIHDIVLVGGSTRIPKIQKLLQ-DFFN 355
HSC71_Paralichthys      ITRARFEELNADLFRGTLDPVEKSLRDAKMDKGQIHDIVLVGGSTRIPKIQKLLQ-DFFN 355
HSC70_Pimephales        ITRARFEELNADLFRGTLDPVEKSLRDAKMDKSQVHEIVLVGGSTRIPKIQKLLQ-DFFN 355
HSC70_Megalobrama       ITRARFEELNADLFRGTLDPVEKSLRDAKMDKAQIHDIVLVGGSTRIPKIQKLLQ-DYFN 355
HSC70_Danio             ITRARFEELNADLFRGTLDPVEKALRDAKMDKAQIHDIVLVGGSTRIPKIQKLLQQDYFN 355
HSC70_Cyprinus          ITRARFEELNADLFRGTLDPVEKSLRDAKMDKAQIHDIVLVGGSTRIPKIQKLLQ-DYFN 350
HSC70_Carassius         ITRARFEELNADLFRGTLDPVEKSLRDAKMDKAQIHDIVLVGGSTRIPKIQKLLQ-DYFN 355
HSC70_Gallus            ITRARFEKLNADLFRGTLDPVEKALRDAKLDKSQIHDIVLVGGSTRIPKIQKLLQ-DFFN 355
HSC70_Homo              ITRARFEELNADLFRGTLDPVEKALRDAKLDKSQIHDIVLVGGSTRIPKIQKLLQ-DFFN 355
HSC70_Xenopus           ITRARFEELNADLFRGTLDPVEKSLRDAKLDKSQIHDIVLVGGSTRIPKIQKLLQ-DFFN 355
HSP70_Xenopus           ITRARFEELNADLFRGTLDPVEKSLRDAKLDKSQIHDIVLVGGSTRIPKIQKLLQ-DFFN 355
HSC70_Salmo             ITRARFEELNADLFRGTLDPVEKSLRDAKMDKAQVHDIVLVGGSTRIPKIQKLLQ-DFFN 355
HSP70_Gallus            ITRARFEELNADLFRGTLEPVEKALRDAKLDKGQIQEIVLVGGSTRIPKIQKLLQ-DFFN 358
HSP70_Homo              ITRARFEELCADLFRGTLEPVEKALRDAKMDKAKIHDIVLVGGSTRIPKVQRLLQ-DYFN 357
                        *******:: :*******:****:* *::::*.:::::**********::::** *:**

HSP70_Salmo             GRELNKSINPDEAVAYGAAIQAAILSGDKSENVQDLLLLDVAPLSLGIETAGGVMTALIK 417
HSP70_Acanthopagrus     GRELNKSINPDEAVAYGAAVQAAILSGDTSGNVQDLLLLDVAPLSLGIETAGGVMTSLIK 417
HSP70_Megalobrama       GRDLNKSINPDEAVAYGAAVQAAILMGDTSGNVQDLLLLDVAPLSLGIETAGGVMTALIK 417
HSP70_Carassius         GRDLNKSINPDEAVAYGAAVQAAILMGDTSGNVQDLLLLDVARLSLGIETAGGVMTALIK 417
HSP70_Fundulus          GRDLNKSINPDEAVAYGAAVQAAILMGDTSENVQDLLLLDVAPLSLGIETAGGVMTPLIK 417
HSP70_Paralichthys      GRELNKSINPDEAVAYGAAVQAAILMGDTSENVQDLLLLDVAPLSLGIETAGGVMTPLIK 417
HSC70-1_Clarias         GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTALIK 415
HSC70-2_Clarias         GRELNKSINPDEAVAYGAAFQAAILSGDTSGNVQDLLLLDVAPLSLGIETAGGVMTPLIK 415
HSC71_Ictalurus         GKELNKSINPDEAVAYGAAVQAESSLGDKSENVQDLVLLDVTPLSLGIETAGGVMTVLIK 415
HSC70_Fundulus          GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 415
HSC70_Acanthopagrus     GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 415
HSC71_Paralichthys      GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 415
HSC70_Pimephales        GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTILIK 415
HSC70_Megalobrama       GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 415
HSC70_Danio             GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLYLGIETAGGVMTVLIK 415
HSC70_Cyprinus          GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 410
HSC70_Carassius         GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 415
HSC70_Gallus            GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 415
HSC70_Homo              GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 415
HSC70_Xenopus           GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 415
HSP70_Xenopus           GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 415
HSC70_Salmo             GKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLDVTPLSLGIETAGGVMTVLIK 415
HSP70_Gallus            GKELNKSINPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVTPLSLGIETAGGVMTALIK 418
HSP70_Homo              GRDLNKSINPDEAVAYGAAVQAAILMGDKSEKVQDLLLLDVAPLSLGLETAGGVMTALIK 417
                        *::*****************.**    **.* :****:****: * **:******** ***

HSP70_Salmo             RNTTIPSKQTQTFTTYSDNQPGVMIQVYEGERAMTKDNNLLGKFELSGIPPAPRGVPQIE 477
HSP70_Acanthopagrus     RNTTIPTKQTQVFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 477
HSP70_Megalobrama       RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 477
HSP70_Carassius         RNTTIPTKQTQTFTSTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 477
HSP70_Fundulus          RNTTIPTKQTQIFSTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGLPPAPRGVPQIE 477
HSP70_Paralichthys      RNTTIPTKQTQIFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELAGIPPAPRGVPQVE 477
HSC70-1_Clarias         RNTSIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSC70-2_Clarias         RNTTIPTKQTQTFSTYADNLPGVLIQVYEGERAMTKDNNLLGKFDLTGIPPAPRGVPQIE 475
HSC71_Ictalurus         RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSC70_Fundulus          RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSC70_Acanthopagrus     RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTRDNSLLGKFELTGIPPAPRGVPQIE 475
HSC71_Paralichthys      RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTRDNNLLGKFELTGIPPAPRGVPQIE 475
HSC70_Pimephales        RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSC70_Megalobrama       RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSC70_Danio             RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNSLLGKFELTGIPPAPRGVPQIE 475
HSC70_Cyprinus          RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGDVPLTKDNNLLGKFELTGIPPAPRGVPQIE 470
HSC70_Carassius         RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSC70_Gallus            RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSC70_Homo              RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSC70_Xenopus           RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSP70_Xenopus           RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSC70_Salmo             RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIE 475
HSP70_Gallus            RNTTIPTKQTQTFTTYSDNQSSVLVQVYEGERAMTKDNNLLGKFDLTGIPPAPRGVPQIE 478
HSP70_Homo              RNSTIPTKQTQIFTTYSDNQPGVLIQVYEGERAMTKDNNLLGRFDLTGIPPAPRGVPQIE 477
                        **::**:**** *:**:** ..*::*****: .:*:**.***:*:*:*:**********:*
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Figure 19 (Continued)

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HSP70_Salmo             VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQDADKYKAEDEAQREK 537
HSP70_Acanthopagrus     VTFDVDANGILNVSAVDKSTGKENKITITNDKGRLSKEEIERMVQDADRYKAEDDLQREK 537
HSP70_Megalobrama       VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEADQYKAEDDLQREK 537
HSP70_Carassius         VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKDEIERMVQEADKYKAEDDLQREK 537
HSP70_Fundulus          VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKDEIEKMVQDADKYKAEDDQQREK 537
HSP70_Paralichthys      VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKQEIEQMVQDAEKYKADDELKRDT 537
HSC70-1_Clarias         VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDDVQRDK 535
HSC70-2_Clarias         VTFDIDANGILNVYEGDKSTGKENKITITNDKGNLSKEDIERMVQEAEKYKAEDDVQRDK 535
HSC71_Ictalurus         VTFDIDANGIMNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDDVQRDK 535
HSC70_Fundulus          VTFDIDANGIMNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDDVQRDK 535
HSC70_Acanthopagrus     VTFDIDANGIMNVSAVDKSTGKENKIQITNDKGRLSKEDIERMVQEAEKYKAEDDVQRDK 535
HSC71_Paralichthys      VTFDIDANGIMNVSAADKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKTEDDVQRDK 535
HSC70_Pimephales        VTFDIDANGIMNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKSEDDVQREK 535
HSC70_Megalobrama       VTFDIDANGIMNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDDVQRDK 535
HSC70_Danio             VTFDIDANGIMNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDDVQRDK 535
HSC70_Cyprinus          VTFDIDANGIMNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKVEDDVQRDK 530
HSC70_Carassius         VTFDIDANGIMNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKSEDDVQREK 535
HSC70_Gallus            VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDEKQRDK 535
HSC70_Homo              VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDEKQRDK 535
HSC70_Xenopus           VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEADKYKAEDEKQRDK 535
HSP70_Xenopus           VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAETYKAEDEQQRDK 535
HSC70_Salmo             VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKCEDDVQRDK 535
HSP70_Gallus            VTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKDDIDRMVQEAEKYKAEDEANRDR 538
HSP70_Homo              VTFDIDANGILNVTATDKSTGKVNKITITNDKGRLSKEEIERMVLDAEKYKAEDEVQREK 537
                        ****:*****:**   ****** *** ******.***::*::** :*: ** :*: :*:

HSP70_Salmo             IAAKNSLESYAFNMKSSVEDDNMKGKISEEDKKKVVDRCDQTISWLENNQLGDKEEYEHQ 597
HSP70_Acanthopagrus     IAANNSLESYAFNMKSTAQDDNLKGKLSEEEQRKLVEK-DETISWLENNQLADKEEYQHR 596
HSP70_Megalobrama       IAAKNSLESYAFNMKNSVEDENLKGKISEDDKKKVIEKCNDTISWLENNQLADKEEYEHQ 597
HSP70_Carassius         IAAKNSLESYAFNMKNSVEDEDLKGKISEDDKKKVIEKCNEAVSWLENNQLADKEEYEHH 597
HSP70_Fundulus          VAAKNSLESYAYHMKSSVEDESLKGKISEEEKKLVVDKCNQTISWLENNQLAEKDEYEHQ 597
HSP70_Paralichthys      ISARNSLESYVYHMKSSVEDDSMKGKIQEEDKKIVIDKCNQTISWLENNQLAEKDEYEHQ 597
HSC70-1_Clarias         VSAKNGLESYAFNMKSTVEDEKLKDKISDEDKQTILDKCNEVISWLDKNQTAEKEEYEHQ 595
HSC70-2_Clarias         VSAKNGLESYAFNMKSTVEDEKLKDKISDEDKQKILDKCNEVISWLDKNQTAEKEEYEHQ 595
HSC71_Ictalurus         VSAKNGLESYAFNMKSTVEDEKLKGKISDEDKHKILDKCNEVISWLDKNQTAEKDEYEHQ 595
HSC70_Fundulus          VSAKNGLESYAFNMKSTVEDEKLAGKISDDDKRKILDKCNEVISWLDKNQTAEKDEYEHQ 595
HSC70_Acanthopagrus     VSAKNGLESYAFNMKSTVEDEKLAGKISDDDKQKILDKCNEVISWLDKNQTAERDGYEHQ 595
HSC71_Paralichthys      VSAKNGVESYAFNMKSTVEDEKLAGKISDEDKQKILDKCNEVISWLDKNQTAEKDEYEHQ 595
HSC70_Pimephales        VSAKNGLESYAFNMKSTVEDEKLAGKISEEDKQKILDKCNEVISWLDKNQTAEKEEFEHQ 595
HSC70_Megalobrama       VSAKNGLESYAFNMKSTVEDEKLKGKISDEDKQKILDKCNEVISWLDKNQTAEREEFEHQ 595
HSC70_Danio             VSAKNGLESYAFNMKSTVEDEKLKGKISDEDKQKILDKCNEVIGWLDKNQTAEREEFEHQ 595
HSC70_Cyprinus          VSAKNGLESYAFNMKSTVEDEKLKGKISDEDKQKILDKCNEVISWLDKNQTAEKEEFEHQ 590
HSC70_Carassius         VSAKNGLESYAFNMKSTVEDEKLKGKISDEDKQKILDKCNEVISWLDKNQTAEKEEFEHQ 595
HSC70_Gallus            VSSKNSLDSYAFNMKATVEDEKLPGKILDEDHQNILDKCNEIINWLDKNQTAEKEEFEHQ 595
HSC70_Homo              VSSKNSLESYAFNMKATVEDEKLQGKINDEDKQKILDKCNEIINWLDKNQTAEKEEFEHQ 595
HSC70_Xenopus           VSSKNSLESYAFNMKATVEDEKLKGKISDEDKQKILEKCNEVIAWLDKNQTAEKEEFEHQ 595
HSP70_Xenopus           VSSKNSLESYAFNMKSTVEDEKLQGKISDEDKQKILEKCNEVVSWLDKNQTAEKEEFEHQ 595
HSC70_Salmo             VSSKNSLESYSFNMKSTVEDEKLQGKISDEEKTKILDKCNEIIAWLDKNQTAEKEEYEHQ 595
HSP70_Gallus            VGAKNSLESYTYNMKQTVEDEKLKGKISDQDKQKVLDKCQEVISWLDRNQMAEKEEYEHK 598
HSP70_Homo              IAAKNALESYAFNMKSVVSDEGLKGKISESDKNKILDKCNELLSWLEVNQLAEKDEFDHK 597
                        :.:.*.::** ::**  ..*: : .*: :.::  :::: :: : **: ** .::: ::*:

HSP70_Salmo             LKELEKVCQPIITKLYQQGGMPTGC---------CGDQARTSSGASSQGPTIEEVD 644
HSP70_Acanthopagrus     QKELEKVCQPIISRLY-QGGTAAGS---------CGAQA----GAGSQGPTIEEVD 638
HSP70_Megalobrama       LKELEKVCNPIITKLY-QGGMPAGG---------CGAQARGGSGAASQGPTIEEVD 643
HSP70_Carassius         LKELEKVCNPIITKLY-QGGMPAGG---------CGAQTRGGSGPVLRGPTIEEVD 643
HSP70_Fundulus          QKELERVCKPVVTKLY--QGAAAGS---------CGS----QAGDTSQGPTIEEVD 638
HSP70_Paralichthys      QKELEKVCNPIVTKLY--QGAAPPP---------GGSSG--HAGGNVQGPTIEEVD 640
HSC70-1_Clarias         HKELEKVCNPIITKLYQSAGDMPGGMPDGMPGGFPGAGAAP--GGGSSGPTIEEVD 649
HSC70-2_Clarias         HKELEKVCNPIITKLYQSAGGMPGGMPDGMPGGFPGAGAAP--GGGSSGPTIEEVD 649
HSC71_Ictalurus         QKDLEKVCNPIITKLYQSDGGMPGGMPDGMPGGFQELGAAP--GGGSSGPTIEEVD 649
HSC70_Fundulus          QKELEKVCNPIITKLYQ---------------------------------------- 612
HSC70_Acanthopagrus     QKELEKVCNPIITKLYQSAGGMPGGMPEGMAGSFPGAGGAAP-GGGSSGPTIEEVD 650
HSC71_Paralichthys      QKELEKVCNPIITKLYQ---------------------------------------- 612
HSC70_Pimephales        QKELEKVCNPIITKLYQSAGGMPGGMPEGMPGGFPGAGGAPS-GGGSSGPTIEEVD 650
HSC70_Megalobrama       QKELEKVCNPIITKLYQ---------------------------------------- 612
HSC70_Danio             QK-LEKVCNPIITKLYQSAGACLVECPN-VPGGFPGAGAAPAGGGSSSGPTIEEVD 649
HSC70_Cyprinus          QKELEKVCNPIITKLYQSAGGMPGGMPDGMPGGFPGAGSAP--GGASSGPTIEEVD 644
HSC70_Carassius         QKELEKVCNPIITKLYQSAGGMPGGMPEGMPGGFPGAGSAP--GGGSSGPTIEEVD 649
HSC70_Gallus            QKELEKVCNPIITKLYQSAGGMPG----GMPGGFPGGGAPPS-GGASSGPTIEEVD 646
HSC70_Homo              QKELEKVCNPIITKLYQSAGGMPG----GMPGGFPGGGAPPS-GGASSGPTIEEVD 646
HSC70_Xenopus           QKELEKVCNPIITKLYQSAGGMPGGMPGGMPGGFPGAGGAPT-GGASSGPTIEEVD 650
HSP70_Xenopus           QKELEKVCNPIITKLYQGAGGMPG----GMPGGFPGAGAAPS-GGASSGPTIEEVD 646
HSC70_Salmo             QQELEKVCNPIITKLYQ---------------------------------------- 612
HSP70_Gallus            QKELEKLCNPIVTKLYQGAG---------------GAG-----AGGSGGPTIEEVD 634
HSP70_Homo              RKELEQMCNPIITKLYQGGCTGPA----------CGTGYVP--GRPATGPTIEEVD 641
                         : **::*:*:::::**
```

Figure 19 (Continued)

Table 6 The similarity of *HSC70-1*, *HSC70-2* and *HSC70* amino acid sequences between *C. macrocephalus* and other species.

	species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	amino acid
1	Clarias_*HSC70-1*		96	91	91	91	91	96	95	94	95	97	83	93	94	96	89	89	89	88	88	88	93	88	88	649
2	Clarias_*HSC70-2*			89	89	89	89	94	93	91	89	95	80	91	91	93	89	87	87	86	87	87	89	88	87	649
3	Salmo_*HSC70*				92	91	91	91	91	95	90	91	82	91	92	92	90	89	89	88	90	91	91	88	84	612
4	Fundulus_*HSC70*					99	99	97	97	97	98	99	99	98	98	98	95	95	94	94	94	94	98	98	93	612
5	Megalobrama_*HSC70*						99	97	95	98	99	100	98	98	98	98	94	95	95	95	95	94	98	98	98	649
6	Paralichthys_*HSC71*							97	95	97	98	99	99	97	98	98	95	94	94	94	94	93	97	93	96	612
7	Ictalurus_*HSC71*								95	94	96	97	96	95	96	96	96	91	92	91	91	91	95	98	97	649
8	Pimephales_*HSC70*									94	96	97	97	96	95	96	95	91	91	90	90	91	95	87	85	650
9	Danio_*HSC70*										96	96	94	94	95	95	90	91	91	90	90	90	95	95	95	649
10	Cyprinus_*HSC70*											99	97	96	97	97	91	91	92	91	91	91	96	95	96	644
11	Carassius_*HSC70*												98	96	97	98	91	92	92	92	92	91	97	95	96	649
12	Acanthopagrus_*HSC70*													95	96	97	91	91	91	91	90	90	96	96	92	650
13	Gallus_*HSC70*														99	97	92	92	92	92	92	91	98	91	89	646
14	Homo_*HSC70*															98	91	93	93	92	93	92	98	91	93	646
15	Xenopus_*HSC70*																91	92	92	91	91	91	98	90	91	650

Table 6 (Continued)

	species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	amino acid
16	Salmo_*HSP70*																	91	91	91	90	92	96	91	92	644
17	Fundulus_*HSP70*																		95	96	94	96	92	98	97	638
18	Megalobrama_*HSP70*																			94	98	96	92	90	91	643
19	Paralichthys_*HSP70*																				94	94	92	86	86	640
20	Carassius_*HSP70*																					95	92	89	90	643
21	Acanthopagrus_*HSP70*																						91	92	90	638
22	Xenopus_*HSP70*																							93	92	646
23	Gallus_*HSP70*																								94	634
24	Homo_*HSP70*																									641

Table 7 The identity of *HSC70-1*, *HSC70-2* and *HSC70* amino acid sequences between *C. macrocephalus* and other species.

	species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	amino acid
1	Clarias_*HSC70-1*		94	95	95	95	95	94	93	91	93	94	94	90	91	93	83	84	86	84	85	83	94	88	82	649
2	Clarias_*HSC70-2*			90	90	91	90	90	89	87	90	91	89	87	87	89	84	84	87	84	86	84	91	85	82	649
3	Salmo_*HSC70*				95	95	95	92	94	92	93	95	93	93	94	95	85	85	87	85	86	85	94	87	83	612
4	Fundulus_*HSC70*					97	98	94	96	94	95	97	98	93	95	95	85	86	87	85	87	85	94	89	84	612
5	Megalobrama_*HSC70*						96	93	96	96	97	99	96	94	95	95	86	86	88	86	87	85	95	89	85	649
6	Paralichthys_*HSC71*							93	96	93	94	96	97	93	94	94	84	85	86	85	86	84	94	88	84	612
7	Ictalurus_*HSC71*								92	89	91	92	92	89	90	91	82	82	84	82	83	82	90	85	81	649
8	Pimephales_*HSC70*									92	95	97	94	93	94	94	83	84	85	84	83	83	94	87	82	650
9	Danio_*HSC70*										93	94	91	91	92	91	83	83	84	82	83	83	91	86	82	649
10	Cyprinus_*HSC70*											98	94	92	93	94	83	83	85	83	84	82	93	86	82	644
11	Carassius_*HSC70*												95	93	94	95	84	85	86	84	85	84	95	88	83	649
12	Acanthopagrus_*HSC70*													91	93	93	82	83	84	83	82	83	93	87	81	650
13	Gallus_*HSC70*														97	95	84	83	86	83	84	82	95	87	82	646
14	Homo_*HSC70*															96	84	84	87	84	85	83	96	88	82	646
15	Xenopus_*HSC70*																84	84	86	84	86	84	97	88	82	650

Table 7 (Continued)

	species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	amino acid
16	Salmo_*HSP70*																	87	92	87	89	89	84	83	84	644
17	Fundulus_*HSP70*																		89	91	88	87	84	85	84	638
18	Megalobrama_*HSP70*																			87	96	91	86	86	85	643
19	Paralichthys_*HSP70*																				86	84	84	84	83	640
20	Carassius_*HSP70*																					89	85	85	85	643
21	Acanthopagrus_*HSP70*																						84	83	83	638
22	Xenopus_*HSP70*																							88	82	646
23	Gallus_*HSP70*																								82	634
24	Homo_*HSP70*																									641

Phylogenetic analysis was performed based on 15 amino acid sequences of *HSC70* and *HSC71*. A phylogenetic tree showed two distinct braches for fishes and clawed frog, chicken and human (Fig. 20). Among teleosts, the *HSC70-1* and *HSC70-2* sequences of *C. macrocephalus* were clustered with *I. punctatus HSC71*, which would be expected given their membership in Order Siluriformes. A second group of teleost sequences comprised members of Family Cyprinidae, including *C. carpio*, *C. gibelio*, *M. amblycephala*, *D. rerio*, and *P. promelas*, and sequences from *P. olivaceus* and *F. heteroclitus*. The two clusters joined the sequence from *S. salar*. The second major branch consisted of sequences from higher vertebrates, including human, chicken and clawed frog.

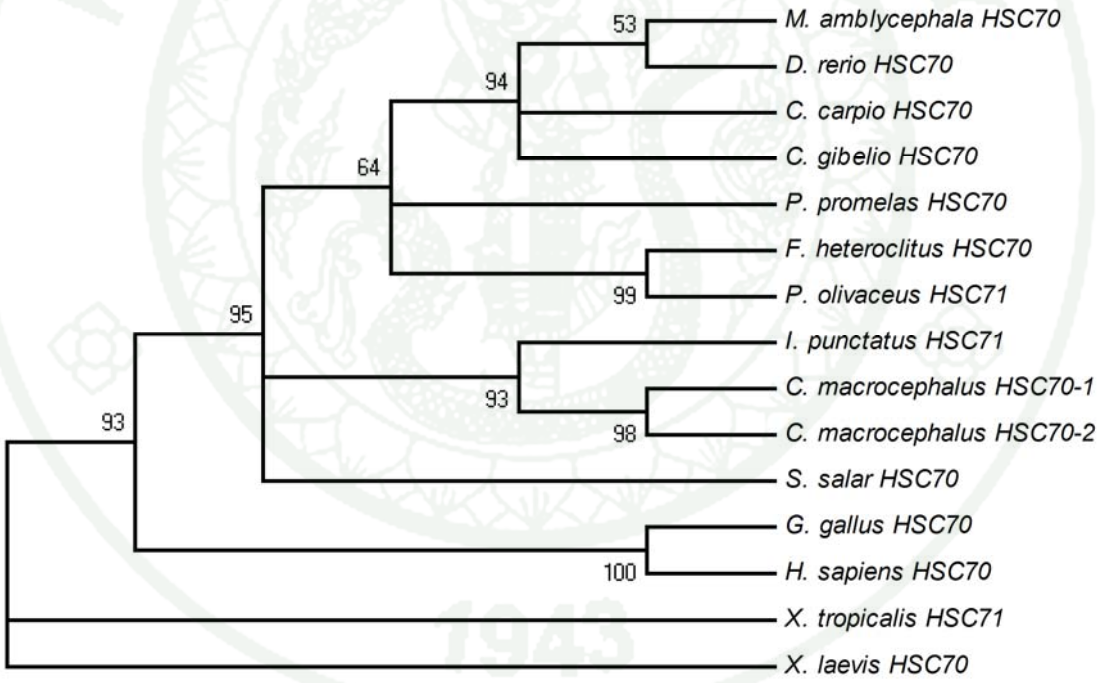


Figure 20 A phylogenetic tree of the amino acid sequences of *HSC70-1* and *HSP70-2* in *C. macrocephalus* and other vertebrates.

Part II

Seasonal expression of *HSC70* and *HSC90* genes and gonad development under pond culture

1. HSC70 and *HSC90* expression and seasonal changes of gonad development

Monthly change in GSI of mature females was monitored over a 1-year period from April 2008 to March 2009 (Fig. 21). Water temperatures during the period varied from 24 to 30° C, with the lowest temperatures in December and January (24-25.5° C). The amount of rainfall varied from 0 mm during December to February to 313 mm in July. Significant variation of mean GSI values ($P < 0.05$) was observed among months. From April until August, mean GSI values ranged from 5.7± 2.0% to 11.3±6.3% with the highest value (13.9±3.6%) in July. The GSI decreased in September and October and remained low in November and December (0.4 ± 0.2% to 0.9±0.5%). From January to March, the GSI steadily increased from 2.2±1.5% to 9.3±2.1%.

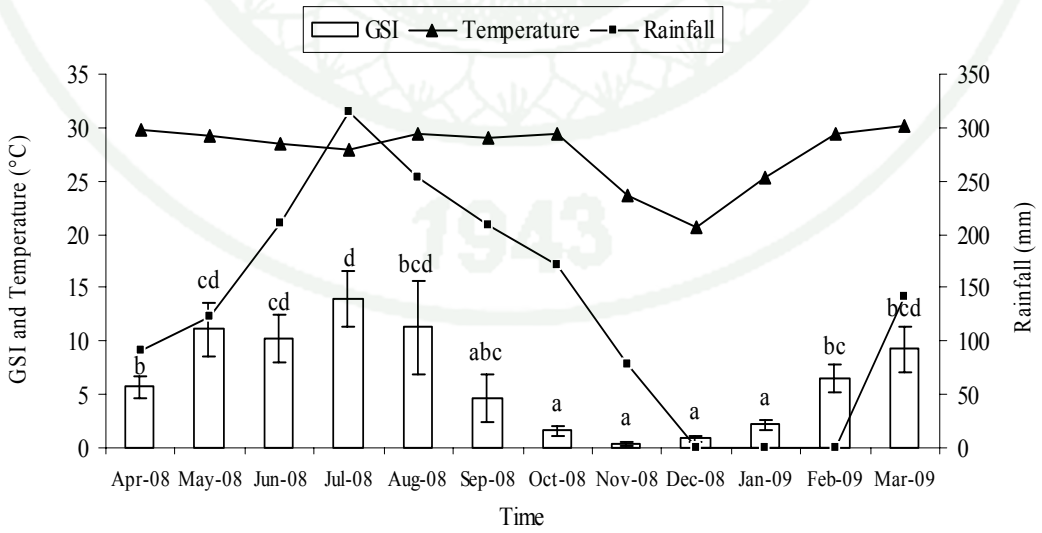
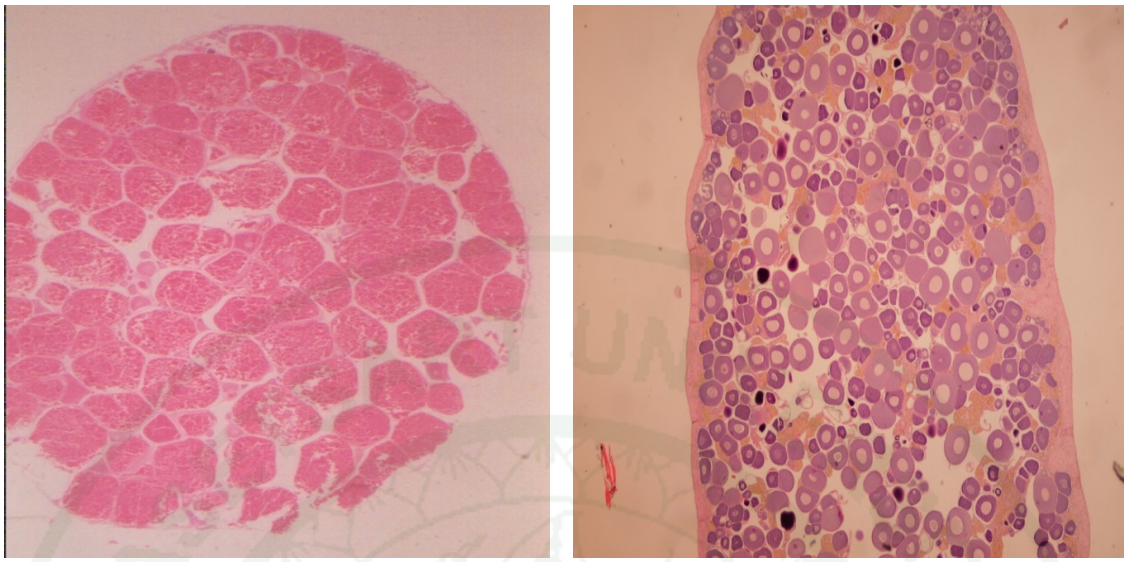


Figure 21 GSI values, temperature and rainfall (mm) throughout the reproductive season of female bighead catfish.



[A] [B]

Figure 22 Ovarian growth of female bighead catfish throughout the reproductive season in April [A] and November [B].

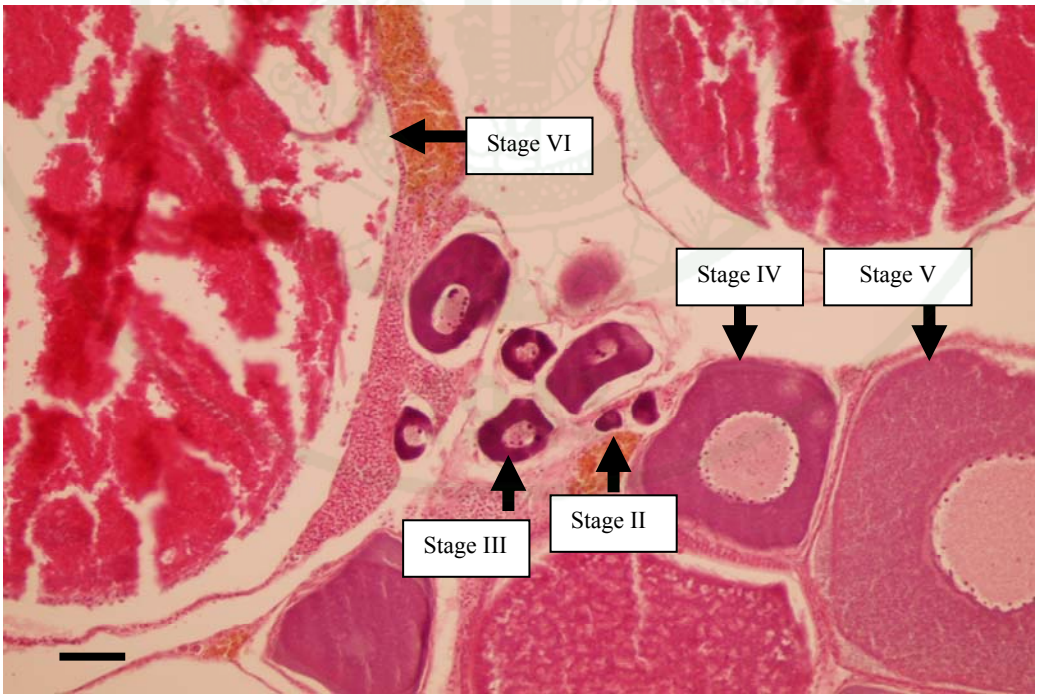


Figure 23 Oocytes at different developmental stages (II-VI) in the ovary of a mature female bighead catfish (scale bar = 200 μm).

Oocyte development of mature females in the earthen pond was examined monthly. The stage I oocytes were undifferentiated and occurred in nests with large nuclei and acidophilic cytoplasm. Oogonia and oocytes were connected or close to ovigerous lamellas (Fig. 24). Stage II oocytes showed increase in size, each contained a large nucleus. Oocytes were loosely associated with ovigerous lamellas. Oocytes were surrounded by follicle cells. Oocyte cytoplasm changed from basifilicus to acidofilifus (Fig. 25). Stage III oocytes showed a well defined follicular epithelium or follicle fully formed with basophilic cytoplasm. Many cytoplasmatic structures or globules of proteins were present (Fig. 26). Stage IV oocytes were characterized by the appearance of euvitellin nucleoli along the nuclear membrane. Nucleus was centrally positioned with many nucleoli. Yolk granules were present in the cytoplasm. A more distinct zona radiata was observed in the cell membrane (Fig. 27). Stage V oocytes were larger with abundant yolk vesicles. Nucleus or germinal vesicle appeared small compared to large cytoplasm. Cytoplasm filled with yolk vacuoles and yolk granules (Fig. 28). Stage VI oocytes were fully grown, containing large amount of acidophilic yolk granules in the cytoplasm. Nucleus migrated to the periphery of the cytoplasm (Fig. 29). Oocytes of *C. macrocephalus* can be classified as immature or pre-vitellogenic (stages I to III), maturing or vitellogenic (stages IV and V), and mature or post-vitellogenic (stage VI).

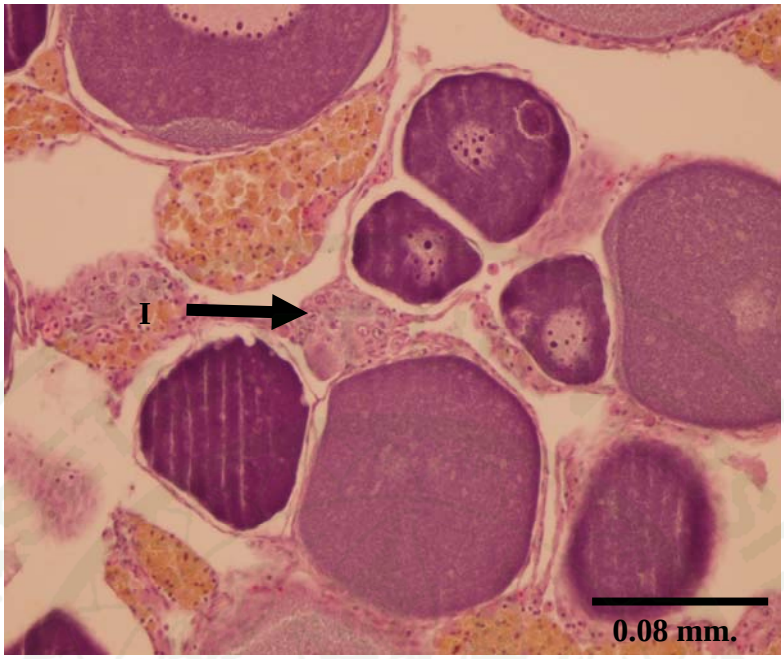


Figure 24 Stage I oocytes. Oogonia and oocytes are connected or close to ovigerous lamellas.

Figure 25 Stage II oocytes. Oocytes are loosely associated with ovigerous lamellas and surrounded by follicle cells. Oocyte cytoplasm changed from basifilicus to acidofilifus.

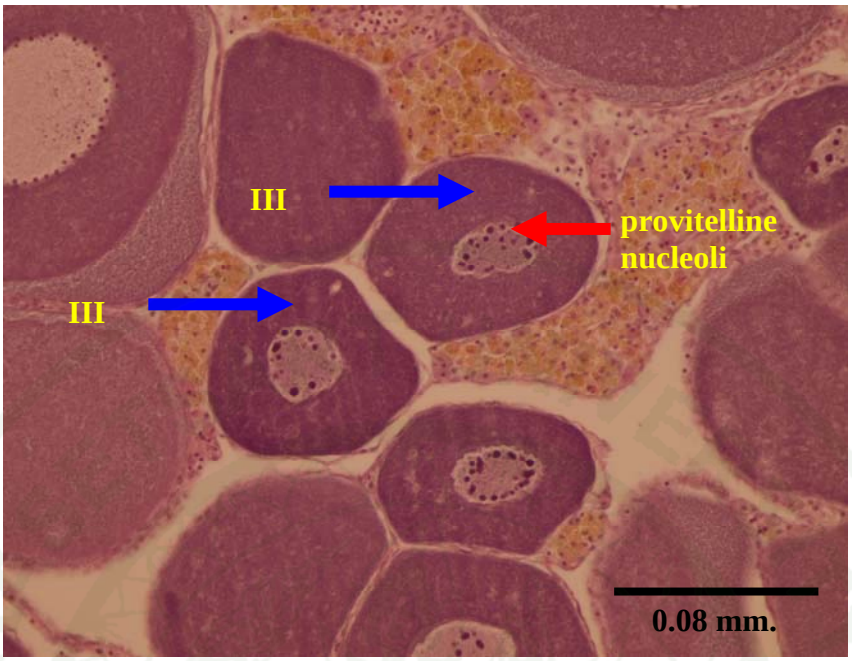


Figure 26 Stage III oocytes. Follicles are fully formed. Many cytoplasmatic structures or globules of proteins are present.

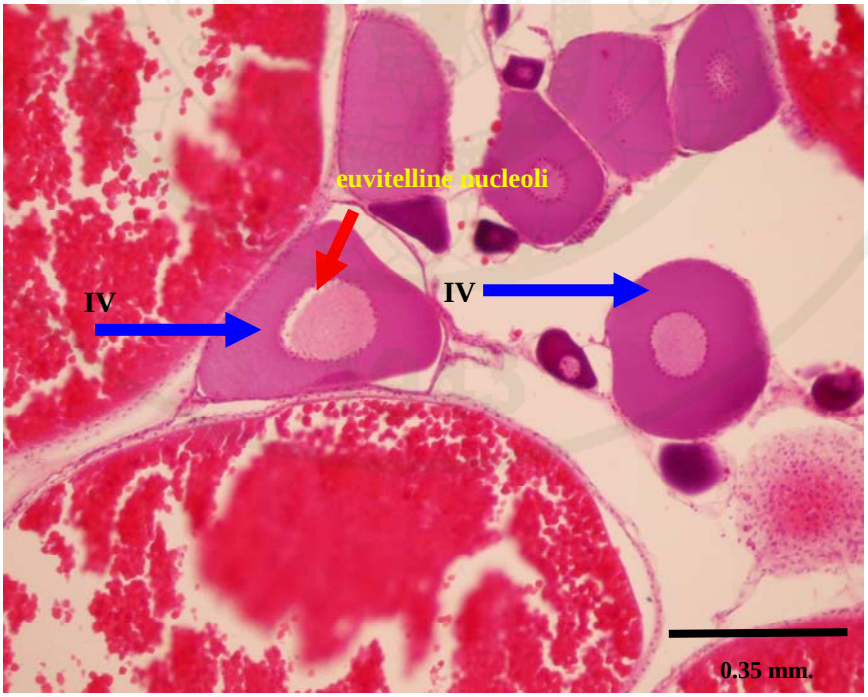


Figure 27 Stage IV oocytes. Nucleus is centrally positioned with many nucleoli close to the nuclear membrane.

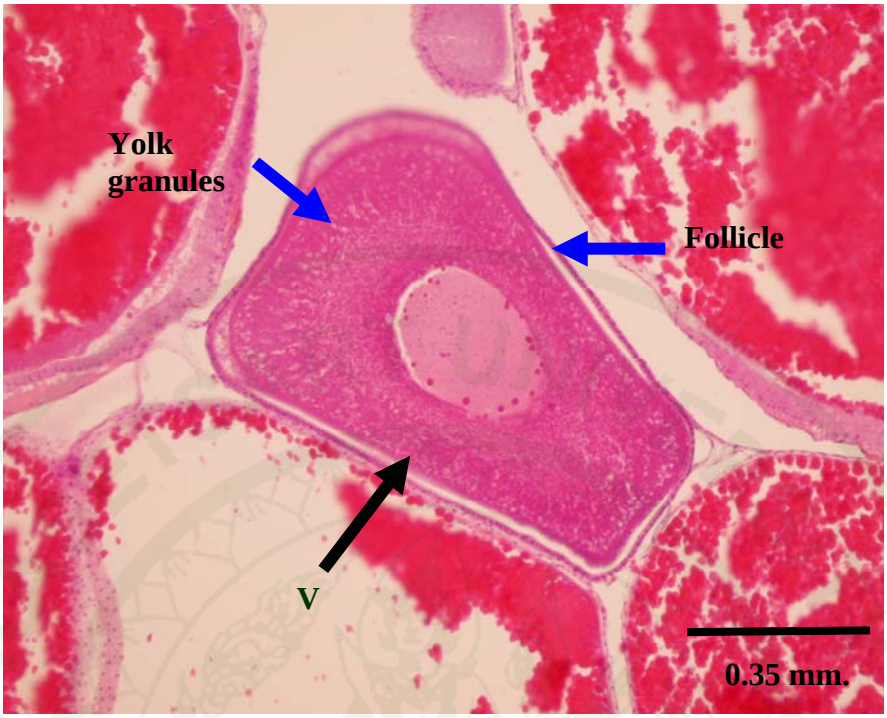


Figure 28 Stage V oocytes. Nucleus or germinal vesicle appears small compared to large cytoplasm. Cytoplasm is filled with yolk vacuoles and yolk granules.

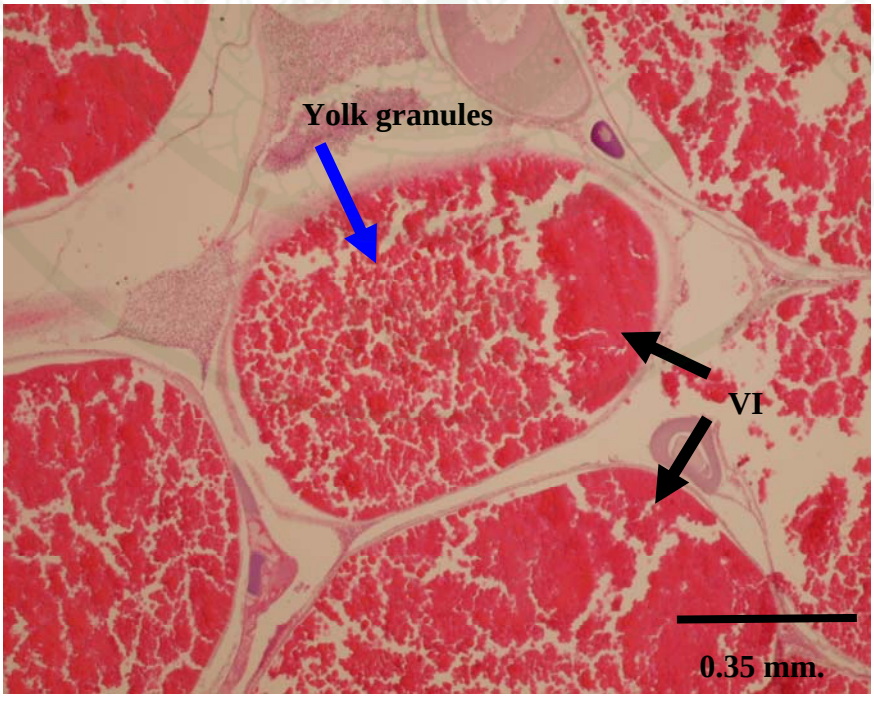


Figure 29 Stage VI oocytes. Nucleus migrated to the periphery of the cytoplasm.

Developing ovaries of mature females exhibited oocytes in all stages (Fig. 30). From April to September when fish were 8 to 13 months old the females contained the highest percent of stage 6 or post-vitellogenic oocytes (34.1±0.7 to 59.0± 1.3%). In October and November, the oocytes remained at stages 2 to 4 with the highest percent in stage 3 (46.8±1.4 to 63.1±1.5 %). Eggs in stages 5 and 6 were observed in December and the proportions of eggs at advanced stages gradually increased until March when ovaries of females contained oocytes at all stages, with high percentage at stages 3 and 4.

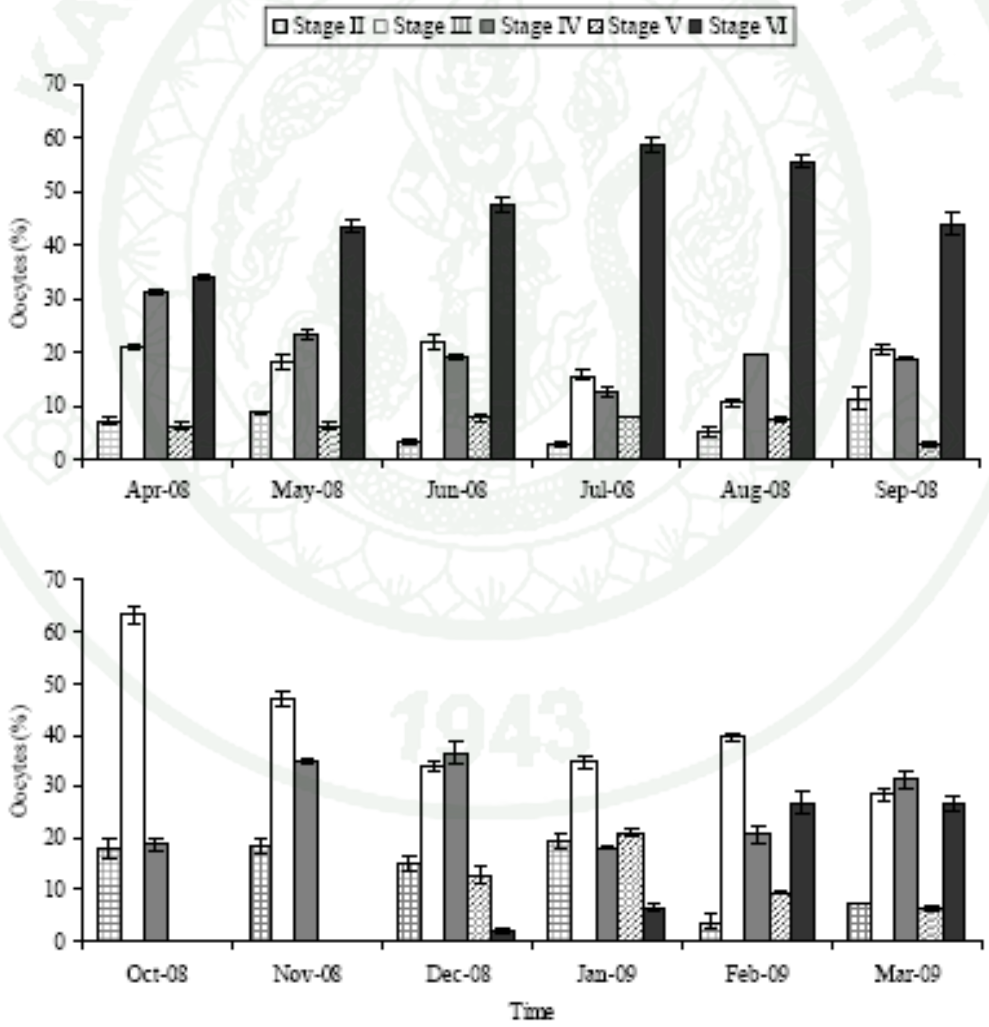


Figure 30 Proportion of oocytes at different developmental stages throughout the reproductive season of female bighead catfish.

2. Expression of *HSC70* and *HSC90* under pond culture conditions

HSC70 and *HSC90* expression, with product sizes of 281 and 567 bp respectively, were highly expressed in gills, brain and liver but only lightly expressed in muscle tissues (Fig. 31). Expression of β-actin was observed in all tissues with product size of 414 bp. Because *HSCs* were only lightly expressed in skeletal muscle, further analysis of expression of these proteins was performed in brain, gills and liver tissues.

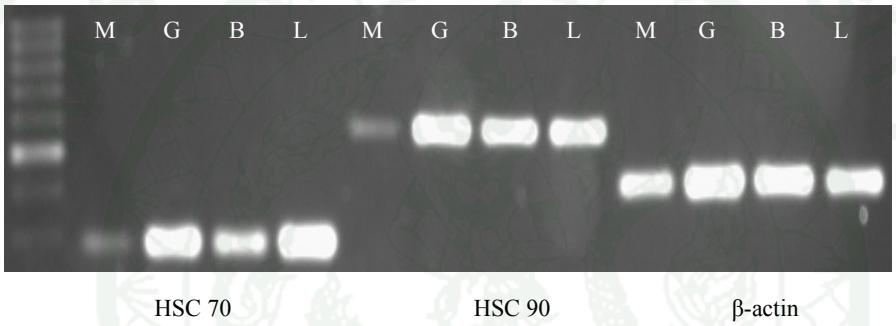


Figure 31 RT-PCR assay for *HSC70* and *HSC90* expression on various tissues. Samples were as follows: skeletal muscle (M), gills (G), brain (B), and liver (L). Lane Marker was a 100 bp DNA marker. β-actin was used as an internal control for relative gene expression.

Relative levels of *HSC70* and *HSC90* mRNA in gills, liver and brain of females varied from 50% to 150% across tissues. Liver showed highest levels of the two *HSC70* and *HSC90* transcripts throughout the breeding season. Percent expression of *HSC70* and *HSC90* was similar in all tissues, with a higher level of *HSC70* from September until March. Despite the lowest temperature (25°C) and no precipitation in December and January, the levels of *HSC70* and *HSC90* did not differ from those of other months (Fig. 32).

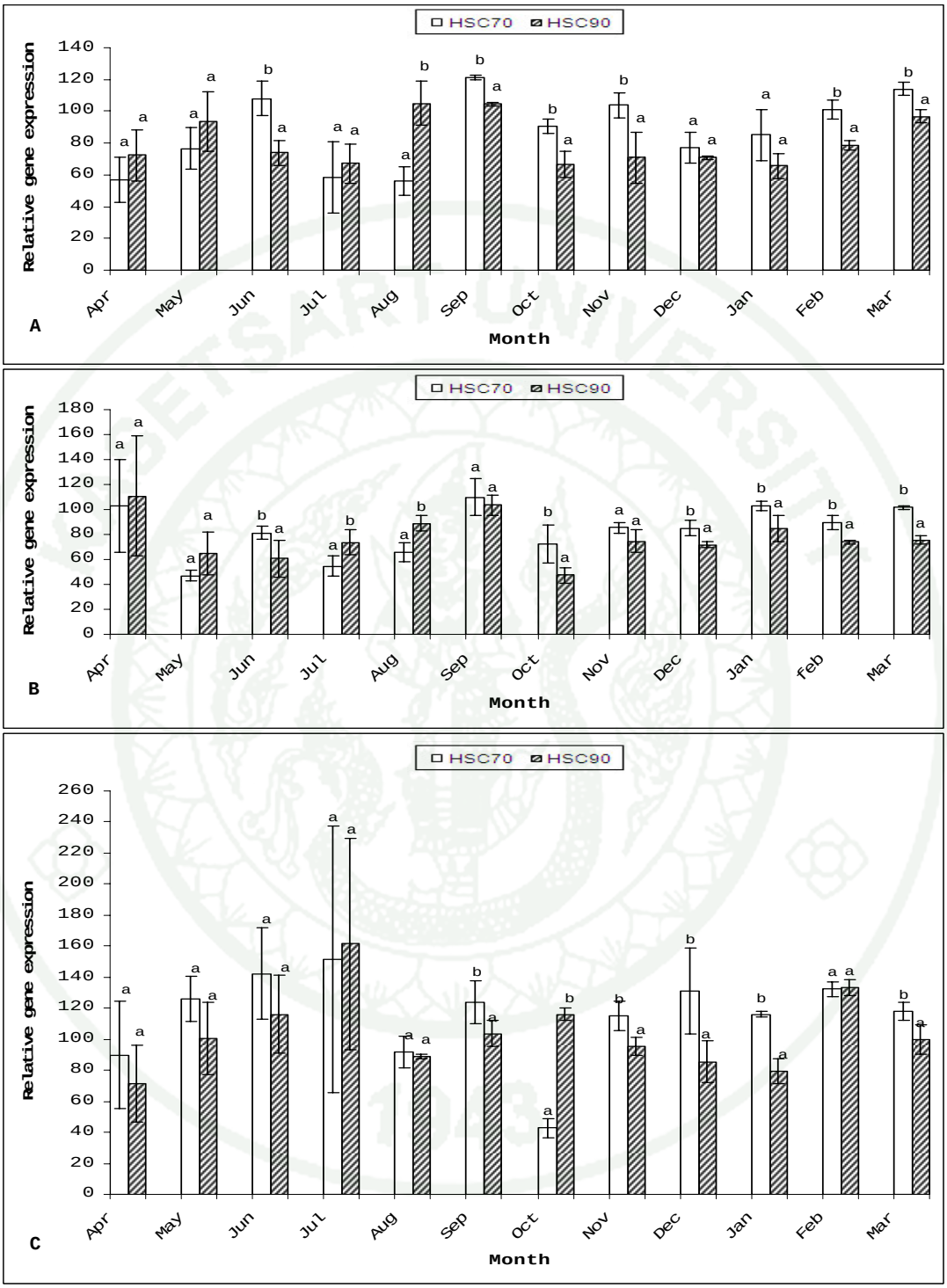


Figure 32 Relative expression levels of *HSC70* and *HSC90* mRNA in tissues of females held under natural pond conditions; A = gills, B = brain, and C = liver. Levels associated with histogram bars with the same letter do not differ significantly at $P < 0.05$.

Part III

Expression of *HSC70* and *HSC 90* genes in response to elevated temperature

The effects of heat exposure (30 and 35°C) on ovarian growth were tested in fish held under hatchery conditions during October to November when water temperature in the earthen pond was 25°C. At 14 days, percentage of eggs at stages 2 to stages 4 was nearly identical between control and the treated fish. At 28 days, oocyte development of females held at warm temperatures proceeded into stage 5 while, oocytes of the females in earthen ponds remained at stages 2 to stages 4. The difference of ovarian growth was pronounced at 48 days, with increased percentage of eggs at stage 5 (19.7±1.1%) in fish held at 35°C compared to 6.5±0.6 and 4.6±1.7% in the control and at 30°C, but ovaries of females in earthen ponds were unchanged and contained only eggs at stages 3 and stages 4 (Fig. 33).

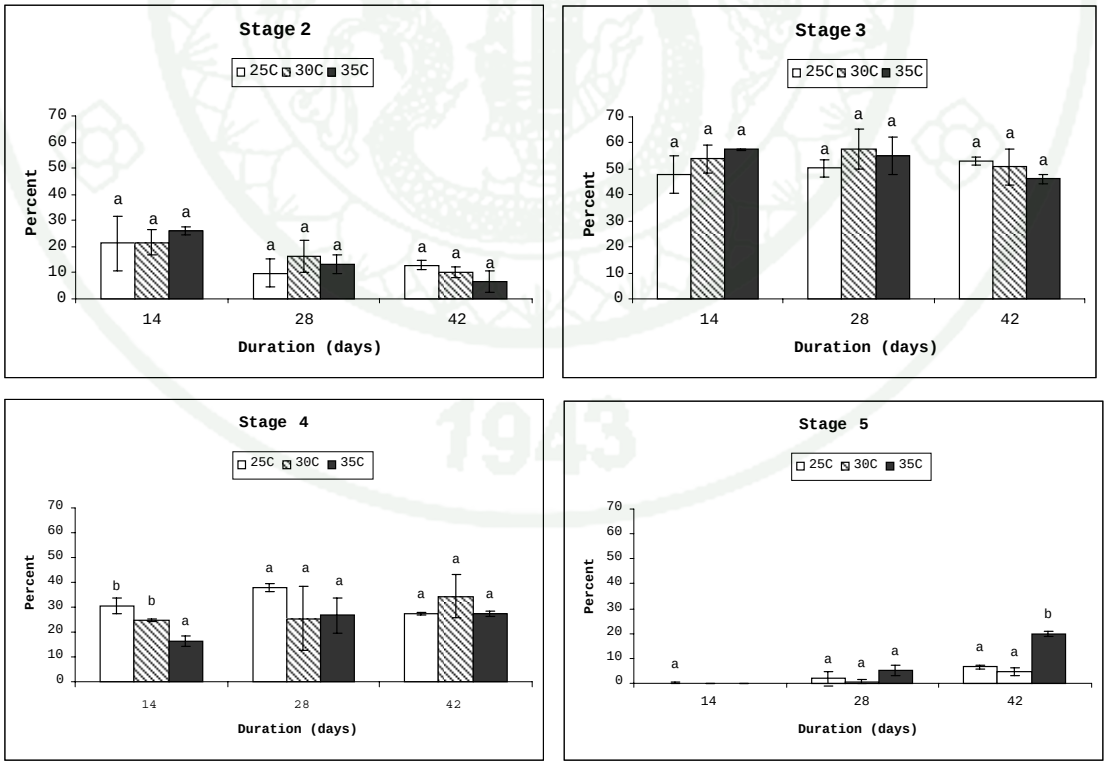


Figure 33 Proportions of eggs at different developmental stages in females held under elevated temperatures for different durations.

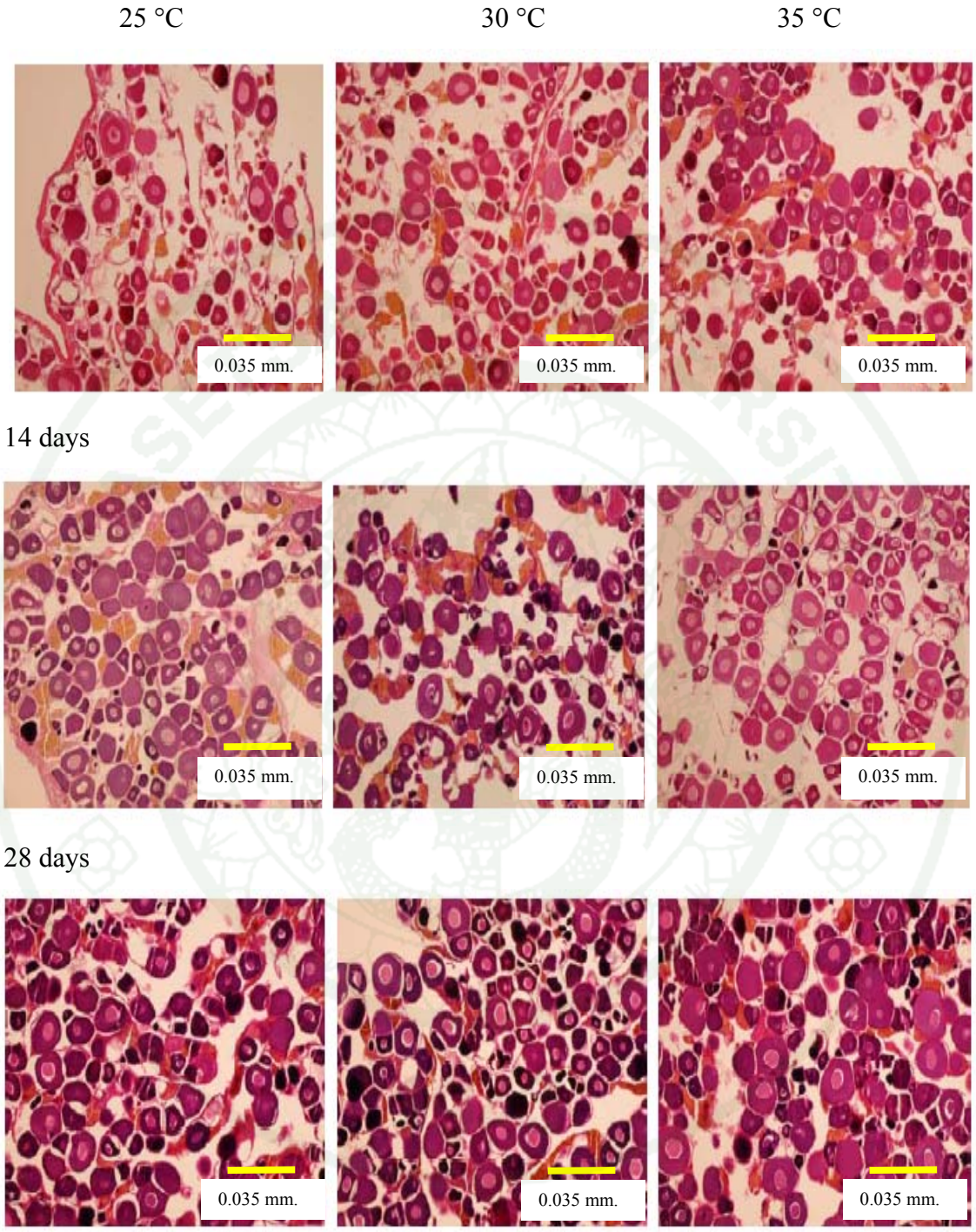


Figure 34 Oocytes at different developmental stages (IV-VI) in the ovary of a mature female bighead catfish under ambient (25°C) and elevated temperatures (30 and 35°C) at 14, 28, and 42 days.

Plasma cortisol levels were higher at elevated temperatures across the experimental period, and remained unchanged at 25 and 30°C. However, at 35°C, cortisol concentration was highest at week 4 (Fig. 35). Although glucose levels were highest at 35°C, the values did not differ significantly across temperature levels or through the experimental period.

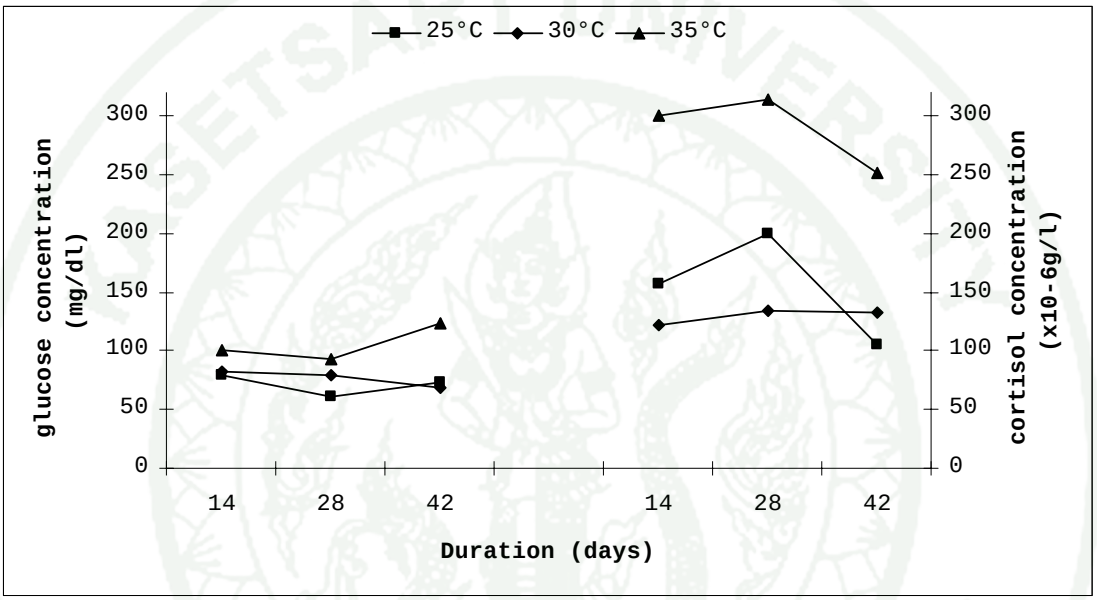
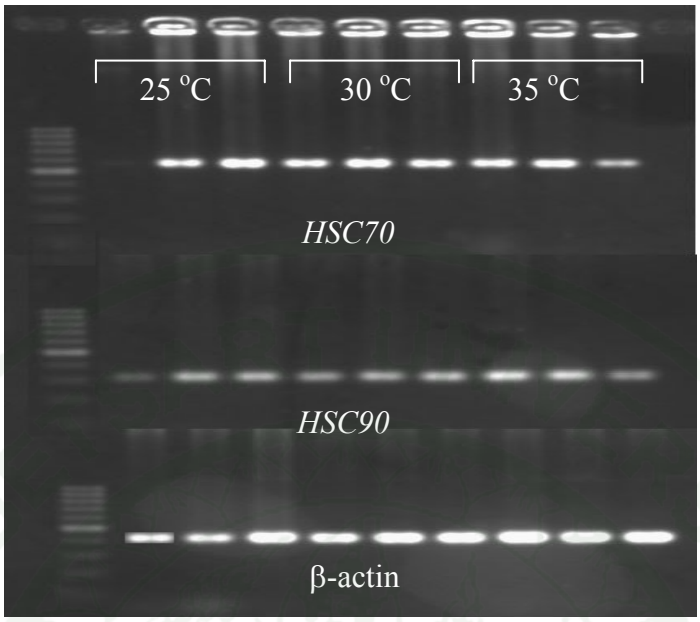
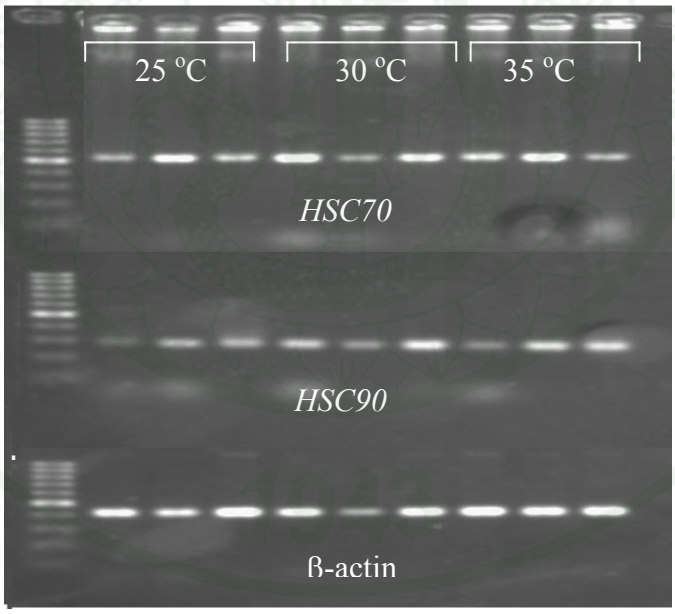


Figure 35 Plasma cortisol and glucose levels in females held under elevated temperatures.

Expression of *HSC70* and *HSC90* with product sizes of 281 and 567 bp respectively was shown in Fig. 36. Expression of *β*-actin was observed in all tissues with product size of 414 bp.



[A]



[B]

Figure 36 RT-PCR assay for *HSC70* and *HSC90* expression on gills tissues [A] and liver tissue [B] of a mature female bighead catfish under ambient (25°C) and elevated temperatures (30 and 35°C). β-actin was used as an internal control for relative gene expression.

The relative levels of *HSC70* and *HSC90* mRNA in gills and liver were similar at different water temperatures at week 2. The expression levels of the *HSC70* and *HSC90* increased with higher temperature after 4 weeks of exposure. However, at week 6, the levels of *HSC70* and *HSC90* decreased significantly (>60 %) in tissues of females held at 35°C (Fig. 37).

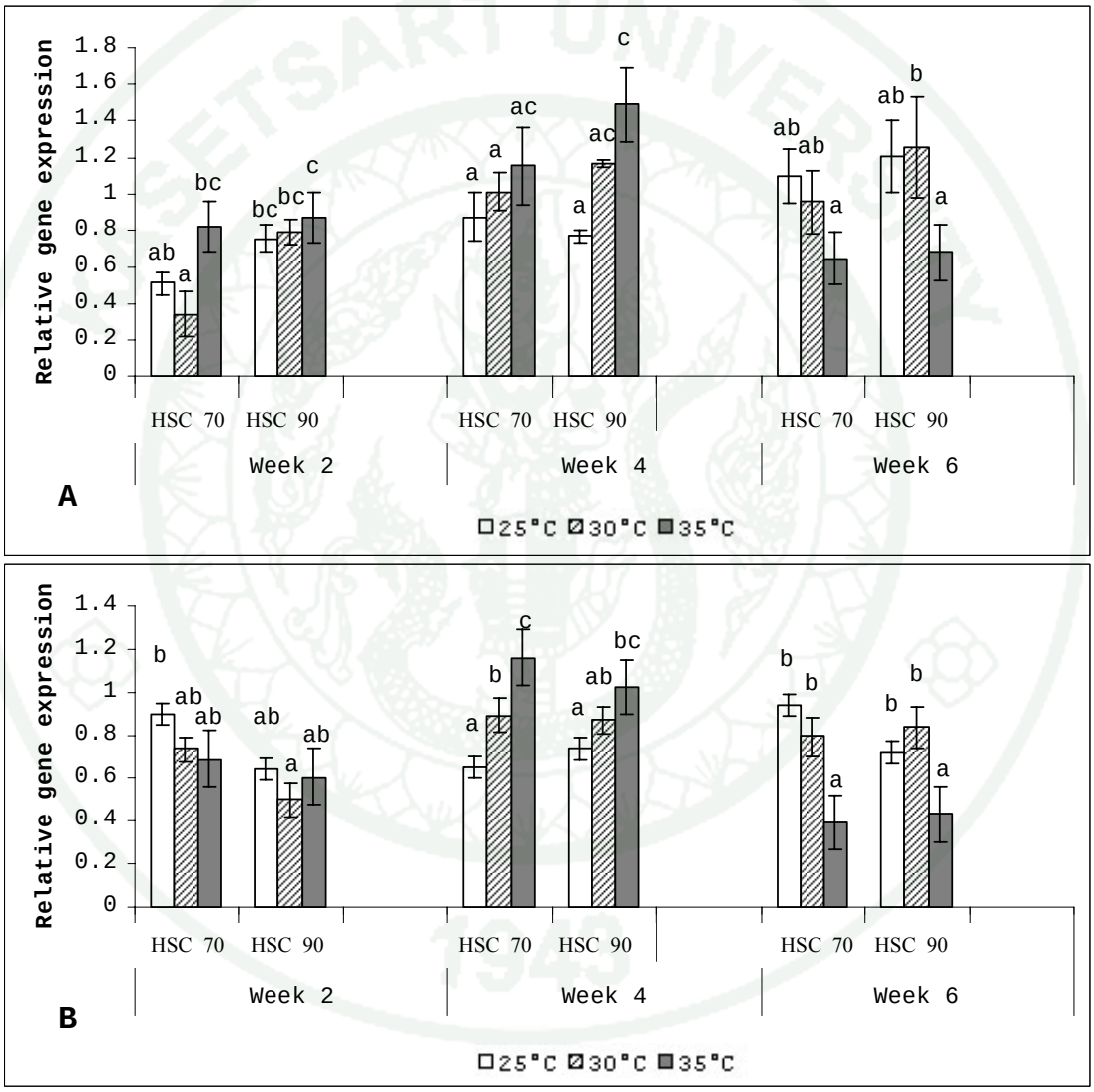
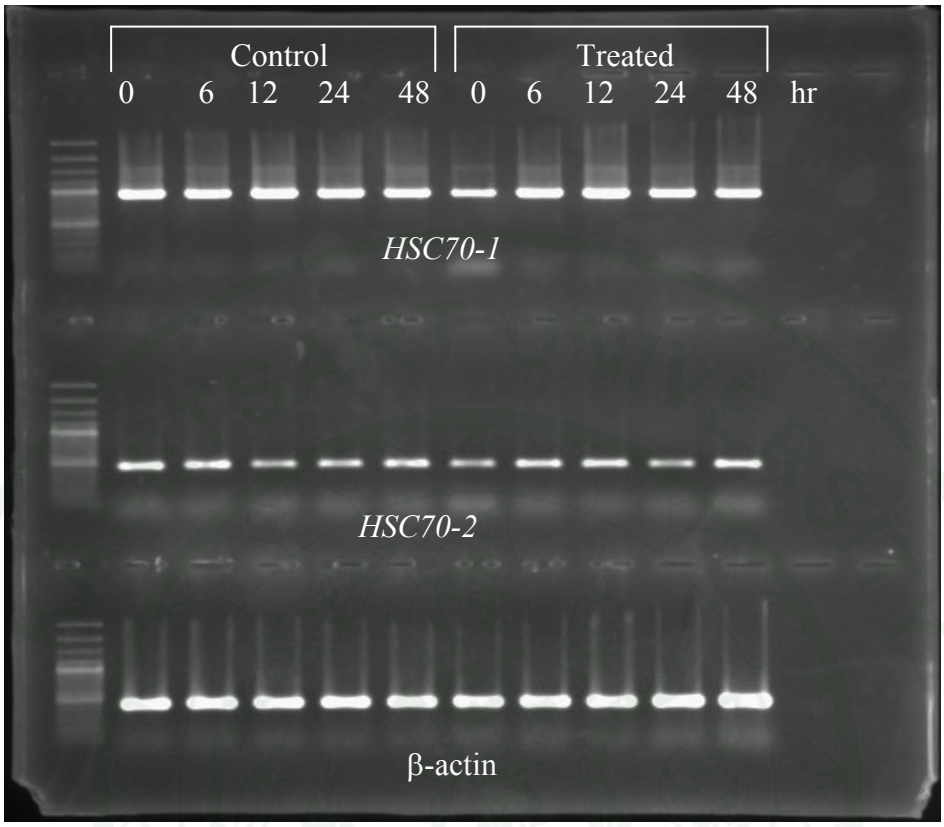


Figure 37 Relative expression of *HSC70* and *HSC90* in tissues of bighead catfish females held under ambient (25°C) and elevated temperatures (30 and 35°C) at 2, 4 and 6 weeks; A = liver and B = gills. Levels associated with histogram bars with the same letters do not differ significantly at $P<0.05$.

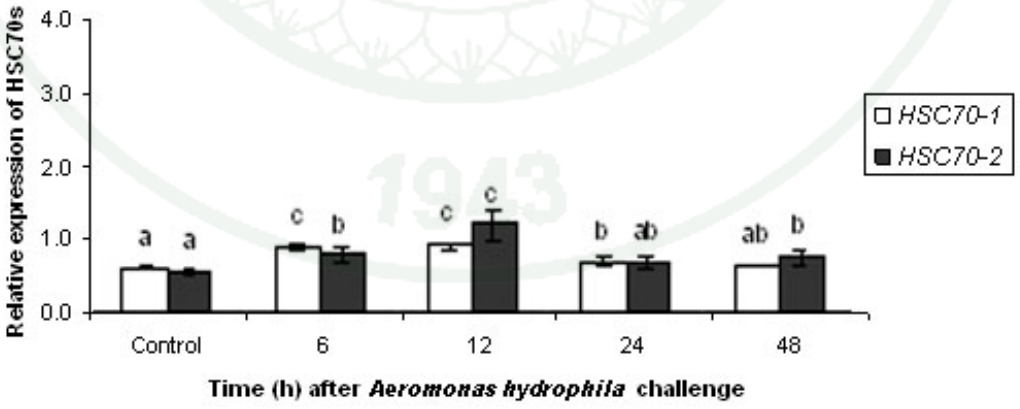
Part IV

Expression of *HSC70s* in response to *Aeromonas hydrophila* infection

The temporal expression of *HSC70-1 and HSP70-2* after a bacterial challenge was analyzed in brain, gills, liver and muscle. *A. hydrophila* challenge resulted in differential expression of the two genes in bighead catfish tissues during 48 h (Fig. 38- 41). The expression levels of the *HSC70-1* and *HSC70-2* in the brain were similar at each time point ($P < 0.05$). However, slight but significant differences in *HSC70-1* mRNA levels were observed in the brain at 12 h, with 0.5-1.0 fold increase ($P < 0.05$) in comparison with the control. The *HSC70-2* was up-regulated significantly in gills at 24 h post-injection and remained unchanged at 48 h, whereas the mRNA level of *HSC70-1* changed only slightly during a 48-h period. In the liver, results of *t*-test showed that the expression levels of *HSC70-1*and *HSC70-2* were significantly different at each time point. While the mRNA levels of *HSC70-1* in liver tissue increased slightly at 12 h and remained unchanged at 48 h, the *HSC70-2* was significantly up-regulated at 24 h and decreased markedly at 48 h post-injection. In skeletal muscle, the *HSC70-2* concentration increased gradually from 6 h and peaked at 48 h post-injection, with 3-fold increase compared to that of the control. In contrast, the mRNA level of *HSC70-1* changed only slightly during a 48-h period.

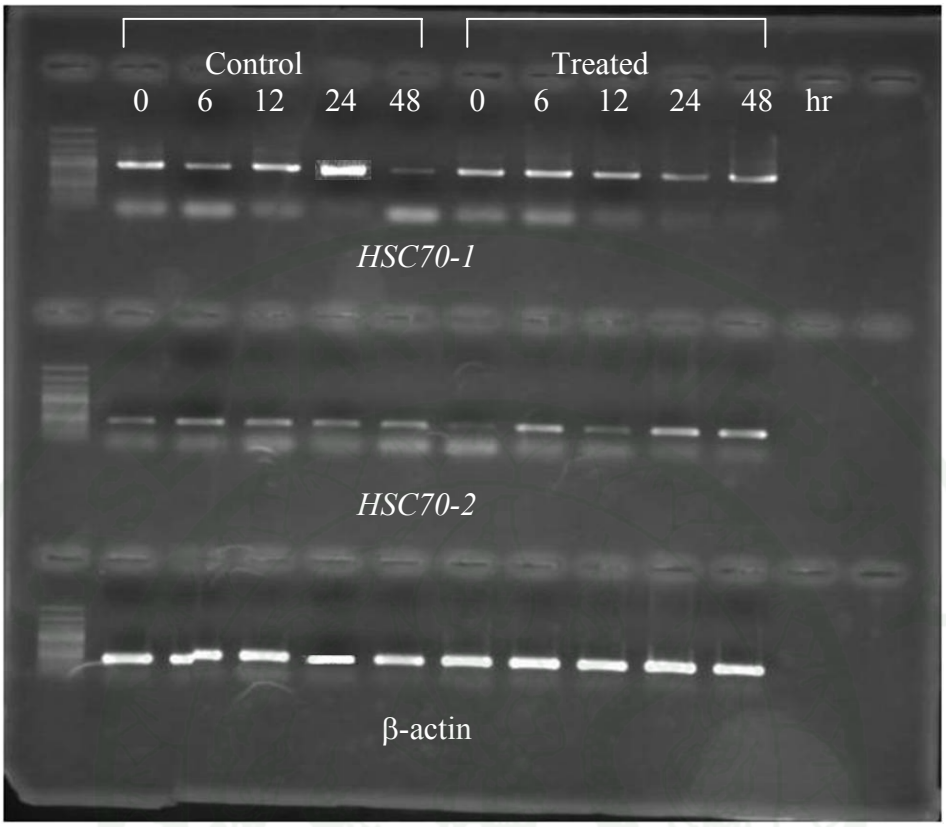


[A]

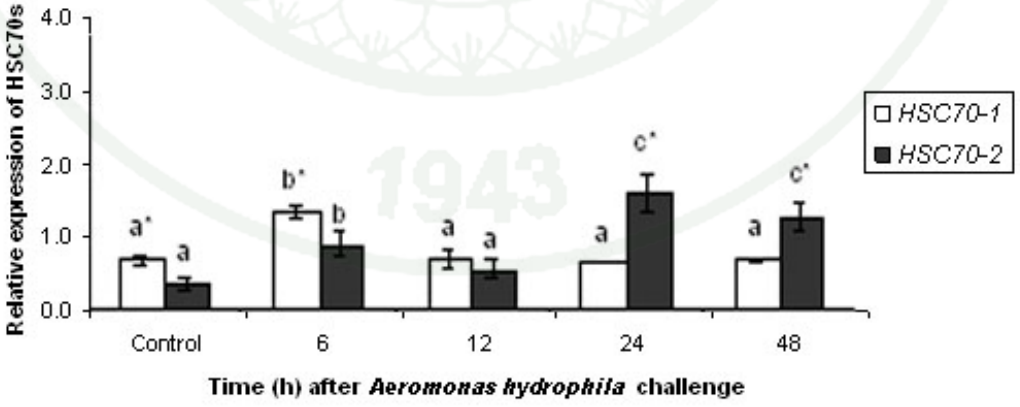


[B]

Figure 38 Expression of *HSC70-1* and *HSC70-2* in brain after *A. hydrophila* challenge.

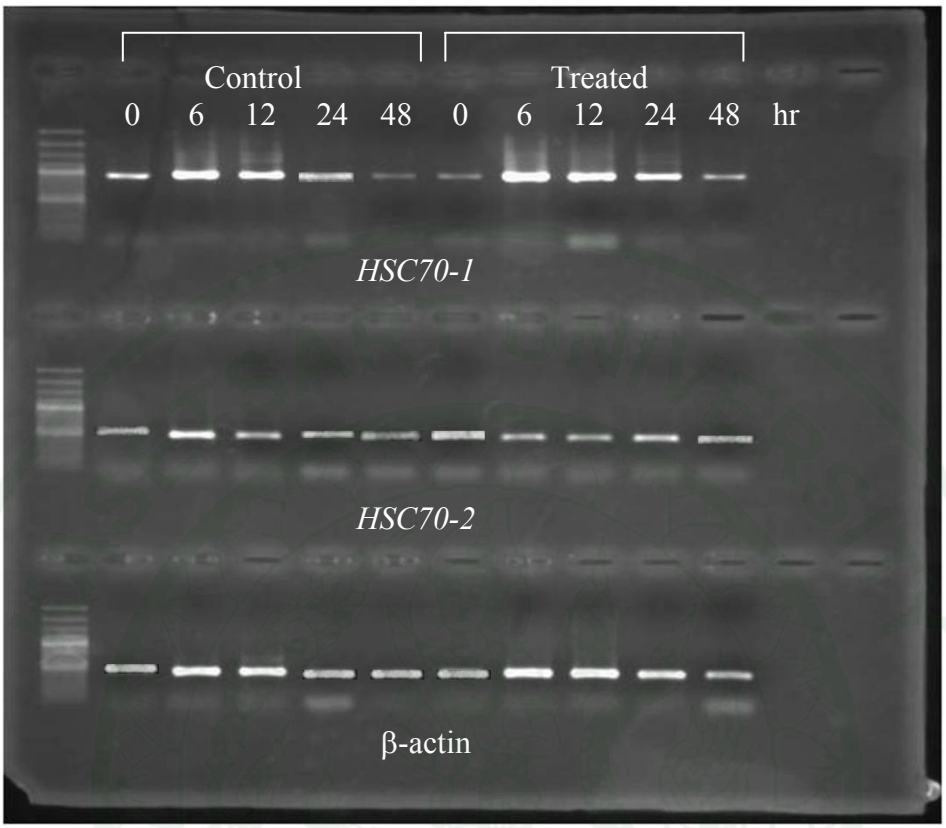


[A]

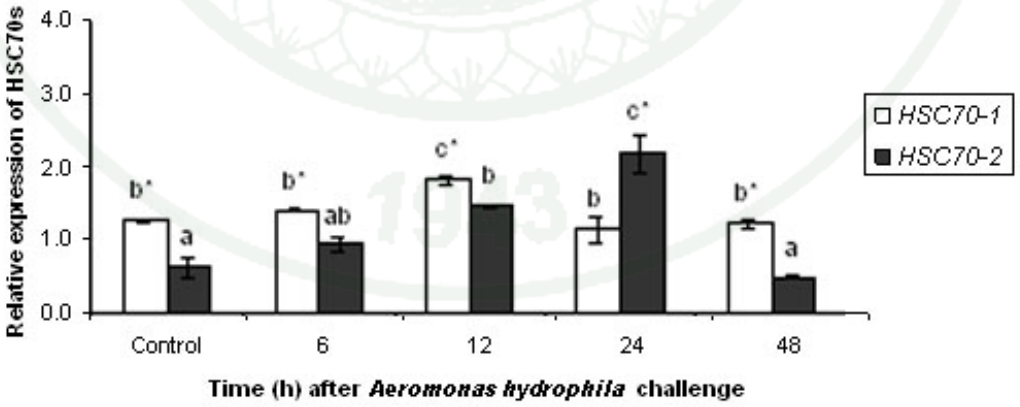


[B]

Figure 39 Expression of *HSC70-1* and *HSC70-2* in gills after *A. hydrophila* challenge.

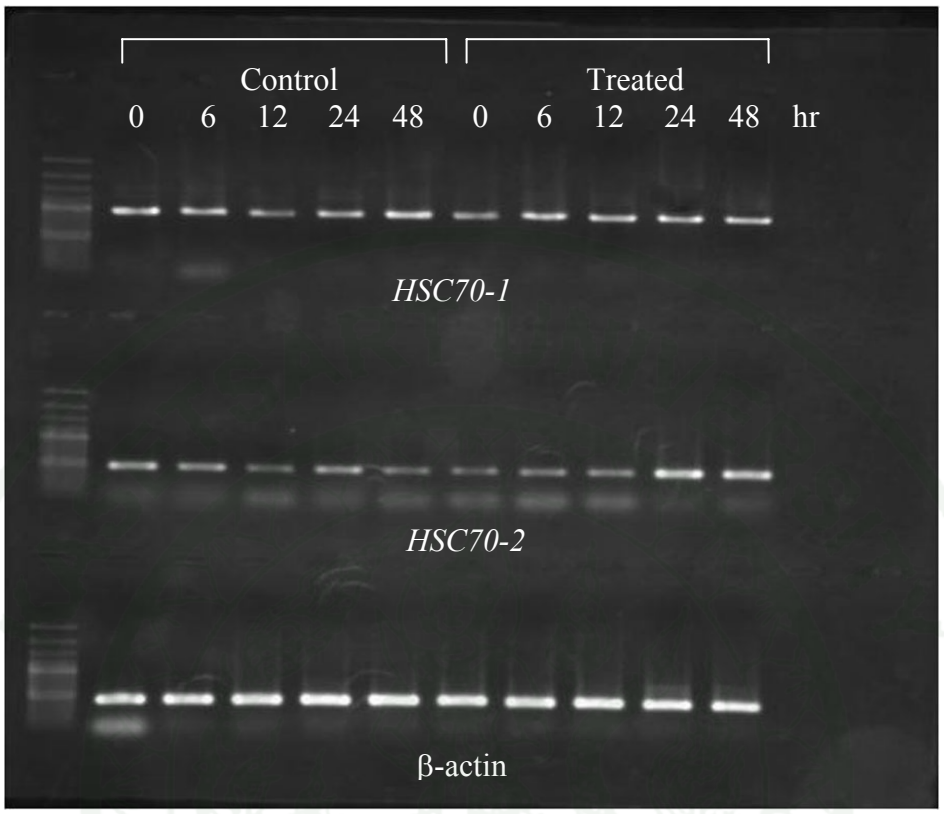


[A]

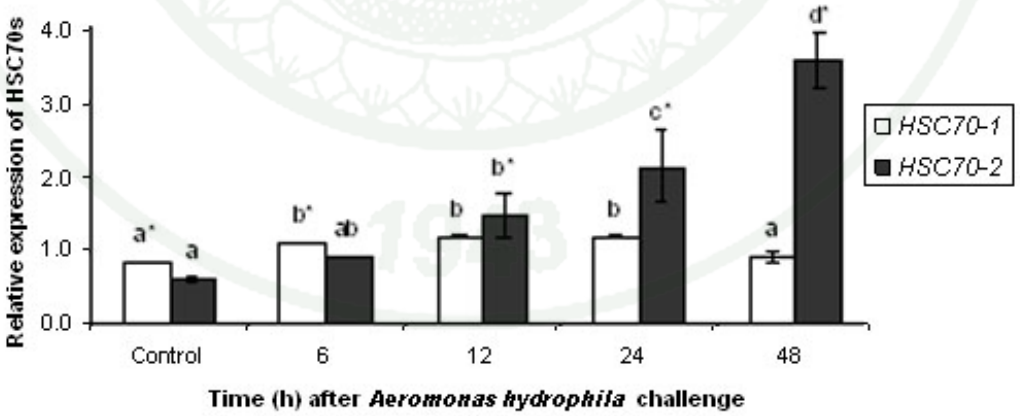


[B]

Figure 40 Expression of *HSC70-1* and *HSC70-2* in liver after *A. hydrophila* challenge.



[A]



[B]

Figure 41 Expression of *HSC70-1* and *HSC70-2* in muscle after *A. hydrophila* challenge.

DISCUSSION

1. Cloning cDNA and genomic DNA of *HSC70-1* and *HSC70-2*

In the present study, I isolated and characterized two cognate heat shock protein 70 cDNAs from liver tissue of bighead catfish. Because band doubles at 70 kDa were observed on agarose gel for purification of cDNA, I cloned two bands separately. I obtained two complete cDNAs and designated *HSC70-1* and *HSC70-2*. The lengths of *HSC70-1* and *HSC70-2* cDNAs were 2,278 bp, with ORFs of 1950 bp encoding 649 amino acids. *HSC70* was placed in the heat shock protein 70 family according to homology with other heat shock proteins.

Characterization of genomic sequences revealed that the bighead catfish *HSC70-1* and *HSC70-2* genes were different in genomic DNA length, with 3,178 and 2,909 bp, respectively and composed of eight exons, and seven introns. Differences in exon and intron sizes were observed between the two loci. The major difference was in the size of intron 4, which was larger (198 bp) in *HSC70-1* than that of *HSC70-2* (64 bp). The presence of introns is a major characteristic of the constitutive forms of the HSP70 family, whereas the inducible forms do not contain introns (Iwama *et al.*, 1998; Basu *et al.,* 2002). The absence of introns enables a rapid translation of mRNA into nascent protein following exposure to stressor. In the *HSP70* family, introns are generally found only in constitutively expressed genes, but not the inducible forms. It has been suggested that the major heat shock genes do not contain introns and so the mRNA can be immediately translated into new proteins within minutes of exposure to the stressor (Iwama *et al.*, 1998). Since most of the inducible heat shock protein genes do not contain introns, the mRNA is rapidly translated into nascent protein within minutes following exposure to a stressor (Basu *et al.*, 2002). In common carp, the similarities of *HSC70-1* and *HSC70-2* were 88% and 78% at protein and DNA level respectively (Ali *et al.*, 2003). There were 69 substitutions between the two common carp proteins, 31 of which located in the C-terminus.

Genomic structures of the two loci were similar to those of other teleost fish, including *Rivulus marmoratus* (Park *et al*., 2001), common carp (Ali *et al.,* 2003), and Wuchang bream (Ming *et al.*, 2010) and mammals. Interestingly, exon sizes were identical between walking catfish *HSC70-1* and Wuchang bream *HSC70*, but the intron sizes were different (Ming *et al.*, 2010). The functional significance of this similarity, however, is not known (Basu *et al.*, 2002). The presence of introns is a major characteristic of the constitutive forms of the *HSP70* family, whereas the inducible forms do not contain introns (Iwama *et al.*, 1998). The lack of introns in the inducible *HSP70s* allows rapid translation of mRNA into nascent protein during stress (Basu *et al.*, 2002).

2. Differential expression of *HSC70-1* and *HSC70-2* in response to bacterial challenge

The heat shock protein (*HSP70*) family contains both inducible and constitutively expressed members. The role of inducible *HSP70* in immune system of fish is well characterized, but the function of *HSC70* during bacterial infection has yet to be elucidated. Until recently, studies reported that bacterial challenge modulated the mRNA levels of the cognate *HSC70* in various tissues of fish and shellfish. Ramaglia *et al.* (2004) reported an increase in protein levels of heat shock cognate, *HSC73* in liver and brain of the western painted turtle infected with *Citrobacter spp*. the causative agent of septicemic cutaneous ulcerative dermatitis. Deane and Woo (2004) reported an increase in expression of *HSC70* in liver of silver sea bream after *Vibrio alginolyticus* challenge. Wuchang bream received an injection of *A. hydrophila* showed that the *HSC70* levels in liver tissue was peaked at 6 h and decreased to baseline after 24 h (Ming *et al.*, 2010). Tiger prawn (*Penaeus monodon*) exposed to *V. harveyi* had increased levels of heat shock cognate *HSP70* in gill tissue after 12 h of treatment and the expression returned to the same levels as that of controls after 72 h (Rungrussamee *et al.*, 2010). In addition, Yue *et al.* (2011) reported the *HSC71* mRNA levels in hepatopancreas of gill of Asiatic hard clam were significantly increased at 12 h post injection of *V. parahaemolyticus* and decreased to the baseline at 24 h.

In this study, bacterial infection differentially induced the expression of both *HSC70*s in bighead catfish tissues during a 48-h period following *A. hydrophila* challenge. The *HSC70-1* was constitutively expressed at low levels and was slightly induced by bacterial infection in most tissues. The *HSC70-2* however, was slightly expressed in brain and gills and moderately up-regulated with stress caused by bacterial challenge in liver and skeletal muscle. The mRNA levels of *HSC70-2* was peaked at 24 h and returned to the same levels as that of the control after 48 h. In contrast, the *HSC70-2* transcript in skeletal muscle gradually increased after 6 h and its level was highest at 48 h. Stress-inducible *HSC70* isoforms have been reported in zebrafish, common carp, and yellowtail. The previous studies have demonstrated that bacterial challenge stimulates up-regulation of the inducible *HSP70* in various immune tissues of teleost fish, including and tissues (haemocytes, hepatopancreas, and muscle) of shellfish (oyster, scallop, and shrimp). Expression patterns of these genes were different under various stressed conditions, e.g., heat stress, cold shock, and heavy metal treatment, and appeared to be tissue-specific. For instance, common carp *HSC70-1* was expressed at high levels in liver after cadmium exposure, while the expression of common carp *HSC70-2* was induced by cold shock in muscle (Ali *et al.*, 2003).

The bacteria *A. hydrophila* is a causative agent of hemorrhagic septicemia, the important disease in freshwater fish. Systemic infection results in mortality within 24-48 h. The most common clinical signs and lesions are hemorrhage in skin, fins, oral cavity and muscles. Histological changes include focal cellular necrosis in the liver and haemopoietic cells, heart and skeletal muscles. With the septicemia, there may be depletion and necrosis of the renal hematopoietic tissue.

Liver plays a significant role in homeostasis and defense mechanism. Ming *et al.* (2010) reported variable levels of *HSP70* and *HSC70* induction in liver tissue of Wuchang bream in response to bacterial injection. It has been suggested that dramatically increased transcription of *HSC70* may indicate their important roles as molecular chaperones under oxidative stress. In cultured cells, yellowtail *HSC70-1* was constitutively expressed under normal and heat-shock conditions whereas the

yellowtail *HSC70-2* was up-regulated by heat shock treatment (Yabu *et al.*, 2010). My study showed similar findings with these reports, suggesting that *HSC70-1*was constitutively expressed in most tissues whereas the expression of *HSC70-2* was induced by bacterial infection in a tissue-specific manner.

The role of *HSP70* in mediating the initiation of an immune response as a result of bacterial infection has been implicated in aquatic species (Triantafilou *et al.*, 2001). Several stimuli that result from bacterial infection and wounding induces an acute phase response (APR) of fish immune system (Bayne and Gerwick, 2001). The APR involves the increase in synthesis and secretion by the liver of several plasma proteins, including the pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and TNFα. During infection, fish macrophages respond by up-regulating the expression and secretion of cytokines to activate a stress response. *HSPs* have been reported to directly induce macrophages to secrete inflammatory cytokines. Studies demonstrated that lipopolysaccharide (LPS) or endotoxin, the major component of the outer membrane of gram-negative bacteria, induces *HSP70* in rat heart. Recombinant *Trypanosoma carassii HSP70* is found to induce up-regulation of inflammatory cytokines and chemokines in macrophages of goldfish (Oladiran and Belosevic, 2009). In addition, *HSPs* are thought to be involved in antigen processing and presentation (Kaufmann, 1990).

In this study, the significant increase in the expression of *HSC70-2* in bighead catfish liver and muscle may correlate with the role of *HSC70-2* in immune response of these tissues after bacterial infection. Ming *et al.* (2010) reported that *A. hydrophila* injection modulated the expression of *HSC70* and *HSP70* in liver of wuchang bream in a time-dependent pattern. Both *HSC70* and *HSP70* mRNA levels significantly increased at 6 h and returned to baseline at 24 h. The similar pattern of *HSP70* expression was observed, after walking catfish were infected with *A. hydrophila* in this study. The *HSC70-2* mRNA concentrations were peaked at 24 h and decreased to the same level of the control at 48 h. Unlike liver, the *HSC70-2* mRNA level was very high in skeletal muscle at 48 h. Because fish infected with *A. hydrophila* showed

severe skin and muscle lesions at the injected site, the high level of *HSC70-2* in muscle may suggest the involvement of *HSC70-2* in cell protection and survival.

The expression of *HSP70-2* in gills of bighead catfish was slightly increased after injection with *A. hydrophila* at 24 h. It was likely that *HSC70-2* was insensitive to the bacteria at the early hours of injection. The increased levels of *HSC70-2* in gills may correlate with the osmotic regulation in bighead catfish.

The *A. hydrophila* infection did not modulate the expression of both *HSC70* isoforms in brain of bighead catfish. The absence of an increase of *HSC70s* in response to bacterial injection might be ascribed to the function of brain tissue. Because brain is the important organ of fish, it is well-protected from pathogenic bacteria. There has been no report on the role of *HSC70* in brain tissues of other teleosts under infectious challenge. Nevertheless, a study in western painted turtle (*Chrysemys picta bellii*) reported that bacterial infection induced the significant mRNA levels of constitutive *HSP73* in the brain (Ramaglia *et al.*, 2004). The concentration of *HSP70* was very high in brain compared to other tissues such as liver, heart and muscle. This correlates well with the high level of *HSP73* expression reported in mammalian brain by Manzerra *et al.* (1997). They suggest that high levels of *HSP73* in neuronal tissue could reflect its involvement in axonal translocation of proteins and synaptic vesicle recycling. In tilapia (*Oreochromis mossambicus*), Yang *et al.* (2009) reported that hyperosmotic stress induced transient expression of brain *HSP70*, which suggested its role in facilitating adaptation to seawater environment.

3. Oocyte development under pond conditions and chronic heat stress

Bighead catfish has a long spawning period due to its asynchronous oocyte development. As a result, the fish can spawn several times in one season (Jalabert, 2005). Reproductive seasonality of tropical freshwater fishes – including bighead catfish – is stimulated by environmental cues, particularly rainfall. Reproductive development of bighead catfish, which can be measured by gonadosomatic index (GSI), usually is associated with seasonal rainfall (Panprommin *et al.*, 2008).

Generally, the onset of reproduction occurs at the beginning of rainy season (May) and continues through October. Results in this study indicated that the highest reproductive investment of 11-month old female fish reared in earthen ponds was in July (GSI = 13.91±3.63%) when the amount of rainfall was highest (313 mm). At the end of the rainy season from October until January, the GSI significantly decreased, with the lowest value in November (0.41±0.26%). Fluctuations in water temperature in the earthen pond, however, did not appear to relate with changes of GSI.

The reproductive cycle of bighead catfish resembles that of African catfish and can be divided into three different phases; a pre-spawning phase (recrudescence of the ovary), a spawning phase, and a resting phase (Jarimopas *et al*., 1995). In the present study, bighead catfish displayed a long breeding season from February to September, during which recruitment of ovarian follicles to maturation resulted in a 115% increase in GSI. The highest numbers of post-vitellogenic (stage VI) oocytes (58.8±1.3%) were observed as the GSI value peaked in July. At the end of breeding between October and November, GSI was lowest, with the absence of advanced-stage eggs (stages V and VI) in the ovaries. Based on GSI values and distribution of oocytes, induced breeding by hormone injection for bighead catfish can be performed as early as February through September. Induced spawning, however, would be difficult outside the natural reproductive season, i.e., from October to January, when previtellogenic oocytes dominate the ovaries. As in other teleost species, endogenous mechanisms of reproduction are well understood in bighead catfish, but little is known about environmental control of their reproductive cycle Nevertheless, results of the present study implied that endogenous mechanisms rather than environmental factors synchronized regression of the ovary in bighead catfish. The inference is based on the observation that ovarian regression occurred in October and November despite favorable conditions to oocyte development, i.e., moderate amount of rainfall and optimal temperature. In some species, ovarian regression coincides with the presence of a refractory period during which ovarian recrudescence is suppressed regardless of environmental conditions and their origin. It has been well recognized that in temperate fishes, the reproductive cycle is strongly affected by seasonal changes in water temperature and photoperiod. These environmental factors may have direct or

indirect effects on initiation of oogenesis, production of vitellogenin, or secretion of pituitary and reproductive hormones.

Although temperature is considered less important in tropical species, several studies have documented the influences of temperature on reproductive maturation. For instance, in African catfish, ovarian development was enhanced and the resting period was shortened from 4-7 months to 2 months when the fish were kept at a constant 25°C water temperature compared to those kept in outdoor ponds with ambient temperature (15° to 30°C). Additionally, the authors suggest that water temperature plays a more significant role in regulating the ovarian cycle than photoperiod. In snakehead, a combination of warm temperature (30°C) and long photoperiod stimulated reproduction and extended the spawning period during the annual reproductive cycle. In this study, ovarian recrudescence was stimulated at least one month ahead of season in the fish kept in the hatchery compared to those kept in earthen pond. Increased quantities of vitellogenic eggs were observed in females held at warm temperatures after six weeks of exposure. Moreover, ovaries of the control fish held at 25°C also contained low proportions of vitellogenic eggs (6.5±0.6%). In contrast, no vitellogenic oocytes were observed in fish held in earthen pond under seasonal temperature regime (27±2.5°C) during October and November. As in other teleost species, endogenous mechanisms of reproduction were well understood in tropical species, but little is known about environmental control of their reproductive cycle. It is not clear how temperature exerts its effects on recrudescence and regression of the ovary in bighead catfish. Studies suggest that temperature may have direct or indirect effects on initiation of oogenesis, production of vitellogenin, or secretion of pituitary and reproductive hormones. For tropical species, warm temperatures were found to stimulate the formation of yolk vesicles in maturing oocytes. Nevertheless, results of the present study implied that endogenous mechanisms rather than environmental factors synchronized regression of the ovary. The inference is based on the observation that ovarian regression occurred in October and November despite favorable conditions to oocyte development, i.e., moderate amount of rainfall and normal temperature. In some species, ovarian regression coincides with the presence

of a refractory period during which ovarian recrudescence is suppressed regardless of environmental conditions.

4. Primary stress response and heat shock protein expression

Bighead catfish exposed to constant temperature at 35°C had higher levels of cortisol compared to fish held at 25°C (control) and 30°C and the cortisol concentration in fish held at 35°C remained high throughout the experiment. Generally, a fish has the ability to adapt to altered environments caused by long-term stress (Barton, 1997). However, a slight reduction in cortisol levels after 6 weeks of heat exposure implied that stress due to high temperature may exceed the adjustment ability of bighead catfish. In such a case, a longer period of thermal stress would severely affect growth, reproduction and immune response in fish (Iwama *et al.*, 1997; Barton, 1997). In addition to its role in physiological stress response, cortisol is known to mediate the effects of stress on fish reproduction (Pankhurst and Van Der Kraak, 1997). The release of cortisol into the circulation can have either stimulatory or inhibitory effects on the reproductive capacity of fish, depending on the stage of gametic development (Pankhurst and Van Der Kraak, 1997). Cortisol is thought to accelerate vitellogenesis, which is inferred from a higher level of plasma cortisol in maturing females than in fully mature females (Billard *et al.*, 1981). In some species, long-term elevation of cortisol suppressed the production of mature oocytes (Pankhurst and Van Der Kraak, 1997). For bighead catfish, increased levels of cortisol may enhance the production of vitellogenin, which was inferred by an increase in number of stage-V oocytes in ovaries of females kept in the hatchery.

The present study demonstrated that temperature played a major role in stimulating ovarian development of bighead catfish during a resting period of their reproductive cycle. The results of this study indicated that ovary recrudescence and formation of mature oocytes were stimulated at least one month ahead of the natural season, when females were kept in the hatchery at constant temperatures (30 and 35°C) for six weeks. Increased plasma levels of cortisol due to heat exposure and the proportions of vitellogenic oocytes implied a stimulatory effect of cortisol on synthesis

of yolk proteins. Although fully matured females could not be obtained by short-term exposure to warm temperatures in the hatchery, the present study suggested that temperature manipulation was probably the most practical way to increase the number of maturing female broodstock at the end of breeding season. Further research is required to determine the effects of cortisol on final maturation as well as the possibility of manipulations of other environmental factors to enhance year-round breeding for breeding catfish.

5. Stress response to heat exposure

Response to stress caused by a 6-week exposure to high temperature was assessed by measuring plasma cortisol and glucose concentrations, and tissue expression of heat shock proteins *HSC70* and *HSC90*. Increased levels of plasma cortisol, which represented initial response to stress were observed in fish held at elevated temperatures and were highest at 35°C after 4 weeks of exposure, followed by a slight decrease at week 6. Cortisol concentrations, however, did not differ at control (25°C) and 30°C and were unchanged throughout the experiment. Cortisol is known to play a role in activating glucose production, particularly in liver by gluconeogenesis and glycogenolysis, to meet increased energy demand of fish (Iwama *et al.*, 1999). Lack of reduction in cortisol levels following 6 weeks of heat exposure implied that chronic heat stress may exceed the adjustment ability of bighead catfish. In such a case, a longer period of heat stress would severely affect growth, reproduction and immune response in fish. Generally, a fish has the ability to adapt to altered environments caused by chronic stress. Adaptation involves a series of physiological responses in metabolic pathways that promote survival of fish under extreme conditions, and varies according to species, developmental stage, nutritional state, as well as severity and duration of stress (Barton, 1997).

In addition to its role in physiological stress response, cortisol is known to mediate the effects of stress on fish reproduction (Pankhurst and Van Der Kraak, 1997). The release of cortisol into the circulation can have either stimulatory or inhibitory effects on the reproductive capacity of fish, depending on the stage of

gametic development. Cortisol is thought to accelerate vitellogenesis, which is inferred from a higher level of plasma cortisol in maturing females than in fully mature females (Billard *et al.*, 1981). In bighead catfish, increased levels of cortisol due to heat stress may enhance the production of vitellogenin, which was inferred by a tremendous increase in yolk granules of stage-V oocytes. However, augmentation of cortisol using implants in mature tilapia *Oreochromis mossambicus* resulted in depression of plasma levels of estrogen and reduced ovary size (Foo and Lam, 1993).

Expression analysis revealed that *HSC70* and *HSC90* proteins were constitutively expressed at high levels in liver tissue under normal pond conditions. The abundance of *HSP* transcript there would be expected because liver is a vital organ regulating homeostasis in the fish body. Similar expression profiles for *HSC70* and *HSC90* were observed in gills, muscle, and brain of female bighead catfish, suggesting that these proteins have a significant functional role in a wide range of normal cells (Deane *et al.*, 1999; Basu *et al.*, 2002; Deane *et al.*, 2002). Fluctuations in *HSC70* and *HSC90* gene expression during a 12-month period indicated that seasonal changes in water temperature and rainfall may affect the response of *HSC70* and *HSC90*. In the present study, chronic heat stress induced accumulation of *HSC70* and *HSC90* mRNA, with the highest levels in liver tissue following 4 weeks of exposure at 35°C. However, the *HSC70* and *HSC90* transcript decreased markedly at week 6 and then was lower than that of control fish. The reduction in *HSP* synthesis during heat exposure has been related to a regulatory role of stress hormones on heat shock proteins. For instance, an increased level of plasma cortisol was found to reduce *HSP70* expression in gill tissue of rainbow trout (Basu *et al.*, 2001). In such a case, plasma cortisol and heat shock proteins are thought to interact through the role of *HSC70* and *HSC90* in regulating the activity, folding, and transportation of nascent glucocorticoid receptor (Pratt, 1993). Under stressful conditions, cortisol binds to glucocorticoid receptor and forms a complex with heat shock proteins. The increase in plasma cortisol results in a decreased amount of unbound glucocorticoid. Therefore, an excess amount of *HSPs* may exert a negative feedback on heat shock protein transcription (Basu *et al.*, 2001).

CONCLUSIONS AND RECOMMENDATIONS

Stress in aquaculture is unavoidable and can have negative impacts on production. Among sources of stress, infectious diseases are major problem which can result in high mortality during developmental and growth stages. The common disease found in bighead catfish culture is bacterial septicemia caused by *A. hydrophila*. The infection occurs most during change in the temperature when cold weather moves in, especially at the end of breeding season. Prevention and cure of this disease are relied on the conventional approaches such as antibiotic treatment. Until recently, alternative treatment strategies based on DNA vaccine derived from heat shock proteins (HSPs) have been demonstrated. These findings have encouraged research on heat shock proteins in other fish and shellfish. In this thesis, I cloned two heat shock cognate protein 70 (HSP70) and determined their expression in response to *A. hydrophila* injection in bighead catfish.

Stress due to increased water temperature is found to have stimulatory or inhibitory effects on fish reproduction. Although temperature is considered less important in tropical species, temperature manipulation has been used to enhance reproductive maturation in a number of farmed fish. In addition, a year round production of fry is possible when a spawning phase can be extended by controlled temperatures. In this thesis, I demonstrated the possibility of using increased temperature to stimulate ovarian development of bighead catfish during post-spawning season. In addition, the manipulation of temperature may induce stressful conditions for the fish. Therefore, the indicators of stress were measured at the physiological and cellular levels.

The first and fourth experiments were conducted to obtain the cDNA sequences and genomic structures of *HSP70-1* and *HSP70-2* in bighead catfish and to determine their expression in response to bacterial infection. The complete cDNA sequences of these two genes were 2,278 bp in length which contained the open reading frame of 1,950 bp, encoding a 649 amino acid protein. The molecular mass was 71.24 kDa for HSC70-1 and 71.27 kDa for HSC70-2. HSC70-1 and HSC70-2

shared 94% similarity in amino acid sequences. The amino acid sequences of bighead catfish HSC70-1 and HSC70-2 shared 82-95% and 82-91% identity of amino acids with other teleosts, chicken and human. The genomic structure of *HSC70-1* (3,179 bp) consisted of seven introns (901 bp) and eight exons (1,870 bp) while that of *HSC70-2* consisted of seven introns (702 bp) and eight exons (1,950 bp). Genomic structures of the two loci were similar to those of other teleost fish and mammals. The presence of introns is the characteristic of the constitutive forms of heat shock protein 70.

An infection by *A. hydrophila* differentially induced the expression of both *HSC70*s in bighead catfish tissues during a 48-h period. The expression of *HSC70-1* was low in most tissues while *HSC70-2* was moderately up-regulated in liver and skeletal muscle. The increased expression of HSC70-2 in these tissues may correlate with the role of HSC70-2 in immune response after bacterial infection. Research on heat shock protein in bighead catfish is in its early stage compared with those in other teleosts. This study provides information on molecular biology of HSC70s and contributes to better understanding of the heat shock protein effects on bacterial infection in this species.

The second and third experiments demonstrated the use of increased water temperature to stimulate ovarian growth of bighead catfish and the effects of heat stress to fish body. Oocyte development was examined in females raised under pond conditions during April 2008 to March 2009 and was used as baseline information. From April to September 2008, the gonadosomatic index (GSI) increased 115%. The GSI value peaked in July and was lowest at the end of breeding period between October and November. In October, 13 month-old female fish were randomly collected from an earthen pond and exposed to different temperatures at 25, 30 and 35 °C under hatchery conditions for 6 weeks. The stimulatory effect of heat stress resulted in an increased proportion of vitellogenic (stage V) oocytes in females held under 30-35°C after 4 to 6 weeks. At the same time, only immature oocytes were observed in ovaries of females held under pond conditions. Nevertheless, it is important to measure the stress indicators during the experiment. In this study, fish exposed to constant temperature at 35°C had higher levels of cortisol which remained

high throughout the experiment. The increased levels of cortisol may enhance the production of vitellogenin, resulting in an increase in number of stage V oocytes in ovaries of females kept in the hatchery. However, the unchanged cortisol levels following 6 weeks of increased temperature may become adversary to the fish if they were to be held under this condition for a longer period.

The measurement of cellular stress response indicated that chronic heat stress induced accumulation of *HSC70* and *HSC90* mRNA, with the highest levels in liver tissue following 4 weeks of exposure at 35°C and decreased markedly at week 6. The reduction in heat shock protein synthesis during heat exposure has been related to a regulatory role of cortisol through transportation of nascent glucocorticoid receptor. Under stressful conditions, cortisol binds to glucocorticoid receptor and forms a complex with heat shock proteins. The increase in plasma cortisol results in a decreased amount of unbound glucocorticoid. Under pond conditions, *HSC70* and *HSC90* were constitutively expressed at a higher level in liver.

Finally, the findings in this thesis have provided recommendations for the application of knowledge on bighead catfish HSC70s and the use of temperature manipulation to enhance reproduction of female brood fish as follows:

1. The role of HSP70 is well characterized and its functions in immune response have been determined in many species of fish and shellfish. Therefore, it would be useful to determine the molecular structure of this inducible form of heat shock protein 70 in bighead catfish.

2. The role of HSC70s in response to other stressors such as heat, heavy metals and other infectious diseases such as viral infection and parasites should be investigated for better understanding of the cellular functions of these stress proteins.

3. New prevention methods to infectious diseases in bighead catfish such as DNA vaccine can be developed based on knowledge of the gene information of HSC70s.

4. The findings of this study suggest the practicality of using warm temperature to extend spawning period of bighead catfish. To obtain higher percent of mature oocytes, fish should be exposed to increased temperature (30-35°C) at least one month before the end of spawning season.

5. Due to the increased levels of cortisol in fish under prolonged heat stress, care must be taken to adjust temperature level and stocking density in the aquaria.

6. Because the reproductive cycle of bighead catfish resembles that of African catfish, it is likely that the methods used to extend spawning period in African catfish can be practiced in bighead catfish. For instance, the experiment can be designed to keep females at a constant 30°C water temperature year round and comparison of ovarian growth monthly should be made to those kept in outdoor ponds with ambient temperature.

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APPENDICES

Appendix A

Effects of Warm Temperatures on Ovarian Development of Walking Catfish *Clarias macrocephalus* (Günther, 1864) During Post-Spawning Season

Kasetsart J. (Nat. Sci.) 46 : 759 - 768 (2012)

Effects of Warm Temperatures on Ovarian Development of Walking Catfish *Clarias macrocephalus* (Günther, 1864) During Post-Spawning Season

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ABSTRACT

This study aimed to determine the possibility of using temperature manipulation to stimulate ovarian activities of *Clarias macrocephalus* during the post-spawning season. The ovarian development and monthly change in the gonado-somatic index (GSI) of females held under pond conditions was monitored over a 1 yr period from April 2008 to March 2009. In October, 13 mth-old female fish (weight ± SD= 108 ± 12.7 g) were randomly collected from an earthen pond and exposed to different temperatures at 25, 30 and 35 °C under hatchery conditions for 6 wk. The results indicated that the change in the GSI appeared to be correlated with the amount of rainfall, but not to the variation in seasonal temperature. A significant ($P < 0.05$) variation of mean GSI values was observed among months with the highest value ± SE (13.91 ± 3.63%) in July. Histological examination of ovaries revealed that in females held under 25, 30 and 35 °C, the number of vitellogenic oocytes progressively increased from 0.5–4.7% at week 2 to 4.6–19.7% at week 6, whereas in females held in the earthen pond, ovaries contained only immature oocytes. Exposure to warm temperatures resulted in significant elevations of plasma cortisol but not glucose concentrations. Although fully matured females could not be obtained, the present study suggested that temperature manipulation was probably the practical way to increase the number of maturing females during the off reproductive season for walking catfish.

Keywords: *Clarias macrocephalus*, gonado-somatic index, ovarian development, reproductive season, vitellogenesis

INTRODUCTION

Aquaculture of catfish *Clarias macrocephalus* (Clariidae) is well established in Thailand and it has been a popular species for domestic consumption for more than 40 yr (Na-Nakorn *et al*., 1993). Since the early 1990s, a hybrid catfish (*C. macrocephalus* × *C. gariepinus*) has replaced the culture of *C. macrocephalus* because it grows rapidly and is more resistant to diseases (Nukwan *et al*., 1990). In 2009, hybrid catfish production was ranked second behind tilapia production with a total of 130,064 t valued at THB 4.9 billion (Department of Fisheries, 2009). The expansion of hybrid catfish farming has led to increased demand for *C. macrocephalus* broodstock. Moreover, the availability of ripe females is low at the end of the breeding season, precluding year-round fry production.

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Received date : 11/06/12 Accepted date : 10/09/12

Female *C. macrocephalus* reach sexual maturity at about one year of age. In Thailand, the spawning season starts at the beginning of the rainy season in May and continues through September (Jarimopas *et al*., 1995; Panprommin *et al*., 2008). It is well understood that induced spawning of catfish by hormone injection outside the natural breeding season is difficult due to low numbers of maturing oocytes (Tan-Fermin *et al*., 1997a; Panprommin *et al*., 2008).

Temperature is the important environmental factor influencing the reproductive cycle and maturation in fish. Long-term manipulation of water temperature has been employed successfully to stimulate ovarian development and to extend the spawning period in African catfish *C. gariepinus* (Richter *et al*., 1987), snakehead *Channa punctatus* (Srivastava and Singh, 1991), and in cold water species such as striped bass *Morone saxatilis* (Clark *et al*., 2005). However, temperature manipulation, has not been practiced to enhance the reproduction capacity of *C. macrocephalus*. The present study determined the possibility of using warm temperatures to stimulate oocyte development at the end of the breeding season. In October 2008, 13 mth-old female walking catfish were randomly selected from an earthen pond and subjected to warm temperatures at 30 °C and 35 °C in the hatchery for 6 wk. The timing and duration of exposure coincided with the post-spawning period of the catfish. The seasonal changes of reproductive development under pond conditions were monitored over 1 yr from April 2008 to March 2009 by measuring the gonado-somatic index (GSI) and histological examination of ovaries. Because exposure to increased water temperatures may be stressful, plasma cortisol and glucose concentrations were measured and compared among different groups of females to determine their responses to temperature stress.

MATERIALS AND METHODS

Fish rearing

Fish used in this study were produced by the artificial fertilization of 40 females (weight ± SD= 210 ± 26 g) and 10 males (weight ± SD = 202 ± 12 g) in August 2007 and were obtained from the Pathumthani Inland Fisheries Research and Development Center, Pathum Thani province. Larvae were reared in concrete tanks (2 × 3 × 1 m) at a stocking density of 3,000 m^{-2} for 2 mth (length ± SD = 5.7 ± 1.6 cm). Thereafter, 3,000 2 mth-old juvenile females were stocked in a 20 × 40 × 1 m earthen pond. They were fed commercial catfish pellets with 25% protein (2% bodyweight) twice daily at 0700 and 1600 hours. Water quality was measured every month throughout the experiment. Fifteen females were collected from the earthen pond monthly from April 2008 (age = 8 mth) to March 2009, measured and weighed. Ovaries were collected and weighed for calculating the GSI (gonad weight per bodyweight × 100).

Ovarian tissues were fixed in Bouin solution for 24 hr, washed in 79% ethanol, and dehydrated using an automated tissue processor. The tissues were embedded in paraffin, sectioned to 5–10 µm, mounted on slides and stained with haematoxylin and eosin. At least 300 oocytes from the anterior, middle and posterior parts of the ovary of each female were examined using a light microscope. Oocytes were classified into six stages of maturation according to Groman (1982): stage I, oocytes are undifferentiated and occur in nests with large nuclei and acidophilic cytoplasm; stage II, oocytes increase in size, each contains a large nucleus with basophilic chromatin; stage III, oocytes show a well defined follicular epithelium and basophilic cytoplasm; stage IV, oocytes are characterized by the appearance of euvitellin nucleoli along the nuclear membrane, the presence of yolk granules in the cytoplasm and a more distinct zona radiata in the cell membrane; stage V, oocytes are larger with abundant yolk vesicles,

showing fewer euvitellin nucleoli in the nucleus and a degenerating nuclear membrane; stage VI, oocytes are fully grown, containing large amount of acidophilic yolk granules in the cytoplasm. Additionally, oocytes of *C. macrocephalus* can be classified as immature or pre-vitellogenic (stages I to III), maturing or vitellogenic (stages IV and V), and mature or post-vitellogenic (stage VI) (Richter *et al*., 1987).

Ovarian development under warm temperatures

Three hundred female fish (age = 13 mth; weight ± SD = 108 ± 12.7 g) were collected from the earthen pond during October 2008 and transferred to 5 × 10 × 1 m concrete tanks 7 d prior to the experiment. One hundred and eighty fish were stocked randomly over fifteen 50 L aquaria (length, 90 cm; width, 45 cm; depth, 45 cm) at a density of 12 fish per aquarium. The fish were divided into three groups. Fish in the first group were maintained at an ambient temperature of 25 ± 2°C. Fish in the second and third groups were raised in aquaria containing aerated water heated by an immersion heater to 30 and 35 °C, respectively. The experimental fish were fed commercial pellets containing 25% protein at 2% bodyweight twice daily and maintained for 6 wk. The dissolved oxygen and pH were measured daily. Blood samples and ovaries were collected from 15 individuals from each group after 2, 4 and 6 wk of rearing. Based on information from the preliminary study, the range in temperature and the duration of exposure were nonlethal for this species under hatchery conditions.

Before tissue and blood collection, fish were anesthetized with 2 phenoxyethanol (0.75 $mg.L^{-1}$). Blood samples were taken from the caudal vein using 1 mL syringes coated with Na_2-EDTA (Titriplex III; Merck; Darmstadt, Germany), placed into 1.5 mL microcentrifuge tubes, and centrifuged at 1,500 rpm at 4 °C for 10 min. The plasma was stored at -20 °C until use. Cortisol levels were quantified by radioimmunoassay using a commercial kit (Cortisol Bridge kit, TKCO1; L&R Enterprise Co. Ltd.; Bangkok, Thailand). Plasma glucose was measured by a colorimetric test using a commercial kit (no. RA 122-10: End point; BIOTECH; Bangkok, Thailand).

Statistical analysis

Data on GSI, the percentage of eggs in different stages and the glucose and cortisol levels were presented as means ± SE. Statistical differences between groups were analyzed by one-way analysis of variance, followed by Duncan's multiple range tests for comparisons of means by the stage of development. Simple linear regression analysis was used to evaluate the relationship between GSI and rainfall as well as between GSI and the proportion of post-vitellogenic eggs. Analyses were performed using the Statistical Package for Social Sciences (SPSS 11.0; Chicago IL, USA). Results were tested for significance at the 95% level and highly significant results at the 99% level.

RESULTS

Reproductive cycle of walking catfish under pond conditions

Monthly changes in the GSI of mature females, the water temperature and rainfall were monitored over 1 yr (Figure 1). Water temperatures varied from 25 to 30 °C, with the lowest temperature in December and January. The amount of rainfall varied from 0 mm during December to February to 313 mm in July. Significant variation of mean GSI values was observed among months. From April until August, mean GSI values ranged from 5.7 ± 2.0% to 11.3 ± 6.3% with the highest value (13.9 ± 3.6%) in July. The GSI decreased in September and October and remained low in November and December (0.4 ± 0.2% to 0.9 ± 0.5%). From January to March, the GSI gradually increased from 2.2 ± 1.5% to 9.3 ± 2.1%.

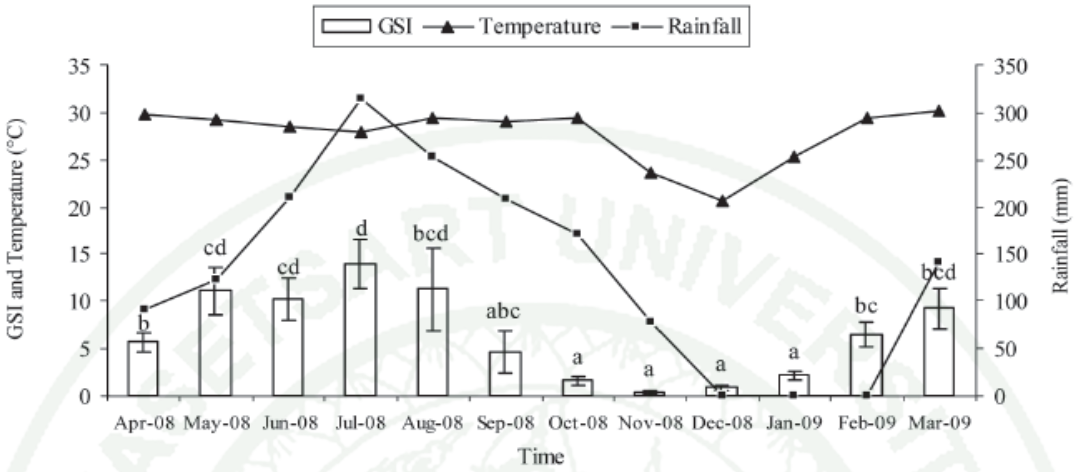

Figure 1 Gonado-somatic index (GSI) values, temperature (°C) and rainfall (mm) throughout the reproductive seasonality of female walking catfish. GSI levels associated with histogram bars with the same letters do not differ significantly ($P < 0.05$). Vertical bars represent ± SE.

Histological analysis of ovaries exhibited seasonal patterns of oocyte development that were classified into six stages. Mean oocyte diameters were 0.046, 0.062, 0.185, 0.35 and 1.0 mm for stages II to VI, respectively (Figure 2). During the natural breeding season from April to September, the ovaries contained large quantities of post-vitellogenic (stage VI) oocytes (34.1 ± 0.7 to 59.0 ± 1.3%; Figure 3). During the post-spawning season in October and November, no advanced oocytes (stages V and VI) were observed, but the ovaries contained high proportions of previtellogenic (stage III) oocytes (46.8 ± 1.4 to 63.1 ± 1.5 %). In December, oocytes in stages V (12.8 ± 1.6%) and VI (1.9 ± 0.4%) were observed and the proportions of stage VI oocytes gradually increased until March (26.7 ± 1.4%) when ovaries of females contained oocytes at all stages. A positive correlation was found between the GSI and the proportion of stage VI oocytes (r = 0.803, $P < 0.01$, Figure 4).

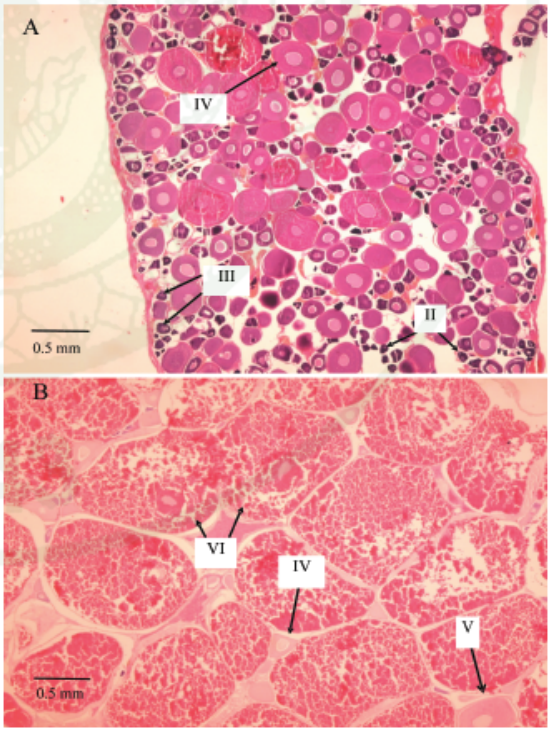

Figure 2 Oocytes at different developmental stages (II–VI) in the ovary of a mature female walking catfish: (A) Stages II–IV; (B) Stages IV–VI.

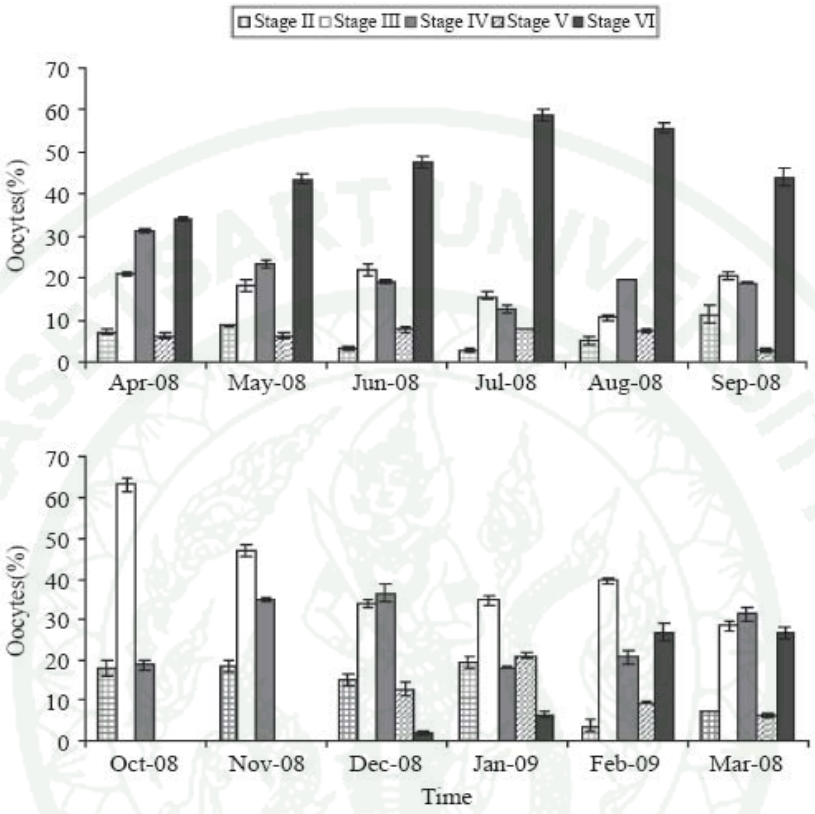

Figure 3 Proportion of oocytes at different developmental stages through the reproductive seasonality of female walking catfish. Vertical bars represent ± SE.

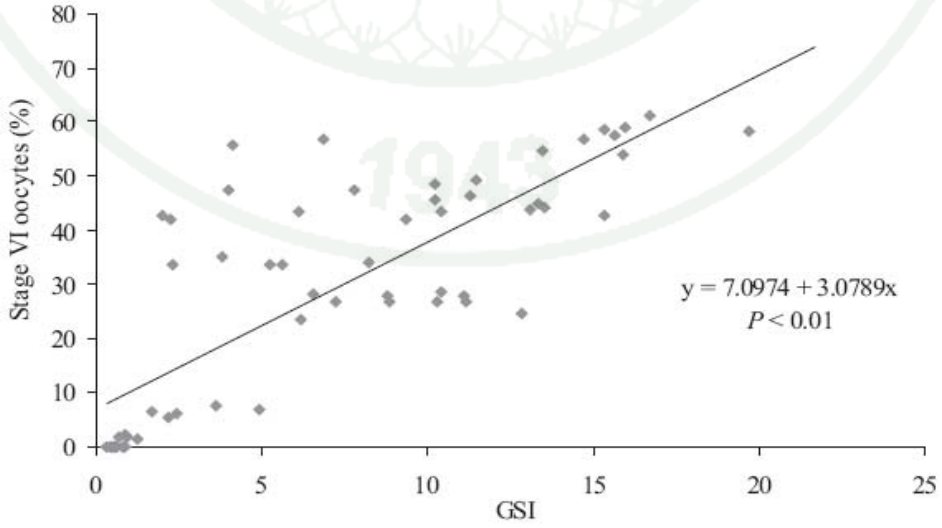

Figure 4 Correlation between gonado-somatic index (GSI) and stage-VI oocytes in female walking catfish.

Oocyte development under warm temperatures

Oocyte development was examined in fish exposed to different levels of warm temperature and at an ambient temperature of 25 °C (Figure 5). At week 2, the percentage of oocytes at stages II to IV was nearly identical among groups. At week 4, the oocyte development of females in the treated groups proceeded into stage V, while oocytes of females held at 25 °C as well as those of females in the pond remained at stages II to IV. The difference in ovarian growth was pronounced at week 6, with an increased percentage of vitellogenic (stage V) oocytes (19.7 ± 1.1%) in fish held at 35 °C compared to 6.5 ± 0.6 and 4.6 ± 1.7% in the 25 and 30 °C groups, respectively, but ovaries of females in the earthen pond were unchanged and contained only previtellogenic oocytes (Figure 4). Similarly, at week 6, the GSI values were higher (0.64–0.68 %) for fish in the 30 and 35 °C groups compared to 0.42–0.47% for those in the pond and fish held at 25 °C.

Plasma cortisol levels were higher at elevated temperatures during the 6 wk of exposure (Table 1). The cortisol concentration was unchanged at 25 °C and 30 °C. However, at 35 °C, the cortisol level was highest at week 4 (313.6 ± 30.5 ng.mL^{-1}) and slightly decreased to 251.5 ± 18.5 ng.mL^{-1} at week 6. Although glucose concentrations (123.4 ± 12.7 mmol.L^{-1}) were highest at 35 °C in week 6, the values did not differ significantly across temperature levels throughout the experimental period.

DISCUSSION

Reproductive seasonality of tropical freshwater fishes—including walking catfish—is synchronized by environmental cues, particularly rainfall (Freund *et al*., 1995; Tan-Fermin *et al*., 1997b). In Thailand, the onset of *C. macrocephalus* reproduction occurs at the beginning of the rainy season (May) and continues through October. The results in the current study indicated that the highest reproductive investment of female catfish reared in an earthen pond was in July (GSI = 13.91 ± 3.63%) when the amount of rainfall was highest (313 mm). At the end of the rainy season from October until January, the GSI significantly

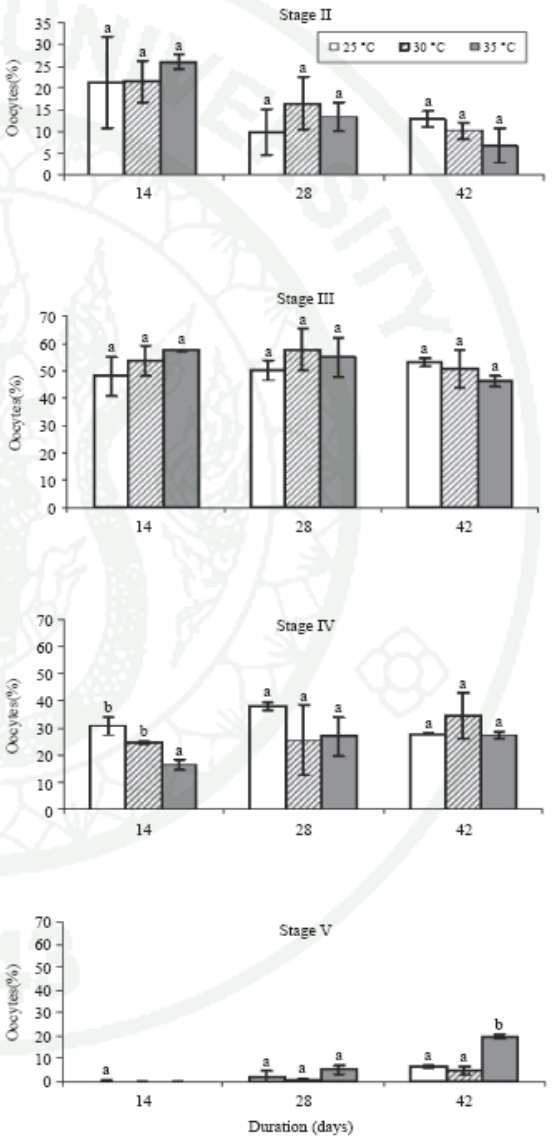

Figure 5 Proportions of oocytes at different developmental stages (II–V) in females held under elevated temperatures for different durations. Values with different letters for each developmental stage indicate significant differences ($P < 0.05$). Vertical bars represent ± SE.

decreased, with the lowest value in November (0.41 ± 0.26%). These results were in agreement with those of Panprommin *et al*., (2008). However, the GSI values in the present study were lower than those reported by Tan-Fermin *et al*., (1997b), most likely due to the differences in age and size of the females and the culture conditions.

During February to September, recruitment of ovarian follicles to maturation resulted in a 115% increase in the GSI. The highest numbers of post-vitellogenic (stage VI) oocytes (58.8 ± 1.3%) were observed as the GSI value peaked in July. At the end of breeding between October and November, the GSI was lowest, with the absence of advanced-stage eggs (stages V and VI) in the ovaries. Based on the GSI values and distribution of oocytes, induced spawning by hormone injection can be performed as early as February and continue through September. However, induced spawning would be difficult from October to January, when pre-vitellogenic oocytes dominate the ovaries. Generally, only stage VI eggs can be induced by hormone treatment in which ovulation occurs within 16–18 hr post injection (Ngamvongchon *et al*. 1988). A study by Tan-Fermin *et al*. (1997a) reported that induced spawning of 2 yr-old females throughout the reproductive cycle was possible, because the ovaries contain a large number of post-vitellogenic oocytes for which development is arrested during the off-season.

As in other teleost species, endogenous mechanisms of reproduction are well understood in walking catfish, but little is known about the environmental control of their reproductive cycle (Ngamvongchon *et al*., 1988; Freund *et al*., 1995; Tan-Fermin *et al*., 1997a; Tan-Fermin *et al*., 1997b). Nevertheless, the results of the present study implied that endogenous mechanisms rather than environmental factors may suppress reproductive activity. The inference is based on the observation of reduced ovarian size in October and November despite favorable conditions for oocyte development, that is, a moderate amount of rainfall and optimal temperature. In some species, ovarian regression coincides with the presence of a refractory period during which ovarian recrudescence is suppressed regardless of the environmental conditions and their origin (Lam, 1983; Richter *et al*., 1987; Freund *et al*., 1995).

Although temperature is considered less important in tropical species (Lam, 1983), several studies have documented the influence of temperature on reproductive maturation. For

Table 1 Cortisol and glucose data (mean ± SE) for female walking catfish exposed to different temperatures for 14, 28, and 42 d.

	Cortisol ($ng.mL^{-1}$)	Glucose ($mmol.L^{-1}$)
Week 2		
25 °C	157.46 ± 9.53	79.57 ± 5.01
30 °C	121.43 ± 3.14	81.86 ± 11.12
35 °C	299.67 ± 29.30*	101.25 ± 8.17
Week 4		
25 °C	199.72 ± 10.75	61.72 ± 5.46
30 °C	133.69 ± 17.18	79.13 ± 3.98
35 °C	313.61 ± 30.50*	92.67 ± 3.83
Week 6		
25 °C	104.93 ± 8.01	73.84 ± 10.53
30 °C	133.16 ± 5.18	71.92 ± 7.19
35 °C	251.54 ± 18.62*	123.41 ± 12.70

* = Significant difference ($P < 0.05$) within week.

instance, in African catfish (*C. gariepinus*), ovarian development was enhanced and the resting period was shortened from 4–7 mth to 2 mth when the fish were kept at a constant 25 °C water temperature compared to those kept in outdoor ponds with ambient temperature fluctuations of 15° to 30 °C (Richter *et al*., 1987). Additionally, the authors suggested that water temperature plays a more substantial role in regulating the ovarian cycle than photoperiod. In snakehead fish, a combination of warm temperature (30 °C) and long photoperiod stimulated reproduction and extended the spawning period during the annual reproductive cycle (Srivastava and Singh, 1991). In the present study, increased quantities of vitellogenic eggs were observed in females held at warm temperatures after 6 wk exposure. Moreover, ovaries of fish held at 25 °C also contained low proportions of vitellogenic eggs (6.5 ± 0.6%). In contrast, no vitellogenic oocytes were observed in fish held in the earthen pond under a seasonal temperature regime (27 ± 2.5 °C) during October and November. It is not clear how an elevated temperature exerts its effects on oocyte recruitment in walking catfish. Studies have suggested that the temperature may have direct or indirect effects on the initiation of oogenesis, the production of vitellogenin or the secretion of pituitary and reproductive hormones (Lam, 1983; Richter *et al*., 1987). For tropical species, warm temperatures were found to stimulate the formation of yolk vesicles in maturing oocytes (Lam, 1983). In snakehead fish, high temperatures were reported to induce a release of gonadotropin, resulting in complete maturation of the ovary (Srivastava and Singh, 1991).

Walking catfish exposed to a constant temperature at 35 °C had higher levels of cortisol compared to fish held at 25 and 30 °C and the cortisol concentration in fish held at 35 °C remained high throughout the experiment. Generally, a fish has the ability to adapt to altered environments caused by long-term stress (Barton, 1997). However, a slight reduction in cortisol levels after 6 wk of heat exposure implied that stress due to high temperature may exceed the adjustment ability of walking catfish. In such a case, a longer period of thermal stress would severely affect growth, reproduction and the immune response in fish (Barton, 1997; Iwama *et al*., 1997). Cortisol is known to mediate the effects of stress on fish reproduction in addition to its role in physiological stress response (Pankhurst and Van Der Kraak, 1997). The release of cortisol into the circulation can have either stimulatory or inhibitory effects on the reproductive capacity of fish, depending on the stage of gametic development (Pankhurst and Van Der Kraak, 1997). Cortisol is thought to accelerate vitellogenesis, which is inferred from a higher level of plasma cortisol in maturing females than in fully mature females (Billard *et al*., 1981). In some species, the long-term elevation of cortisol suppressed the production of mature oocytes (Pankhurst and Van Der Kraak, 1997). For walking catfish, increased levels of cortisol may enhance the production of vitellogenin, which was inferred by an increase in number of stage-V oocytes in the ovaries of females kept in the hatchery.

CONCLUSION

The present study demonstrated the use of warm water temperatures to stimulate the ovarian development of female catfish at the end of the natural breeding season. Significant proportions of vitellogenic oocytes were observed when females were kept in the hatchery at constant temperatures (30 and 35 °C) for 6 wk compared to fish held at 25 °C. Increased plasma levels of cortisol in treated fish may have a stimulatory effect on yolk protein synthesis. Although post-vitellogenic eggs could not be obtained by short-term exposure to warm temperatures in the hatchery, this study suggested that temperature manipulation was probably the practical way to increase the proportion of maturing oocytes during the off-season. It would be useful to determine whether long-term exposure, that is 3 mth of heat stress during the dry season, can

induce the formation of post-vitellogenic oocytes. However, factors such as stocking density and water quality also can cause additional stress in fish and may affect their reproductive performance. Further research is required to determine the effects of cortisol on final maturation as well as the possibility of manipulating other environmental factors to enhance the year-round breeding of walking catfish.

ACKNOWLEDGEMENTS

This work was supported by the Agricultural Research Development Agency (ARDA) and the Thailand Research Fund (Grant # RMU5180015).

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Appendix B

Cloning and Expression of Two HSC70 Gene in Walking Catfish *Clarias macrocephalus* (Günther, 1864) Challenged with *Aeromonas hydrophila*

Aquaculture Research

Aquaculture Research, 2012, 1–13 doi:10.1111/are.12076

Cloning and expression of two HSC70 genes in walking catfish *Clarias macrocephalus* (Günther, 1864) challenged with *Aeromonas hydrophila*

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Abstract

Two different cDNAs encoding heat shock cognate protein 70 genes were isolated from the liver of walking catfish. The complete cDNA sequences for walking catfish *HSC70-1* and *HSC70-2* were of identical length (2278 bp), with an open reading frame of 1950 bp and a predicted 649 amino acid protein. Genomic sequences of the walking catfish *HSC70-1* and *HSC70-2* genes were composed of eight exons and seven introns, but exon and intron sizes were different. Amino acid sequences of both proteins shared 94% similarity with 38 substitutions. The walking catfish HSC70-1 and HSC70-2 proteins shared 82–95% identity of amino acids with those of other teleosts, chicken and human. Phylogenetic analysis revealed that walking catfish HSC70-1 and HSC70-2 clustered with channel catfish HSC71. Under normal conditions, *HSC70-1* and *HSC70-2* transcripts were expressed at similar levels in liver, gills, brain and skeletal muscle of walking catfish. Bacterial infection by *Aeromonas hydrophila* differentially induced the expression of *HSC70*s in these tissues during 48 h. *HSC70-1* was constitutively expressed at low levels in most tissues, whereas *HSC70-2* was up-regulated at moderate and high levels in liver and skeletal muscle respectively. The significant increase in the expression of HSC70-2 in these tissues may relate to the role of HSC70-2 in the immune response of walking catfish.

Keywords: *Clarias macrocephalus*, HSC70, *Aeromonas hydrophila*, cDNA cloning, genomic cloning, bacterial infection

Introduction

Exposure to stress causes protein disruption and results in an accumulation of denatured and aberrantly folded proteins within the cell (Krone, Sass & Lele 1997). Heat shock or stress proteins (HSPs) are ubiquitous, highly conserved proteins that play a central role in controlling cellular homeostasis in response to a wide range of stresses, including heat stress, oxidative stress, heavy metals and bacterial infection (Iwama, Thomas, Forsyth & Vijayan 1998). HSPs are found in all organisms from bacteria to fish and mammals, among which heat shock protein 70 (HSP70) is the most widely studied group of HSPs (Feder & Hofmann 1999). HSP70 is not expressed in most tissues, but is highly inducible under stress conditions. However, some members of the HSP families are constitutively expressed in cells under normal conditions, such as the 70-kDa heat shock cognate protein (HSC70) (Iwama *et al.* 1998). HSC70 acts primarily as molecular chaperone for other proteins to facilitate protein synthesis, folding, translocation, assembly and degradation (Feder & Hofmann 1999). HSC70 and HSP70 have received attention in fish and shellfish due to increasing evidence of their involvement in the immune system (see reviews by Basu, Todgham, Ackerman, Bibeau, Nakano, Schulte & Iwama 2002; Robert 2003; Roberts, Agius, Saliba, Bossier & Sung 2010; Sung, MacRae, Sorgeloos & Bossier 2011). HSC70 and HSP70 levels in liver and hepatopancreas tissues were modulated during bacterial and viral infections (Deane, Li & Woo 2004; Ming, Xie, Xu,

Liu, Ge, Liu, He, Cheng, Zhou & Pan 2010; Rungrussamee, Leelatanawit, Jiravanichpaisal, Klinbunga & Karoonuthaisiri 2010; Yue, Liu, Sun & Tang 2011; Zhang; Deane, Jia, Qu, Chen, Zhang & Woo 2012). It has been suggested that the role of HSC70 during infection is associated with various pathways of protein translocation, whereas HSP70 is able to stimulate innate and adaptive immune response and protect cells against apoptosis (Basu *et al.* 2002; Robert 2003; Deane *et al.* 2004, 2012).

Disease is a major problem in aquaculture causing economic losses worldwide. Prevention and cure of infectious diseases are relied on the conventional approaches such as antibiotic treatment. Until recently, studies of HSP70s and infectious diseases have suggested the potential of using heat shock proteins derived from fish and shellfish pathogens as recombinant DNA vaccines (Plant, LaPatra & Cain 2009; Sung, Ashame, Chen, MacRae, Sorgeloos & Bossier 2009; Baruah, Ranjan, Sorgeloos & Bossier 2010; Roberts *et al.* 2010; Ryckaert, Pasmans, Tobback, Duchateau, Decostere, Haesebrouck, Sorgeloos & Bossier 2010; Baruah, Ranjan, Sorgeloos, MacRae & Bossier 2011; Sung *et al.* 2011). *HSC70* genes have been cloned from several fish species, including platyfish *Xiphophorus maculatus* (Yamashita, Hirayoshi & Nakata 2004), Wuchang bream *Megalobrama amblycephala* (Ming *et al.* 2010), grass carp *Ctenopharyngodon idella* (Zhang, Zhou, Wang & Zhou Zhang, Zhou, Wang, and Zhou 2011) and humphead snapper *Lutjanus sanguineus* (Zhang, Wu, Yang, Pang, Jian, and Lu 2011). In addition, two *HSC70* genes were identified in zebrafish *Danio rerio* (Graser, Malnar-Dragojevic & Vincek 1996; Santacruz, Vriz & Angelier 1997), common carp *Cyprinus carpio* (Ali, Dorgai, Abraham & Hermesz 2003) and yellowtail *Seriola quinqueradiata* (Yabu, Imamura, Mohammed, Touhata, Minami, Terayama & Yamashita 2011).

Walking catfish *Clarias macrocephalus* (Clariidae) is an important species for domestic consumption in Thailand. It commands high retail prices due to its superior meat quality. Although *C. macrocephalus* is widely distributed throughout Southeast Asia, culture of this species is well established in Thailand for more than 50 years. However, during the culture period, walking catfish may suffer from haemorrhagic septicaemia or ulcerative disease caused by the Gram-negative bacterium *Aeromonas hydrophila* (Angka, Lam & Sin 1995). The outbreak of the disease appears to be secondary infection of stressed fish. *Aeromonas hydrophila* was reported to be the cause of high mortality in walking catfish from nursing to grow-out periods, resulting in heavy economic losses (Areerat 1987; Angka *et al.* 1995).

In this study, we cloned cDNA and genomic sequences of two cognate HSC70s from walking catfish to predict amino acid sequences and determine their evolutionary distances from those of other teleosts and higher vertebrates. The HSC70 EST clone obtained from previous work (Panprommin, Poompuang & Srisapoome 2007) was used to design primers for RACE techniques. To investigate the role of HSC70s in the walking catfish immune system, the fish were injected intraperitoneally with *A. hydrophila*. The expression profiles of the two HSC70 genes determined using semi-quantitative RT-PCR were compared in brain, gill, liver and muscle tissues during 48 h post injection. This is the first report describing the cloning and characterization of *HSC70* genes in walking catfish.

Materials and methods

Rapid Amplification of cDNA Ends (RACE)

The 371-bp HSC70 EST clone (EB360505) containing the partial open reading frame and the 3′ untranslated region (UTR) was obtained from the previous work (Panprommin *et al.* 2007); therefore, only 5′ RACE was performed. Total RNA was extracted from liver tissue of an adult female (152 g) using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The extracted RNA was purified using a QuickPrep Micro mRNA Purification kit (Amersham Biosciences, Buckinghamshire, UK). The 5′ RACE technique was utilized to recover the 5′ information of target cDNA using a BD Smart RACE cDNA Amplification kit (BD Biosciences ClonTech, San Jose, CA, USA) according to the manufacturer's instructions. Approximately, 1 μg of mRNA was used for first-strand cDNA synthesis. For the 5′-RACE-ready cDNA, 1 μg of mRNA, 1 μL of BD Smart II A oligo (5′-AAGCAGTGGTATCAACGCAGAGTACG CGGG-3′) and 1 μL of 5′-CDS primer [5′-$(T)_{25}$VN-3′, N = A, C, G or T; V = A, G or C] were used to generate the nucleotide sequence at the 5′ end. After first-strand cDNA synthesis, the 5′-RACE-ready cDNA was used as a template to

generate the nucleotide sequence at the 5′ end. For RACE PCR reactions, a 50-μL PCR reaction mixture consisted of 2.5 μL of 5′-RACE-ready cDNA, 5 μL of 10× BD Advantage 2 PCR buffer, 1 μL of 10 mM dNTP Mix (each at 2.5 mM), 1 μL of 50× BD Advantage 2 Polymerase Mix, 5 μL of 10× Universal Primer Mix [UPM: Long (0.4 μM), 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTAT-CAAC G CAGAGT-3′ and Short (2 μM), 5′-CTAATA CGACTCACTATA GGGC-3′], and 1 μL of 10 μM of gene-specific primers, HSC70-1R1, HSC70-1R2, HSC70-1R3, HSC70-1R4 for HSC70-1 and HSC70-2R1, HSC70-2R2, HSC70-2R3, HSC70-2R4 for HSC70-2 (Table 1), under the following conditions for 25 cycles: 94°C for 3 min, 94°C for 30 s, 60°C for 30 s and 72°C for 3 min.

The RACE products were gel-purified using the GeneJET Plasmid Miniprep kit (Fermentas, Germany) according to the manufacturer's instructions. Band doubles at 1000 bp were observed on the agarose gel; therefore, two bands were cloned separately. The DNA fragment was ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into *E. coli* strain JM109. The positive clones were selected for extraction of the plasmid using GeneJET Plasmid Miniprep kit (Fermentas) according to the manufacturer's instructions. Three microlitre of extracted plasmid DNA was sequenced using the Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The M13 forward and reverse primers were used for nucleotide sequencing of the inserts.

Genomic cloning of two HSC70 cDNAs

The two heat shock cognate cDNAs obtained in this study were called walking catfish *HSC70-1* and *HSC70-2*. Primers for genomic cloning of walking catfish *HSC70-1* and *HSC70-2* were designed based on the full-length cDNAs (Table 1). Genomic DNA was extracted from the liver tissue (20–30 mg) of a female fish using a Genomic DNA purification kit (Fermentas). The extracted DNA was subjected to PCR amplification using eight primers (Table 1). A 25-μL PCR mixture consisted

Table 1 List of gene-specific primers, nucleotide sequences and positions of primers used in this study

Primer	Primer sequence (5′ to 3′)	Position*		Objective
		HSC70-1	*HSC70-2*	
HSC70-1R1	CCAATGCCTGGTTTGCCTTGAA	2210–2231		5′RACE
HSC70-1R2	GTCTCTGCTGTTGACAAGAGC	1495–1515		5′RACE
HSC70-1R3	CCAGTATTGAGATCGACTCCC	872–892		5′RACE
HSC70-1R4	GCTCTGAGAGAAATGTCCTTATTTTCGA	602–629		5′RACE
HSC70-2R1	CCAATGCCTGGTTTGCCTTGAA		2210–2232	5′RACE
HSC70-2R2	GGAGTTCCTCAAATCGAAGTG		1441–1461	5′RACE
HSC70-2R3	CTACAGCAGGAGACACTCACC		695–715	5′RACE
HSC70-2R4	CTCAGCGTCAAGCCACAAAAGATGCTGG		491–518	5′RACE
HSC70F1	CAGATTGAGGTCACATTTGAC	2085–2106	1820–1841	Genomic cloning
HSC70R1	CCAATGCCTGGTTTGCCTTGAA	3088–3110	2822–2845	Genomic cloning
HSC70F2	GCTATTGCCTATGGGTTG	800–818	800–818	Genomic cloning
HSC70-1R2	GTCTCTGCTGTTGACAAGAGC	2119–2130		Genomic cloning
HSC70-2R2	GGAGTTCCTCAAATCGAAGTG		1843–1865	Genomic cloning
HSC70F3	CCAGCTGTTGGCATTGATCTG	46–67	46–67	Genomic cloning
HSC70-1R3	CCAGTATTGAGATCGACTCCC	1169–1190		Genomic cloning
HSC70-2R3	CTACAGCAGGAGACACTCACC		903–924	Genomic cloning
RTHSC70-1F	GCTATTGCCTATGGGTTGGAC	571–591		Semi-quantitative RT-PCR
RTHSC70-1R	GTCTCTGCTGTTGACAAGAGC	1495–1515		Semi-quantitative RT-PCR
RTHSP70-2F	GCTATTGCCTATGGGTTGGAC		571–591	Semi-quantitative RT-PCR
RTHSP70-2R	CAGAAACTTCTGCAGGACTTC		1072–1092	Semi-quantitative RT-PCR
Ipβ-actin-F†	AGAGAGAAATTGTCCGTGACATC			Semi-quantitative RT-PCR
Ipβ-actin-R†	CTCCGATCCAGACAGAGTATTTG			Semi-quantitative RT-PCR

*Nucleotide positions are based on walking catfish DNA sequences submitted to GenBank (Accession number JX273642 for *HSC70-1* and accession number JX273643 for *HSP70-2*).

†Primers for β-actin are based on GenBank sequence (Accession number AY555575).

of 1μl of genomic DNA, 2.5 μL of 10× *Taq* buffer, 0.75 μL of $MgCl_2$, 1.5 μL each of dNTPs, 1 μL each of forward and reverse primers, 0.2 μL of 1 U *Taq* DNA polymerase, and 17.05 μL of sterile water under the following conditions for 25 cycles: 95°C for 30 s, 58°C for 30 s, 72°C for 1 min 30 s. PCR products were cloned and sequenced as described above. The genomic nucleotide sequences were compared with cDNA sequences to determine the exon–intron boundaries of the genes.

Sequence analysis and phylogenetic analysis

The nucleotide and deduced amino acid sequences were searched for homology and compared using the BLAST programs (NCBI, http://www.ncbi.nlm.nih.gov) (Altschul, Gish, Miller, Meyers & Lipman 1997). The Clustal W program (Thompson, Higgins & Gibson 1994) was used for multiple sequence alignment of amino acids from HSC70 and HSC71 proteins of other teleost species. These amino acid sequences were used to construct the *HSC70* phylogenetic tree using the neighbour-joining method (Saitou & Nei 1987) and bootstrapped for 1000 replicates using MEGA version 3.1 (Kumar, Tamura & Nei 2004).

Tissue distribution using RT-PCR

Total RNA was extracted from gill, liver, brain and muscle tissues of five walking catfish (150 ± 5.3 g in weight) using TRIzol reagent (Molecular Research Center) according to the manufacturer's instructions. Approximately, 1 μg of total RNA was used for first-strand cDNA synthesis using iScript Select cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

PCR amplifications were performed using the primers listed in Table 1. The level of expression of β-actin was used as an internal control. Primers for the β-actin gene were based on information obtained from channel catfish *Ictalurus punctatus* (AY555575). PCR was carried out in a 25-μL reaction mixture containing 1× *Taq* buffer, 0.75 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each forward and reverse primer, 1 U *Taq* DNA polymerase (Fermentas) and 0.5 μL of first-strand cDNA. The PCR was performed under the following conditions: pre-denaturation at 96°C for 3 min, and 26 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and 72°C for 1 min. The last extension at 72°C was extended for 5 min. Ten microlitre of each PCR product was subjected to electrophoresis through a 1.5% agarose gel in 1× TBE buffer at 100 volts. A 100-bp DNA ladder was used to estimate the molecular weight. The gel was stained with ethidium bromide (0.5 mg/mL) and amplification products were observed under UV light.

Bacterial challenge

One hundred, 4-month-old healthy fish (body weight = 29.7 ± 6.4 g) were collected from an earthen pond at the Department of Aquaculture, Kasetsart University, and transferred to a 6 × 4 m^2 concrete tanks 7 days prior to the experiment. The fish were stocked randomly into two 250-L fibreglass tanks at 26–28°C of 50 individuals each. Fish in the first group were injected intraperitoneally with 0.1 mL of 0.85% NaCl and served as a control. Fish in the second group were injected with 0.1 mL of 10^9 CFU/mL of *A. hydrophila* by the same route as control. The experimental fish were fed commercial pellets at 5% body weight twice daily. Tissue samples, including brain, gills, liver and skeletal muscle, were randomly collected from injected and control fish at 6, 12, 24 and 48 h post injection for total RNA extraction.

Expression of HSC70-1 and HSC70-2 mRNA using semi-quantitative RT-PCR

Total RNA was extracted from brain, gills, liver and muscle tissues using the TRIzol reagent (Molecular Research Center) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out using a RevertAid First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. Approximately, 1 μg of total RNA was incubated with 1 μL of oligo$(dT)_{18}$ at 70°C for 5 min. Four microlitre of 5× reaction buffer, 1 μL of RiboLock Ribonuclease inhibitor and 2 μL of 10 mM dNTP mix were added and the reaction was incubated at 37°C for 5 min. One microlitre of RevertAid M-MuLV Reverse Transcriptase (Fermentas) was added and the mixture was incubated at 42°C for 60 min, followed by 70°C for 10 min.

PCR reactions were performed using two sets of gene-specific primers, RTHSC70-1F, RTHSC70-1R, RTHSC70-2F and RTHSC70-2R (Table 1). PCR amplification was carried out in a 25-μL reaction volume containing 0.5 μL of cDNA first-strand

template, 0.2 mM of each dNTPs, 1× *Taq* buffer, 1 U of *Taq* DNA polymerase, 1 μM of forward primer and 1 μM of reverse primer. PCR profiles for 26 cycles were as follows: denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min. The level of expression of β-actin was used as an internal control.

The PCR products were separated and visualized on a 1.5% agarose gel containing ethidium bromide (0.5 mg/mL). The density of bands was measured using FluorChem 8000 Advanced Fluorescence, Chemiluminescence and Visible Light Imaging (Alpha Innotech Corporation, San Leandro, CA, USA), which corresponded to pixel density values (Integrated Density Value, or IDV). Expression of the *HSC70-1* and *HSC70-2* genes was measured as pixel density values. The pixel density value of each product was used to calculate the ratio of the selected gene's expression relative to β-actin expression. The expression of β-actin was normalized to 100.

Statistical analysis

Data on relative expression levels of the two *HSC70* genes at four time points were presented as means ±SE. for each tissue, where $n = 20$ fish. Statistical differences in gene expression levels between groups were analysed using one-way analysis of variance (ANOVA) using SPSS 11.0 (Chicago, IL, USA). A student's *t*-test was used to compare means between groups of *HSC70* genes at the same time point. For all statistical tests, significance was set at $P < 0.05$.

Results

Cloning of two full-length HSC70 cDNAs

The full-length cDNA sequences of the walking catfish *HSC70-1* and *HSC70-2* genes were 2278 bp, with an open reading frame of 1950 bp encoding 649 amino acids. Both cDNAs contained 33 and 295 bases of 5′- and 3′-untranslated regions (Figs 1 and 2). The 3′-untranslated region of both cDNAs included the termination signal (TAA) and a conserved polyadenylation signal (AATAAA) located 19 bases before the poly(A) tail. Molecular masses determined from the deduced amino acid sequences were approximately 71.24 and 71.27 kDa, respectively, for HSC70-1 and HSC70-2. A homology search of these proteins using the BLAST program revealed highest homology to the cytosolic and constitutively expressed HSC70 family.

Figure 1 Nucleotide and deduced amino acid sequences of walking catfish *HSC70-1*. Three signatures of the heat shock protein 70 family are shown in boxes, a putative nuclear localization signal is underlined and a consensus sequence EEVD at the C-terminus is indicated in italics. The numbers on the right are positions of the nucleotides and amino acids. The polyadenylation signal (AATAAA) is underlined.

Amino acid sequence analysis identified three signature sequences of the heat shock protein 70 family: IDLGTTYS in the N-terminal domain, IFDLGGGTFDVSIL and IVLVGGSTRIPKIQK in the middle domain, and the EEVD cytoplasmic motif at the C-terminus for both HSC70-1 and HSC70-2 (Figs 1and 2). One repeat of the tetrapeptide motif (GGMP) was present in the C-terminal region of HSC70-1, whereas HSC70-2 contained two GGMP motif repeats. Overall, HSC70-1 and HSC70-2 sequences were 94% identical, with 38

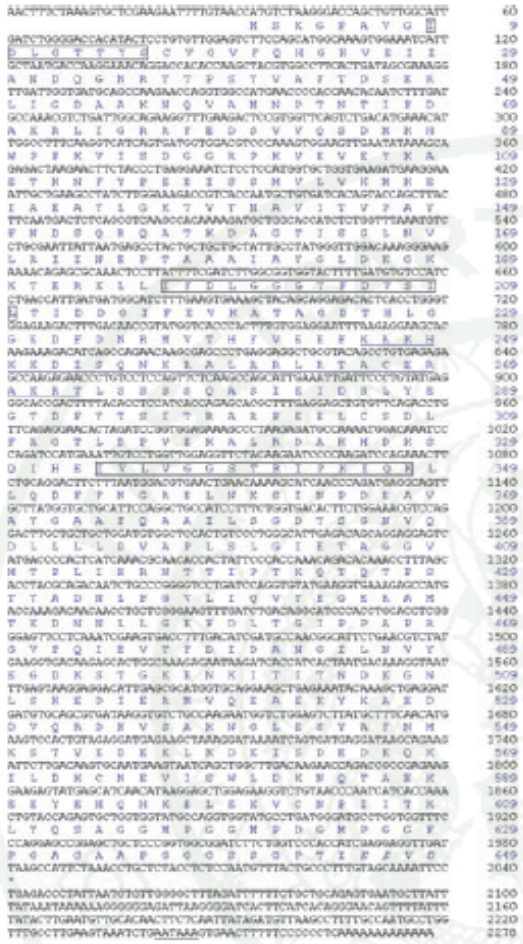

Figure 2 Nucleotide and deduced amino acid sequences of walking catfish *HSC70-2*. Three signatures of the heat shock protein 70 family are shown in boxes, a putative nuclear localization signal is underlined, and a consensus sequence EEVD at the C-terminus is indicated in italics. The numbers on the right are positions of the nucleotides and amino acids. The polyadenylation signal (AATAAA) is underlined.

substitutions between the two predicted proteins (Fig. 3).

Alignment of the amino acid sequences of HSC70-1 and HSC70-2 with HSC70 and HSC71 from other species revealed that the three signature sequences of heat shock protein 70 family and the EEVD motif were highly conserved. The two HSC70 proteins of walking catfish contained 649 amino acids, similar to those of zebrafish HSC70, channel catfish HSC71 and Wuchang bream HSC70. The HSC70 amino acid sequences of all fish species varied from 612 to 650 residues, whereas clawed frog HSC70 and human HSC70 contained 646 amino acids (Table 2). The HSC70-1 and HSC70-2 shared 82–95% identity of amino acids with other teleosts, chicken and human (Table 3). The amino acid sequences of HSC70-1 and HSC70-2 were submitted to the GenBank database with accession numbers JX112294 and JX112295 respectively.

Genomic DNA structure of HSC70-1 and HSC70-2

Initially, primers (HSC70F1-R1) were designed based on information of a 371-bp EST clone (EB360505) of *C. macrocephalus*. These primers were used to clone a fragment of 1048 bp. Subsequently, primers HSC70F2-HSC70-1R2 and HSC70F3-HSC70-1R3 were designed and were used to clone a 1351-bp and a 1165-bp fragment respectively. A genomic sequence of *HSC70-1* (3179 bp) was obtained. The coding region consisted of seven introns (144, 85, 89, 203, 199, 233 and 195 bp) and eight exons (205, 206, 153, 556, 203, 199, 233 and 195 bp) (Fig. 4). The genomic sequence of the walking catfish *HSC70-1* gene was deposited in GenBank under accession number JX273642.

Primers HSC70F1-R1, HSC70F2-HSC70-2R2 and HSC70F3-HSC70-2R3 were used to clone three genomic fragments (921 bp, 1094 bp and 1110 bp respectively) of *HSC70-2*. A genomic sequence of *HSC70-2* (2980 bp) was obtained. The coding region of *HSC70-2* consisted of seven introns (144, 85, 22, 64, 117, 126 and 144 bp) and eight exons (205, 206, 172, 539, 201, 213, 219 and 195) (Fig. 4). The genomic sequence of the walking catfish *HSC70-2* gene was deposited in GenBank under accession number JX273643. Comparisons of genomic structure revealed that the exon and intron sizes of the two genes were different for exons 3, 4, 5, 6 and 7 and for introns 3, 4, 5 and 6.

Phylogenetic analysis

Phylogenetic analysis was performed based on 15 amino acid sequences of HSC70 and HSC71. A phylogenetic tree showed two distinct branches for fish and clawed frog, chicken and human (Fig. 5). Among teleosts, the HSC70-1 and HSC70-2 sequences of walking catfish were clustered with channel catfish HSC71, which would be expected given their membership in Order Siluriformes. A second group of teleost sequences comprised mem-

Figure 3 Alignment of amino acid sequences of HSC70-1 and HSP70-2. Identical amino acids are marked with asterisk (*), highly related amino acids with a colon (:), and related amino acids with single dot (.). Numbers refer to the position in amino acid sequence.

bers of Family Cyprinidae, including common carp, gibel carp, Wuchang bream, zebrafish and fathead minnow, and sequences from Japanese flounder and killifish. The two clusters joined the sequence from Atlantic salmon. The second major branch consisted of sequences from higher vertebrates, including human, chicken and clawed frog.

Expression analysis of HSC70-1 and HSC70-2 in response to A. hydrophila challenge

Under normal conditions, the mRNA levels of *HSC70-1* and *HSC70-2* were similar in liver, gills, brain and skeletal muscle of walking catfish. The temporal expression of *HSC70-1* and *HSP70-2* after a bacterial challenge was analysed in the same tissues. To distinguish the two HSC 70s cDNA sequences, gene-specific primers were designed for *HSC70-1* (RTHSC70-1F and RTHSC70-1R) and *HSC-2* (RTHSC70-2F and RTHSC70-2R) (Table 1). PCR products of size 945 bp and 522 bp were obtained, respectively, for *HSC70-1* and *HSC70-2*. The positions of these primers were chosen to prevent amplification of the *HSP70* cDNA. The *A. hydrophila* challenge resulted in differential expression of the two genes in walking catfish tissues during a period of 48 h (Fig. 6). The expression levels of the *HSC70-1* and *HSC70-2* in the brain were similar at each time point ($P < 0.05$). However, slight but significant differences in HSC70-1 mRNA levels were observed in the brain at 12 h, with 0.5–1.0 fold increase ($P < 0.05$) in comparison with the control. The *HSC70-2* was up-regulated significantly in gills at 24 h post injection and remained unchanged at 48 h, whereas the mRNA level of *HSC70-1* changed only slightly during a 48-h period. In the liver, results of *t*-test showed that the expression levels of *HSC70-1* and *HSC70-2* were significantly different at each time point. Whereas the mRNA levels of *HSC70-1* in liver tissue increased slightly at 12 h and remained unchanged at 48 h, the *HSC70-2* was significantly up-regulated at 24 h and decreased markedly at 48 h post injection. In skeletal muscle, the HSC70-2 concentration increased gradually from 6 h and peaked at

Table 2 Comparison of HSC70 amino acid sequence of walking catfish with those of teleosts and other species

Species	ORF (bp)	Amino acid residues
Clarias macrocephalus HSC70-1	1950	649
Clarias macrocephalus HSC70-2	1950	649
Ictalurus punctatus HSC71	1950	649
Fundulus heteroclitus HSC70	1839	612
Acanthopagrus schlegelii HSC70	1953	650
Paralichthys olivaceus HSC71	1839	612
Pimephales promelas HSC70	1953	650
Megalobrama amblycephala HSC70	1950	649
Danio rerio HSC70	1950	649
Cyprinus carpio HSC70-2	1935	644
Carassius auratus gibelio HSC70	1950	649
Xenopus laevis HSC70	1953	650
Gallus gallus HSC70	1941	646
Homo sapiens HSC70	1941	646

48 h post injection, with threefold increase compared with that of the control. In contrast, the mRNA level of *HSC70-1* changed only slightly during a 48-h period.

Discussion

In this study, we isolated and characterized two cognate heat shock protein 70 cDNAs from the liver of walking catfish. Band doubles at 70 kDa were observed on an agarose gel for purification of cDNA; therefore, the two bands were cloned separately. We obtained two complete cDNAs and were designated as *HSC70-1* and *HSC70-2*. The lengths of *HSC70-1* and *HSC70-2* cDNAs were 2278 bp, with ORFs of 1950 bp encoding 649 amino acids. The walking catfish HSC70-1 and HSC70-2 proteins shared high amino acid sequence identity (85–90%) with those of HSC70s from other fish species and, therefore, were placed in the HSP70 gene family. The amino acid sequences of fish HSC70s showed a high degree of similarity at the N-terminus, whereas the sequences at the C-terminus were more variable. Characterization of genomic sequences revealed that the walking catfish *HSC70-1* and *HSC70-2* genes were composed of eight exons and seven introns. Differences in exon and intron sizes were observed between the two loci. The major difference was in the size of intron 4, which was larger (198 bp) in *HSC70-1* than in *HSC70-2* (64 bp). In common carp, the similarities of HSC70-1 and HSC70-2 were 88% and 78% at the protein and DNA levels respectively (Ali *et al.* 2003). There were 69 substitutions between the two common carp proteins, 31 of which located in the C-terminus. Walking catfish HSC70-1 and HSC70-2 sequences were 94% identical, with 38 substitutions between the two predicted proteins. Nucleotide sequences of the two cDNAs shared 91% identity.

Genomic structures of the two loci were similar to those of other teleost fish, including *Rivulus marmoratus* (Park, Lee, Yoon, Lee, Choe, Choe, Park & Kim 2001), common carp (Ali *et al.* 2003) and Wuchang bream (Ming *et al.* 2010). Interestingly, exon sizes were identical between walking catfish *HSC70-1* and Wuchang bream *HSC70*, but

Table 3 Per cent identity and similarity of walking catfish HSC70-1 and HSC70-2 amino acid sequences to those of other fish, clawed frog, chicken and human HSC70 and HSC71 amino acid sequences

	HSC70-1		HSC70-2		
Species	Identity	Similarity	Identity	Similarity	Accession number
Ictalurus punctatus HSC71	94	96	90	94	P47773
Fundulus heteroclitus HSC70	95	91	90	89	DQ202278
Salmo salar HSC70	95	91	90	89	BT059361
Acanthopagrus schlegelii HSC70	94	83	89	80	AAX07833
Paralichthys olivaceus HSC71	95	91	90	89	AB006814
Pimephales promelas HSC70	93	95	89	93	AAS46619
Megalobrama amblycephala HSC70	95	91	91	89	EU623471
Danio rerio HSC70	91	94	87	91	CAA72216
Cyprinus carpio HSC70-2	93	95	90	89	AAP51388
Carassius auratus gibelio HSC70	94	97	91	95	AAO43731
Gallus gallus HSC70	90	93	87	91	NP_990334
Homo sapiens HSC70	91	94	87	91	NP_006588
Xenopus laevis HSC70	93	96	89	93	AAH41201

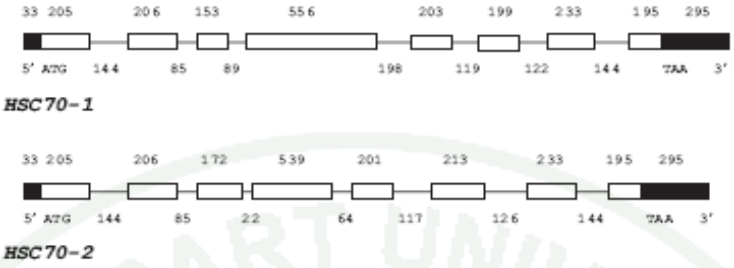

Figure 4 Predicted gene structures of walking catfish *HSC70-1* and *HSC70-2*. White boxes represent exons, black boxes are 5'- and 3'untranslated regions and lines represent introns. The sizes (base pairs) of each exon and intron are indicated. The start codon (ATG) and stop codon (TAA) are also indicated.

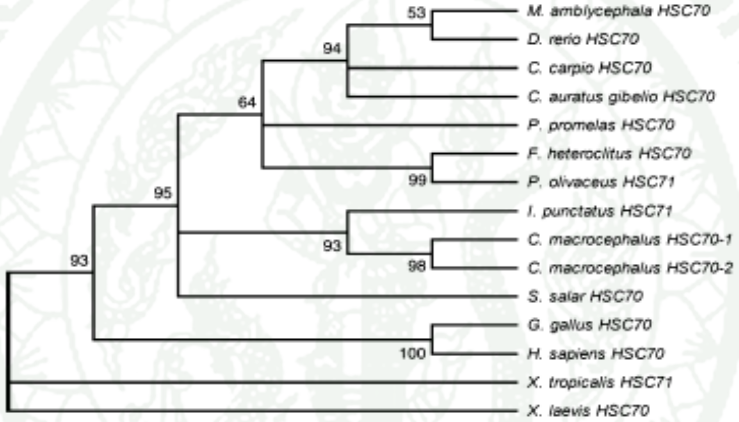

Figure 5 Phylogenetic tree showing relationships of HSC70-1 and HSC70-2 deduced amino acid sequences of walking catfish and amino acid sequences of HSC70/HSC71 from other species. GenBank accession numbers are as follows: *Clarias macrocephalus* HSC70-1 (JX112294), *C. macrocephalus* HSC70-2 (JX112295), *Cyprinus carpio* HSC70 (AAP51388), *Carassius auratus gibelio* HSC70 (AAO43731), *Danio rerio* HSC70 (CAA72216), *Ictalurus punctatus* HSC71 (P47773), *Pimephales promelas* HSC70 (AAS46619), *Megalobrama amblycephala* HSC70 (EU623471), *Salmo salar* HSC70 (BT059361), *Fundulus heteroclitus* HSC70 (DQ202278), *Paralichthys olivaceus* HSC71 (AB006814), *Xenopus laevis* HSC70 (AAH41201), *X. tropicalis* HSC71 (XM002937528), *Gallus gallus* HSC70 (NP_990334) and *Homo sapiens* HSC70 (NP_006588). The numbers at branches indicate bootstrap values after 1000 replications.

the intron sizes were different (Ming *et al.* 2010). The functional significance of this similarity, however, is not known. The presence of introns is a major characteristic of the constitutive forms of the HSP70 family, whereas the inducible forms do not contain introns (Iwama *et al.* 1998). The lack of introns in the inducible HSP70s allows rapid translation of mRNA into nascent protein during stress (Basu *et al.* 2002). It has been suggested that multiple forms of HSP70/HSC70 in fish genomes arose by gene duplication during vertebrate evolution (Yamashita *et al.* 2004; Yabu *et al.* 2011).

The heat shock protein (HSP70) family contains both inducible and constitutively expressed members. The role of inducible *HSP70* in immune system of fish is well characterized, but the function of *HSC70* during bacterial infection has yet to be elucidated. Studies reported that bacterial challenge modulated the mRNA levels of the cognate *HSC70* in various tissues of fish and shellfish. Ramaglia, Harapa, White and Buck (2004) reported an increase in protein levels of heat shock cognate, *HSC73* in liver and brain of the western painted turtle infected with *Citrobacter* spp., the causative agent of septicaemic cutaneous ulcerative dermatitis. Deane *et al.* (2004) reported an increase in expression of *HSC70* in liver of silver sea bream after *Vibrio alginolyticus* challenge. Wuchang bream that received an injection of *A. hydrophila* showed

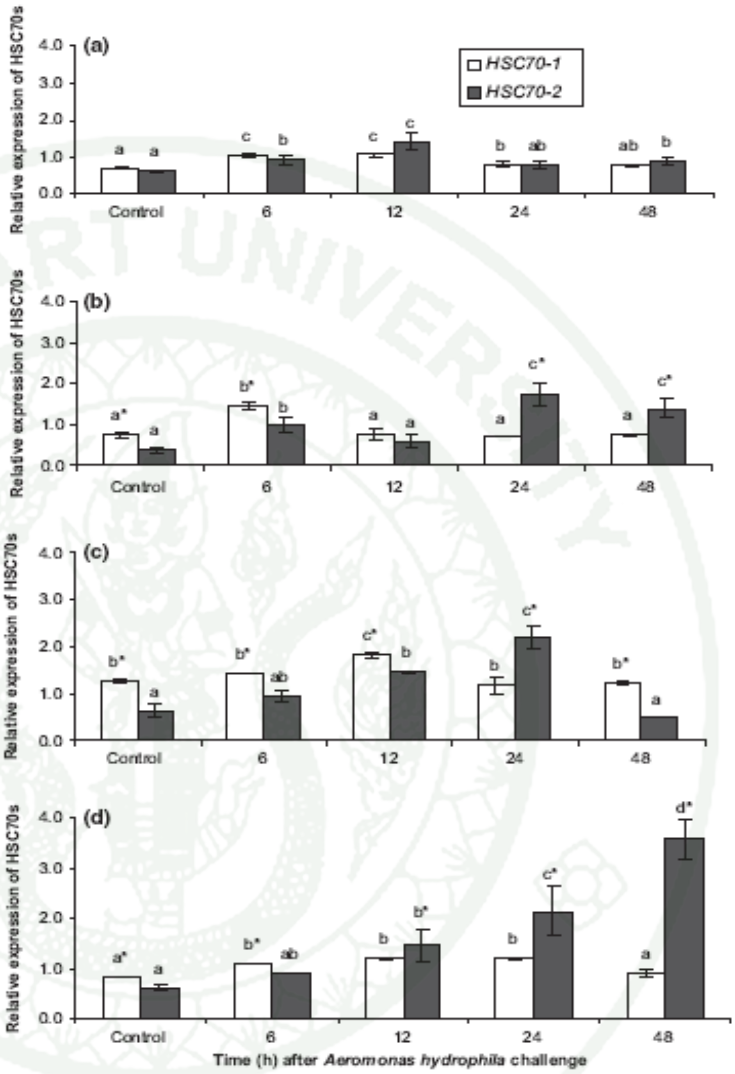

Figure 6 Relative expression of HSC70-1 and HSC70-2 in brain (a), gills (b), liver (c) and muscle (d) after bacterial challenge. Levels associated with histogram bars with the same letters do not differ significantly at $P < 0.05$. Asterisks indicate significant differences in expression levels between HSC70-1 and HSC70-2 at the same time point.

that HSC70 levels in liver tissue peaked at 6 h and decreased to baseline after 24 h (Ming *et al.* 2010). Tiger prawn (*Penaeus monodon*) exposed to *V. harveyi* had increased levels of heat shock cognate HSP70 in gill tissue after 12 h of treatment, and expression returned to the same levels as controls after 72 h (Rungrussamee *et al.* 2010). In addition, Yue *et al.* (2011) reported that the HSC71 mRNA levels in hepatopancreas of gill of Asiatic hard clam (*Meretrix meretrix*) were significantly increased at 12 h post injection of *V. parahaemolyticus* and decreased to baseline at 24 h.

In this study, the expression of *HSC70-1* and *HSC70-2* was detected in various tissues of walking catfish under normal conditions (data not shown). By contrast, bacterial infection differentially induced the expression of the two genes in brain, gills, liver and skeletal muscle of adult fish during a 48-h period after *A. hydrophila* challenge. *HSC70-1* was constitutively expressed at low levels and was slightly induced in most tissues. *HSC70-2*, however, was slightly expressed in brain and gills and moderately up-regulated in liver and skeletal muscle. The mRNA levels of *HSC70-2* peaked at 24 h and returned to the same levels as controls after 48 h. In contrast, the *HSC70-2* transcript in skeletal muscle gradually increased after 6 h and its level was highest at 48 h. Stress-inducible *HSC70* isoforms have been reported in zebrafish, common carp and yel-

lowtail. Expression patterns of these genes were different under various stress conditions, e.g., heat stress, cold shock and heavy metal treatment, and appeared to be tissue-specific. For instance, common carp *HSC70-1* was expressed at high levels in liver after cadmium exposure, while the expression of common carp *HSC70-2* was induced by cold shock in muscle (Ali *et al.* 2003). In culture tailfin cells, yellowtail *HSC70-1* was constitutively expressed under normal and heat-shock conditions, whereas *HSC70-2* was up-regulated by heat shock treatment (Yabu *et al.* 2011). Our results showed similar findings, suggesting that HSC70-1 was constitutively expressed in most tissues, whereas the expression of HSC70-2 was induced by bacterial infection in a tissue-specific manner.

The bacterium *A. hydrophila* is a causative agent of haemorrhagic septicaemia, an important disease in freshwater fish. Systemic infection results in mortality within 24–48 h (Areerat 1987; Angka *et al.* 1995). The most common clinical signs and lesions are haemorrhage in skin, fins, abdominal cavity and muscles (Silva, Mouriño, Viera, Jatobá, Seiffert & Martins 2012). Histological changes include focal cellular necrosis in the liver and haemopoietic cells, heart and skeletal muscles (Rey *et al.* 2009). In this study, the significant increase in the expression of *HSC70-2* in walking catfish liver and muscle may relate to the role of *HSC70-2* in immune response of these tissues after bacterial infection. Ming *et al.* (2010) reported that *A. hydrophila* injection modulated the expression of HSC70 and HSP70 in liver of Wuchang bream in a time-dependent pattern. Both HSC70 and HSP70 mRNA levels significantly increased at 6 h and returned to baseline at 24 h. A similar pattern of HSC70 expression was observed after walking catfish were infected with *A. hydrophila* in this study. The HSC70-2 mRNA concentrations peaked at 24 h and decreased to level of the control at 48 h. Unlike liver, the HSC70-2 mRNA level was very high in skeletal muscle at 48 h. Because fish infected with *A. hydrophila* showed severe skin and muscle lesions at the injected site, the high level of HSC70-2 in muscle may suggest the involvement of *HSC70-2* in cell protection and survival.

Aeromonas hydrophila infection did not modulate the expression of both HSC70 isoforms in brain of walking catfish. Similar results were reported in the brain of humphead snapper infected with *V. harveyi* (Zhang, Zhou *et al.* 2011, Zhang, Wu *et al.* 2011). There has been no report on the role of HSC70 in brain tissues of other teleosts under infectious challenge. Nevertheless, a study in western painted turtle (*Chrysemys picta bellii*) reported that bacterial infection induced significant mRNA levels of constitutive HSP73 in the brain (Ramaglia *et al.* 2004); high levels of HSP73 in the brain may suggest the role of this protein in translocation and synaptic vesicle recycling.

The expression of *HSP70-2* in the gills of walking catfish slightly increased after injection with *A. hydrophila* at 24 h. It was likely that *HSC70-2* was insensitive to the bacteria at the early hours following injection. The increased levels of HSC70-2 in gills may correlate with osmotic regulation in the walking catfish.

In conclusion, we cloned and characterized two cDNAs encoding 649 amino acid heat shock cognate proteins designated *HSC70-1* and *HSC70-2* in walking catfish. The two *HSC70* genes showed high levels of similarity of protein products, but less similarity at the DNA level. Gene structure and deduced amino acid sequences of these heat shock cognate protein 70s in walking catfish were similar to those of homologous genes from other species. *A. hydrophila* infection caused a tissue-specific expression of HSC70-2. The mRNA levels of *HSC70-2* were modulated in liver and skeletal tissues, but not in brain and gills. The expression of *HSC70-2* might be associated with the immune response of walking catfish. Further research is needed to elucidate the role of the two copies of *HSC70*s in response to other stressors such as heat, and to determine the functional significance of the two copies of *HSC70* in the walking catfish genome. Future studies of the inducible HSP70 would enhance knowledge of this gene and its role against infectious diseases for walking catfish.

Acknowledgments

This study was supported by the Thailand Research Fund (Grant no. RMU5180015) and the Agricultural Research Development Agency (ARDA). We thank Saowalak On-ming for technical assistance.

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