

## Heat Shock Protein and their Significance in Fish Health

Addisu Demeke\*, Asmelash Tassew

Department of Microbiology, Immunology and Public health, College of Veterinary medicine and Agriculture, Addis Ababa University, Debre Zeit, Ethiopia

### Review Article

**Received date:** 23/12/2015

**Accepted date:** 24/02/2016

**Published date:** 29/02/2016

#### \*For Correspondence

Addisu Demeke, Department of Microbiology, Immunology and Public health, College of Veterinary medicine and Agriculture, Addis Ababa University, Debre Zeit, Ethiopia, Tel: +251 111 239 752.

**E-mail:** ademeke16@yahoo.com

**Keywords:** Stress proteins, hsp, Gene families, Functional significance, Fish.

#### ABSTRACT

Despite decades of intensive investigation, important questions remain regarding the functional, ecological, and evolutionary roles of heat shock proteins. In this paper, we review the relevant studies of heat shock protein genes and the functional use in fish health. Although molecular studies of the heat shock proteins in fish are still in their early descriptive phase, data are rapidly being collected. More is known about the biotic and abiotic factors regulating heat shock proteins. We briefly review these studies and focus on the role of heat shock proteins in development and their importance in fish in nature. Functional genomic approaches will provide the tools necessary to gain a comprehensive understanding of the significance of heat shock proteins in the cellular stress response, in the physiological processes at higher levels of organization, and in the whole animal in its natural environment.

### INTRODUCTION

Heat shock proteins are a family of highly conserved cellular proteins present in all organisms that have been examined<sup>[1]</sup>, including fish. Extensive studies on model species have revealed three major families of heat shock proteins: Hsp90 (85–90 kDa), Hsp70 (68–73 kDa), and low molecular weight heat shock proteins (16–47 kDa). In the unstressed cell, these proteins have constitutive functions that are essential in various aspects of protein metabolism<sup>[1]</sup>. Hsp90 is active in supporting various components of the cytoskeleton and steroid hormone receptors<sup>[2]</sup>. Hsp70 is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured proteins<sup>[3]</sup>. The low molecular weight heat shock proteins have diverse functions that are species-specific. Unlike other heat shock proteins, these proteins have no known constitutive function and are only induced during stress<sup>[4]</sup>.

Classical studies of stress in fish have focused on the organism stress response. The characteristic feature of this organism stress response is the rapid release of stress hormones, including cortisol and catecholamines, resulting in the mobilization of energy reserves in an attempt to re-establish homeostasis<sup>[5]</sup>. In addition to this stress response, a generalized stress response system exists at the cellular level, which includes the actions and functions of various heat shock proteins<sup>[6]</sup>. While the term 'heat shock protein' arose from early observations on *Drosophila* exposed to a severe heat stress, heat shock proteins can be up-regulated in cells that are exposed to a wide variety of stressors, particularly those that denature proteins<sup>[7]</sup>. In fish, the induction of heat shock protein families, a component of the cellular stress response, has been reported in cell lines, primary cultures of cells, as well as in various tissues from whole animals<sup>[6]</sup>. Most of these studies demonstrated a correlation between increased levels of heat shock proteins and exposure to stressors within relevant ecological range. These observations suggest that the cellular stress response is likely to be playing some role in enhancing the survival and health of the stressed fish.

The mechanisms underlying the sensing of a stressor and the induction of heat shock proteins are far from clear. Studies on Hsp70 have demonstrated that the regulation of hsp70 gene expression occurs mainly at the transcriptional level<sup>[1]</sup>. Analysis of heat shock protein genes and a comparison of their promoter sequences from a variety of organisms led to the identification of a

palindromic heat shock element (HSE), CNNGAANN TTCNNG<sup>[8]</sup>. It has been demonstrated that heat shock protein induction results primarily from the binding of an activated heat shock transcription factor (HSF) to HSEs upstream of heat shock protein genes<sup>[4]</sup>. 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.

While most of our knowledge regarding the biology of heat shock proteins has been derived from work on a limited number of model systems, fish represent an ideal organism in which to resolve the regulation and functional significance of heat shock proteins. In particular, fish offer an alternate and excellent model system in which to investigate the functional, ecological, and evolutionary genomics of heat shock proteins. Fish are ectothermic vertebrates that inhabit an aquatic environment with high temperature conductivity. As a result, temperature is an important factor influencing their biogeographic distribution over evolutionary time. In addition, daily and seasonal temperature fluctuations have an important impact during the lifetime of individual fish. Therefore, fish are a convenient model to study the effects of thermal stress in the intact organism on both short and long time scales. Fish have also emerged as an important developmental model<sup>[9]</sup>, since many species have external fertilization, and large manipulable eggs and embryos. Thus, heat shock protein expression and regulation can be studied at all life-history stages in fish. In this paper we review what is known regarding the sequence and genomic structure of the major heat shock protein gene families in fish. We then address the physiological roles of heat shock proteins in fish and their importance as part of the integrated response to environmental change. Studies into the functional genomics of heat shock proteins in fish will provide substantial insight into the physiological and ecological roles of these highly conserved proteins.

## HEAT SHOCK PROTEIN FAMILY

The HSPs have been extensively studied, especially with regard to their cellular localization, regulation, and functions<sup>[10]</sup>. HSPs are present in both prokaryotic and eukaryotic cells, and their high level of conservation suggests that they play an important role in fundamental cell processes. HSPs were initially discovered in *Drosophila melanogaster* larvae that were exposed to “heat shock”<sup>[11]</sup>, and subsequent studies identified several subsets of these proteins in the 70 kDa range. Over the past 30 years, a large number of additional proteins have been discovered within this family, and these are collectively referred to as “HSPs”.

The principal HSPs range in molecular mass from ~ 15 to 110 kDa and are divided into groups based on both size and function<sup>[12]</sup>. They are present in the cytosol, mitochondria, endoplasmic reticulum, and nucleus, although these locations vary depending on the particular protein. The most well-studied and understood HSPs in mammals are those with molecular masses of ~ 60, 70, 90, and 110 kDa. These HSPs are expressed at euthermic body temperatures (~ 37 °C) and in conditions of stress (e.g., heat shock) and have distinct locations and functional properties. Small-molecular-mass proteins also termed small HSPs, exhibit tissue-specific expression and include heme oxygenase, Hsp32, Hsp27,  $\alpha$ B-crystallin, and Hsp20 chaperone.

## HEAT SHOCK PROTEIN GENES IN FISH

In order for functional genomics to be successfully applied in fish, a substantial amount of basic molecular information must first be collected. Most studies of heat shock proteins in fish have been performed exclusively at the protein level, and thus relatively little is known about the sequence, genomic structure, or organization of the genes encoding heat shock proteins in fish. Indeed, heat shock protein genes have only been cloned from a modest number of different fish species. Hsp70 has been cloned from rainbow trout<sup>[13]</sup>, zebrafish<sup>[14]</sup>, tilapia *Oreochromis mossambicus*;<sup>[15]</sup> and pufferfish *Fugu rubripes*;<sup>[16]</sup> and heat stress-related increases in mRNA levels have been documented for many of these genes. As is the case for hsp70 genes in other organisms, the hsp70 genes of fish are highly conserved at the amino acid level. Zafarullah et al.<sup>[17]</sup> isolated and characterized another member of the rainbow trout hsp70 multigene family, the constitutively expressed heat-shock cognate, hsc71. Santacruz et al.<sup>[18]</sup> cloned and characterized a zebrafish hsc70. Recently, a heat shock cognate (hsc71m) has also been isolated from the hermaphroditic teleost *Rivulus marmoratus*<sup>[19]</sup>. This gene is not induced by stress, but is enriched in *Rivulus* muscle, suggesting that there may be multiple isoforms of the heat-shock cognate, with differing tissue-specific distributions. A fragment of an hsc70 gene has also been sequenced in carp – *Cyprinus carpio*;<sup>[20]</sup>

Currie and Tufts<sup>[21]</sup> detected a band corresponding to Hsp90 while profiling the Hsp70 response in rainbow trout red blood cells. Mammalian genomes encode two closely related hsp90 genes (alpha and beta). Both have been sequenced in zebrafish and both have been shown to be differentially regulated in developing embryos. A complete sequence of an hsp90 $\alpha$  has also been obtained from the chinook salmon (*Oncorhynchus tshawytscha*;<sup>[22]</sup>). The expression of this gene was studied in a chinook salmon embryonic cell line and it was shown to be heat inducible. A fragment of hsp90 $\alpha$  has been cloned from the Japanese flounder (*Paralichthys olivaceus*; Nam, Hirono, and Aoki, unpublished data; accession number AU090921). An hsp90 sequence from Atlantic salmon (*Salmo salar*) was characterized by Pottinger<sup>[23]</sup> that corresponded to the hsp90 $\beta$  of zebrafish with a 92% amino acid identity. Atlantic salmon hsp90 $\beta$  expression, *in vitro* and *in vivo*, was shown to be upregulated in gill and kidney tissues, but the magnitude of induction was not as great as for the inducible hsp70 gene.

Several members of the low molecular weight heat shock protein family have been cloned in fish. An hsp30 has been cloned from the chinook salmon (Hargis, Goff, Hickey and Weber, unpublished data; accession number U19370). Pearson et al.<sup>[24]</sup> cloned and characterized an hsp47 in zebrafish. Pearl and Prodromou<sup>[25]</sup> cloned two low molecular weight heat shock proteins,

hsp27 and hsp30, in the desert pupfish, *Poeciliopsis lucida*. Sequence analysis indicated that these genes are members of the  $\alpha$ -crystallin / small heat shock protein superfamily. The hsp30 genes appear to have diverged more rapidly than hsp27 and were most similar to homologs in *Xenopus*, while hsp27 was highly similar to mammalian and avian homologs [25].

The mechanisms regulating the expression of heat shock protein genes in fish have not yet been extensively studied, although some work has been initiated on hsp70 genes. Currie and Tufts [21] first suggested that Hsp70 in rainbow trout is regulated primarily at the level of transcription. Subsequently Airaksinen et al. [26] reported that an HSF1-like factor was involved in the induction of hsp70 mRNA in rainbow trout. Recently, this transcription factor has been cloned in zebrafish. These same authors also cloned a fragment of HSF from bluegill sunfish (*Lepomis macrochirus*). Interestingly, the HSF from bluegill sunfish was more similar to human, mouse and chicken HSF than that of the zebrafish gene. This suggests that either the HSF genes of fishes are surprisingly divergent, or that fish genomes encode multiple genes for HSF. Two forms of HSF1 transcript were detected using reverse transcription-polymerase chain reaction (RT-PCR) in zebrafish. Expression of both transcripts was confirmed by RT-PCR analysis of control and heat shocked hepatic, gonad, and gill tissue. There were differences in the amounts of these two transcripts among tissues, and in their responses to heat stress. The two transcripts were highly similar, except for a 78 bp insertion / deletion, and thus appear to be splice variants. However, there was a single nucleotide change that caused a substitution of lysine to asparagine adjacent to the alternative splice site, which opens the possibility that these two transcripts represent distinct isoforms of HSF1. However, the high degree of similarity between these two zebrafish HSF variants cannot account for the surprising degree of divergence between the zebrafish and bluegill sunfish genes.

## GENOMIC STRUCTURE OF FISH HEAT SHOCK PROTEIN GENES

At present remarkably little is known about the genomic organization of any of the genes encoding heat shock proteins in fish. For most species, there is little or no information regarding even the total number of heat shock family members encoded in the genome, or their linkage relationships. Lim and Brenner [16] found five intronless hsp70 genes in a region of approximately 42 kb in the pufferfish (*Fugu rubripes*). However, the linkage relationships of the Fugu hsp70 genes bore no similarity to those in other organisms. Complete sequences were obtained only for hsp70-2 and hsp70-4, while the partial sequences were obtained for hsp70-1, hsp70-3, and hsp70-5. The Fugu hsp70-4 was most similar to the hsp70-1 of mammals, the sequence linked to the major histocompatibility complex (MHC), but there was no evidence of this linkage relationship in Fugu.

The five Fugu hsp70 genes were found to be arranged in a head to head, tail to tail, and head to tail orientation. The genes were fairly similar to each other (94% similarity at the amino acid level) and amino acid differences were distributed broadly across the molecules, with no obvious regions of lower similarity. The primary exception was the hsp70-2 gene, which contained a substantial deletion at the 3' end relative to the other copies. The significance of this deletion is not known. Putative HSEs were identified in the 5' regions adjacent to each of the Fugu hsp70 genes, suggesting the possibility that all of these genes are heat-inducible. There were multiple stretches of sequence similarity within the upstream regions of the genes, suggesting some additional similarity in their regulation. However, there were also substantial differences between genes in the promoter regions, suggesting the possibility of tissue or stressor-specific regulation. A functional genomics analysis has not yet been attempted for these genes, so the question of their relative roles and regulation remains to be addressed.

Little is known regarding the total number of hsp70 genes in other fish species. Lele et al. [14] used degenerate PCR from genomic DNA in an attempt to identify variants of hsp70 in zebrafish (*Danio rerio*). Only two genes were identified: hsp70-4 and hsp70-15. Hsp70-4 was most similar to the hsp70 of rainbow trout (*Oncorhynchus mykiss*; [27]), and was strongly heat inducible during embryonic development, suggesting that this gene represents the inducible hsp70 gene in zebrafish. Hsp70-15 was similar to the heat shock cognate (hsc71) of rainbow trout [14] were not able to find any evidence that this gene was actually expressed in zebrafish, at least in the developing embryo. Santacruz et al. [18] studied the expression of an hsc70 gene in the developing zebrafish embryo, indicating that this gene was expressed. Although the evidence presented by [14] is not conclusive, it does suggest that either there is only one copy of hsp70 in the zebrafish genome, or that the sequences of these gene copies have been homogenized through gene conversion. This process has occurred in other species. For example, in the rat, the hsp70-1 and hsp70-2 gene products, although encoded by different genes, are identical at the amino acid level [28].

Genomic sequence information is available for a few other fish heat shock protein genes. The complete gene encoding an hsp70 has been cloned in tilapia (*Oreochromis mossambicus*; [15]). Typical of the inducible hsp70 genes in other species, this gene does not contain introns. Three copies of the HSE consensus sequence were present in the promoter of the gene, and transfection experiments were used to show that these elements could confer heat-induction on a reporter gene. The promoter of an hsp70 gene from zebrafish has also been cloned [18]. These authors made stable transgenic lines of zebrafish expressing green fluorescent protein (GFP) under the control of the zebrafish heat shock promoter. At normal temperatures GFP activity was not detectable in developing embryos (except in the eye lens), but was expressed in all tissues following heat shock.

Genomic sequences have been obtained for a heat shock cognate (hsc71) from *Rivulus marmoratus* [19] and rainbow trout [29]. Like the homologous mammalian genes, the fish hsc71 gene contained eight introns. Exon sizes were identical between these two fish species, although their intron sizes differed. The most striking difference was in the size of intron 4, which was 1580 bp in *Rivulus*, but only 225 bp in the rainbow trout. The first intron was larger in both the fish species than in the homologous genes in

mammals (1.6–1.8 kb in the fish vs. 0.56–0.73 kb in mammals). The functional or evolutionary significance of these differences is not known.

Much remains to be studied regarding the functional genomics of heat shock proteins in fish, but sufficient sequence information is now available that these experiments can be productively attempted. Important questions to be addressed include: (a) what is the functional significance of the multiple somewhat divergent copies of heat shock protein family members in fish genomes; (b) does the regulation of, for example, multiple similar copies of hsp70, differ in response to heat stress or among tissues; and (c) what is the relationship between the thermal habitat of fish species and the structure, number, or regulation of heat shock protein genes?

Previous studies on the roles of heat shock proteins in fish provide important insights that can guide functional genomic investigation. In the remainder of this review we summarize this work on heat shock proteins in fish in the context of factors regulating heat shock proteins, the roles of heat shock proteins in development, the effects of hormones on heat shock proteins, and the importance of these proteins for environmental adaptation in fish. Taken together, these studies indicate that the regulation and roles of heat shock proteins in fish are complex. This complexity makes fish an ideal model for studying the significance of heat shock proteins in the cellular stress response using functional genomics.

## FACTORS REGULATING HEAT SHOCK PROTEINS IN FISH

Understanding the factors that regulate heat shock proteins in fish is providing researchers with substantial insight into their functional significance and roles within the cellular and organismal stress responses. Heat shock protein expression is influenced by a wide variety of abiotic and biotic factors, and in this section we discuss some of the factors that influence them.

### Abiotic factors and their effects on heat shock proteins

The majority of studies on heat shock proteins in fish have been limited to *in vitro* examinations conducted in laboratory environments. Furthermore, most of these studies reported the induction of heat shock protein families following exposure to stress, without elucidating the functional significance underlying their observations<sup>[6]</sup>. Studies in fish have demonstrated that heat stress can induce various heat shock proteins in cell lines<sup>[30]</sup>, primary cell culture<sup>[31]</sup>, and in tissues from whole animals<sup>[31]</sup>. Osmotic stress has recently been demonstrated to induce hsp90 mRNA in chinook salmon (*Oncorhynchus tshawytscha*;<sup>[32]</sup>) and Atlantic salmon (*Salmo salar*;<sup>[33]</sup>), and Hsp54 and Hsp70 in Atlantic salmon<sup>[34]</sup>. Elevated levels of various heat shock proteins have been measured in tissues of fish exposed to environmental contaminants, such as heavy metals<sup>[34]</sup>, industrial effluents<sup>[35]</sup>, pesticides<sup>[36]</sup>, and polycyclic aromatic hydrocarbons<sup>[36]</sup>. It is noteworthy that while many indicators of fish stress (e.g. plasma cortisol concentrations) are altered by handling and sampling procedures,<sup>[36]</sup> demonstrated that handling stress does not alter levels of hepatic hsp70 in rainbow trout (*Oncorhynchus mykiss*). The effects of abiotic factors on heat shock protein expression in fish have been extensively reviewed<sup>[36]</sup>.

### Biotic factors and their effects on heat shock proteins

Less is known regarding the effects of biotic factors on heat shock proteins in fish. It is reported that levels of Hsp70 were significantly raised in the brains of goldfish (*Carassius auratus*) that were reared in the presence of a predator, the bluegill sunfish (*Lepomis macrochirus*). More is known about the effects of pathogenic exposure on heat shock proteins. Pathogens are common in natural environments and can have detrimental impacts on the health of fish populations. Heat shock proteins are known to be involved in the immune response following pathogenic exposures in mammals. Cho et al.<sup>[37]</sup> were the first to observe a cellular stress response (Hsp90) in fish cells, following exposure of cells to infectious haematopoietic necrosis virus (IHNV). Forsyth et al.<sup>[38]</sup> observed increased Hsp70 in hepatic and head kidney tissues of coho salmon (*Oncorhynchus kisutch*) infected with *Renibacterium salmoninarum*, the causative agent of a slowly developing, chronic disease (bacterial kidney disease) of salmonids. Subsequent experiments demonstrated that juvenile rainbow trout (*Oncorhynchus mykiss*) infected with *Vibrio anguillarum*, the causative agent of the acute disease vibriosis, had elevated levels of Hsp70 in hepatic and head kidney tissues prior to clinical signs of disease. Collectively, these data provide early evidence that a relationship exists between heat shock proteins and disease in fish.

There are several plausible links between heat shock proteins and the immune system in organisms that are faced with bacterial challenges, including fish. Perhaps the simplest connection is that virulent pathogens may damage components within a cell through the release of cytolytic substances, thus altering cellular homeostasis and inducing heat shock proteins. In fish, the inflammatory pathology caused by pathogenic exposure may alter physiological processes at the cellular level, such as ion regulation and acid-base balance. Host immunocytes (phagocytes and granulocytes) release extracellular substances such as reactive oxygen species, cationic peptides, lysozyme, and cytokines that are known inducers of various heat shock proteins. The exogenous administration of heat shock proteins can also upregulate two major macrophage / monocyte differentiation markers, and studies have demonstrated that host cells can recognize small tumour-related peptides when complexed with Hsp70. Therefore, heat shock proteins may be an integral part of the MHC-class II peptide complex assembly lending support to the hypothesis that heat shock proteins are involved in antigen presentation. Heat shock proteins may also be important in providing maintenance<sup>[39]</sup> and protection to phagocytic cells by repairing damage or protecting against auto-lysis or apoptosis

due to auto-oxidation brought about by the cells own internal defence systems. Further studies are necessary to elucidate the relationship between the immune system and heat shock proteins, and to resolve how their production assists fish in responding at the immunological level to an infectious challenge. Such studies can not only help us to understand disease states more clearly, but can assist in the formulation of strategies to protect against them.

## HEAT SHOCK PROTEINS: AN ALTERNATIVE TO CONTROL DISEASE IN AQUATIC ORGANISM

Hsp70 is the most widely studied stress protein in aquatic organisms and it is thought to function in induced thermo tolerance and cross tolerance<sup>[40,41]</sup>. It is now becoming clear that stress-induced Hsp70 enhances tolerance of aquatic organisms to disease, and work with several pathogenic *Vibrio* challenge models has raised many issues related to the role of Hsps in fish and shrimp pathology. In this context, anon-lethal heat shock (NLHS) of 37 °C for 30 min followed by 6 hr recovery maximally induced endogenous Hsp70 and optimally enhanced the resistance of *Artemia* larvae against *V. campbellii* and *V. proteolyticus*<sup>[42]</sup> *Vibrio* species known to infect brine shrimp. The two-fold increase in larval survival, in concert with stress protein synthesis, suggests a protective role for Hsp70. In a separate experiment, exposure of *Artemia* larvae to a combined hypo- and hyperthermic shock enhanced the amount of a 70 kDa polypeptide which reacted with antibody to Hsp70. Protection against infection by *V. campbellii* was significantly enhanced in these larvae, with the result again supporting a causal link between Hsp70 accumulation induced by heat stress and enhanced resistance to infection<sup>[43]</sup>. Similar observations were made in shrimp other than *Artemia* where Hsp70 build-up after a 24 hr hyperthermic stress from 29 °C to 37 °C correlates with attenuation of gill-associated virus (GAV) replication in the black tiger prawn. The most frequently used protocol to stimulate Hsp expression in these experiments entails a short NLHS followed by incubation for several hours under non-stress conditions<sup>[44]</sup>.

Other methods that enhance Hsp70 synthesis and prime aquatic organisms against disease include exposure to chemical inducers of Hsp70. Pro-TeX<sup>®</sup>, a soluble variant of Tex-OE<sup>®</sup>, a patented extract from the skin of the prickly pear cactus *Opuntia ficus-indica*, is a non-stressful inducer of high levels of endogenous or host-derived Hsps which has become available for use in fish and shellfish. Stimulation of salmon and gilthead sea bream *Sparus aurata* L. with Pro-TeX<sup>®</sup> in the laboratory before exposure to *Vibrio anguillarum* infection reduces loss of fish to half of what occurs in fish not exposed to Pro-TeX<sup>®</sup>. When Pro-TeX<sup>®</sup> was used to stimulate fish before exposure to infection, circulating Hsp levels were detectable after incubation with little or no delay<sup>[45]</sup>. Treatment of *Artemia* with Tex-OE<sup>®</sup> (152 ppb) for 1 hr promoted accumulation of Hsp70 and enhanced survival when subjected to *V. campbellii* challenge. Protection is perhaps due to enhanced prophenoloxidase (proPO) and nitric oxide (NO) production, important components of the innate immune system<sup>[46]</sup>.

Supplying exogenous Hsps, either by feeding with Hsps encapsulated in bacteria or injecting recombinant Hsp70, represents another way to limit *Vibrio* infection in aquatic organisms. As one example, feeding with *E. coli* YS2 over-producing DnaK, the prokaryotic equivalent of Hsp70, enhances gnotobiotic *Artemia* larvae survival approximately two- to three-fold upon challenge with pathogenic *V. campbellii*<sup>[47]</sup>. Similar results were obtained when larvae were fed with heated bacterial strains LVS 2 (*Bacillus* sp.), LVS 3 (*Aeromonas hydrophila*), LVS 8 (*Vibrio* sp), GR 8 (*Cytophaga* sp.) and GR 10 (*Roseobacter* sp.), all of which produce increased amounts of DnaK when compared to non-heated bacteria. Improvement in larval resistance to *V. campbellii* infection correlates with escalating amounts of DnaK, suggesting a protective role for this protein, either via chaperoning or by immune enhancement<sup>[48]</sup>. Support for an immunological effect is offered by the observation that feeding DnaK-enriched bacteria stimulates the Pro PO cascade system of *Artemia*, a mechanism important for pathogen melanisation by the innate immune system. In a related study, feeding white leg shrimp *Litopenaeus vannamei* larvae with *E. coli* YS2 over-producing DnaK protects against pathogenic *V. harveyi*, boosting survival beyond 30% in a standardized challenge assay. As revealed by RT-q PCR, administration of DnaK enhances crustin mRNA transcript 7-fold more in whole larvae homogenates than those fed with YS2 cells that do not produce DnaK. Crustins are cationic cysteine-rich antimicrobial peptides and their up-regulation may protect shrimp larvae by suppressing *Vibrio*<sup>[49]</sup> In fish, intra-coelomal injection with DnaK and GroEL, proteins equivalent to mammalian Hsp70 and Hsp60, combined with a non-lethal heat shock, safeguards *Xiphophorus maculatus* from death caused by *Yersinia ruckeri*<sup>[50]</sup>. These studies indicate that the resistance of aquatic organism to *Vibrio* infection is enhanced by endogenous DnaK / Hsp70.

To summarize, there are several mechanisms by which Hsp70 guards against bacterial infection. Hsp70 may stabilize cells against injury due to pathogen proliferation, assist the proper folding of cell proteins synthesized in response to bacterial pathogens and facilitate the storage and re-folding of partially denatured proteins. Hsps have the potential to improve tolerance to *Vibrio* sp. via immune stimulation. Hsps are thought to influence the production of cell surface peptides which are presented to the immune system, facilitating recognition of diseased cells<sup>[51,52]</sup> and they are involved with Toll-like receptors, a major element of the innate immune system. This possibility is currently under investigation, work that promises to yield findings of fundamental importance with applications in aquaculture, a major method of food production.

## FUNCTIONAL ROLES OF HSP'S

The precise functions of proteins in the HSP70 family have not been completely delineated. However, the high degree of conservation of these proteins across species, coupled with their importance in cell survival in various conditions, suggests that

these HSPs are critical for both normal cellular function and survival after a stress. Therefore, one of the primary means to gain insight into HSP70 function in both *in vitro* and *in vivo* systems has been to assess their cellular responses after a stress-related induction.

### Thermotolerance

One of the first physiological functions associated with the stress-induced accumulation of the inducible Hsp70 was acquired thermotolerance, which is defined as the ability of a cell or organism to become resistant to heat stress after a prior sublethal heat exposure. Data from subsequent studies demonstrated that the induction of Hsp70 was associated with the development of tolerance to a variety of stresses, including hypoxia, ischemia, acidosis, energy depletion, cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>[53]</sup>, and ultraviolet radiation<sup>[54]</sup>. The phenomenon of acquired thermotolerance is transient in nature and depends primarily on the severity of the initial heat stress. In general, the greater the initial heat dose, the greater the magnitude and duration of thermotolerance. The expression of thermotolerance following heating will occur within several hours and last 3–5 days in duration. Additional supporting evidence includes observations that have linked the kinetics of thermotolerance induction and decay with parallel changes in HSP70 induction and degradation. However, these studies have generally been correlative in nature, with no causal link established between induction of HSP70 and acquired thermotolerance.

The similar kinetics of thermotolerance demonstrated by cells, tissues, and animals suggest that the morbidity and mortality associated with whole body heating is due in part to the dysfunction of some critical target tissues. It can be postulated that the development of thermotolerance results from the improved tolerance of the weakest organ and cell systems. Presumably, these tissues are both heat sensitive and vital to the animal. For instance, the small intestine is capable of generating thermotolerance<sup>[55]</sup> and is also reported to be the tissue most sensitive to heat damage. Both the small intestine and whole animal are sensitive to *in vivo* temperatures ranging from 41 °C to 42 °C, whereas gastrointestinal disorders are frequently observed after whole body heating (42 °C for 120 min) and during heat stroke in humans.

Advances in molecular biology techniques have provided researchers with tools to address the issue of a causal link between HSP induction and thermotolerance more directly. Cellular manipulations that either block HSP70 accumulation or overexpress certain HSPs have been shown to either increase or decrease heat sensitivity. For example, transfection of a plasmid containing the *Drosophila* HSP70 gene into a monkey fibroblast cell line produced large increases in HSP70 accumulation in these cells and improved tolerance to a heat shock paradigm. Elevations in cellular HSP27 levels via plasmid transfection have also yielded a state of thermotolerance without the need for a conditioning thermal stress. Conversely, microinjections of monoclonal antibodies specific for HSP70 inhibited the synthesis of these proteins, resulting in a reduction in thermotolerance<sup>[56]</sup>.

As noted, HSPs appear to play a role in protecting cells from damage generated by a variety of stressors. Their synthesis is associated with protection against light-induced damage to the retina<sup>[54]</sup> and ischemia-reperfusion injury to the heart, liver, and kidney. In addition, studies of cardiac shock followed by resuscitation have revealed that hepatocytes synthesize members of the HSP70 family early in the course of recovery. The fact that HSP70 message is preferentially translated by a cell under stress to the exclusion of other messages may result in the inability of the cell to produce some proteins or respond to additional signals. In this model, the cell may “choose” self-preservation over tissue preservation to the detriment of the organ. This model may be particularly relevant in a situation where HSP70 accumulation could be utilized as a biomarker of cellular injury<sup>[57]</sup>. In this scenario, cells of tissues most at risk would also be the cells most likely to accumulate HSP70 during stress, and this HSP70 accumulation could mark a tissue for potential failure.

Although the precise mechanisms for the improvement in cellular thermotolerance in association with an increase in HSP levels have not been delineated, it is tenable to postulate that proteins in the HSP70 family are involved in preventing protein denaturation and / or processing denatured proteins and protein fragments that are produced by stressors such as hyperthermia. Supporting evidence for this scenario comes from a set of *in vitro* experiments by Mizzen and Welch<sup>[58]</sup>, who demonstrated that heat stress results in translational arrest within a cell, and this arrest is proportional to both the intensity and duration of the applied heat stress. Subsequent resumption of translation resulted in HSP mRNA being translated into HSPs before the synthesis of other proteins took place within the cell. Interestingly, the period of translational arrest in response to heat stress could be shortened in these experiments if cells were first made thermotolerant.

One interpretation of these results is that a primary function of HSPs during cellular stress is to maintain translation and protein integrity. Cells that were made thermotolerant also produced less HSP during a second challenge compared with previously unheated cells, suggesting there is a regulation of HSP synthesis that is dependent on the levels of these proteins existing within the cell. Although a majority of data in this area has been derived from *in vitro* methodologies, a unique set of experiments in humans by Moseley and colleagues<sup>[59]</sup> generated data supportive of this concept. Healthy men performed a challenging exercise protocol in either hot (46 °C) or more moderate (30 °C) ambient conditions. Leukocytes obtained from subjects after the protocol were then incubated at 41 °C. The increase in Hsp70 synthesis in heat-stressed leukocytes was inversely proportional to the length of the initial “conditioning” exercise stress, suggesting that cells regulate the amount of these stress proteins in response to repeated challenges.

An additional issue related to the development of thermotolerance deals with the possibility that HSPs, through their interaction

with cellular proteins during translational arrest, play a role in preventing protein denaturation and processing denatured proteins that are generated in response to stressors such as heat. For example, data suggest that the injection of denatured proteins into cells or the generation of abnormal proteins can induce HSP activity.

Although these different sets of data clearly demonstrate a broad range of physiological processes that involve the HSPs, the evidence that the HSPs are responsible for cellular thermotolerance is circumstantial rather than conclusive. The variety of stressors used to condition cells will likely induce other important cellular defense proteins in addition to HSPs, such as antioxidant enzymes <sup>[57]</sup>. It should also be noted that thermotolerance can be generated in the absence of HSPs. In these studies, thermotolerance was manifested under conditions of protein synthesis inhibition (i.e., no HSP accumulation) as well as a chronic exposure to a lower temperature than is required for HSP accumulation. Other studies have demonstrated that inhibition of transcription during the conditioning heat stress also allows the maintenance of thermotolerance <sup>[60]</sup>. In addition, oxidative stresses, which can confer thermotolerance, may not increase the levels of HSPs. In other stresses, such as ischemia, where HSPs are thought to play a role, HSP overexpression has also not been found to confer tolerance. Therefore, generating a scenario in which the development of stress tolerance in a cellular system is causally linked to an increase in Hsp70 expression is difficult because organisms and cells respond to stress in a variety of complex ways <sup>[61]</sup>.

The mechanisms contributing to thermal injury vs. thermotolerance are even less clear in the intact organism. One obvious explanation for thermal injury at the cellular level is direct heat damage <sup>[62]</sup>. However, this cellular damage is likely due in part to functional impairment of a tissue or organ (e.g., reductions in blood flow) and the possible impact of systemic factors such as endotoxin-mediated cytokine production. Moreover, much of the research attempting to gain an understanding of the intact organism's adaptive response to heat has focused on heat acclimatization processes. Because the factors involved in heat injury at the whole organism level are complex and the mechanisms contributing to the protective role of HSPs are not well defined, issues such as these remain a central challenge in this field of research.

### **HSP70 functions associated with stress tolerance**

Although the evidence linking stress-induced HSP70 accumulation with tolerance to heat and other stressors is compelling, the mechanisms by which HSPs confer stress tolerance are not well understood. Attention has primarily been focused on the role of HSP70 as a chaperone and its potential ability to contribute to cellular repair processes in response to interventions such as heat, oxidative stress, activation of proteases, release of lysosomal and proteolytic enzymes, and alterations of the cytoskeleton.

Several important cytoprotective functions have been attributed to HSPs and, in particular, the HSP70 family. These include 1) the folding of proteins in various intracellular compartments, 2) the maintenance of structural proteins, 3) the refolding of misfolded proteins, 4) translocation of proteins across membranes and into various cellular compartments, 5) the prevention of protein aggregation, and 6) the degradation of unstable proteins <sup>[63]</sup>. Interestingly, it has also been noted that HSPs can play a role in apoptosis. HSP27, HSP70, and HSP90 proteins are predominantly antiapoptotic, whereas HSP60 is proapoptotic. Moreover, it appears that these HSPs function at multiple points in the apoptotic signaling pathway to elicit this response.

Although there are numerous studies available demonstrating the broad range of physiological processes that involve HSPs, including protein translocation, receptor regulation, cytoskeleton stabilization, and management of protein folding and repair, evidence directly demonstrating that the HSPs are responsible for stress tolerance is not conclusive. In addition, the complexity of the integrated response to a physiological challenge *in vivo* makes it difficult to ascertain what "stressor" is responsible for stimulating an increase in HSP synthesis. In a situation such as an aerobic exercise bout of moderate intensity and duration, additional signals besides an elevation in core temperature ( $T_c$ ) are present that could potentially activate HSP expression, including acidosis, energy depletion, reductions in blood flow to visceral organs and an associated tissue hypoxia, and generation of ROS <sup>[64]</sup>. Furthermore, in addition to HSPs, cells will express other important stress proteins such as antioxidant enzymes, providing an organism with multiple cytoprotective options.

It is also important to note that there are numerous studies demonstrating that thermotolerance can be generated in the absence of intracellular HSP accumulation. Therefore, it is problematic, especially at the whole organism level, to definitively link an increase in HSP70 expression directly to the acquisition of stress tolerance, partly because mammalian species respond to stress in a multitude of complex, integrated ways.

## **SUMMARY AND FUTURE DIRECTIONS**

In this mini-review, an attempt was made to summarize the physiological factors that modulate HSP responses to stressors at cellular and systemic levels. From the literature presented, it should be evident that the HSP70 family of proteins is essential for cellular survival from heat stress and other types of physiological challenge. It is clear that these proteins are ubiquitously present in cells under both normal and stressful conditions and that their structure is well conserved among species. In addition, there is a large body of evidence to support the role of HSPs for improving cell survival to otherwise lethal challenges.

Despite the significant amount of progress that has been made regarding biochemical and structural features of HSP70, the mechanisms by which these proteins provide protection against cellular stress are still not thoroughly understood. Delineation

of these mechanisms will have significant implications both clinically and at a basic science level. Furthermore, technological advances will enhance the ability of researchers to greatly extend experiments addressing HSP functions and mechanisms from cell culture into animals and humans. On the basis of research performed over the past decade, we have learned that the induction of HSP70 is not confined to heat shock paradigms involving extreme conditions in culture systems and lower species. Instead, HSPs are synthesized in animals and humans in response to many relevant physiological (e.g., heat stress, exercise, energy depletion) and pathological (e.g., viral infections, cytokine release) conditions.

Finally, it is still uncertain whether HSPs can be utilized in a therapeutic setting. Although gene therapy programs have made impressive advances in recent years, the over expression of HSP70 has proven to be problematic. Thus, the answers to many of these questions await further study.

## REFERENCES

1. Morimoto RI and Santoro MG. Stress-inducible responses and heat shock proteins: New pharmacological targets for cytoprotection. *Nat Biotechnol.* 1998;16:833–838.
2. Hahn GM, et al. Mechanistic implications of the induction of thermotolerance in Chinese hamster cells by organic solvents. *Cancer Res.* 1985;45:4138–4143.
3. Kiang JG and Tsokos GC. Heat shock proteins 70 kDa: Molecular biology, biochemistry, and physiology. *Pharmacol Ther.* 1998;80:183–201.
4. Ciocca DR, et al. Fuqua Biological and clinical implications of heat shock protein 27,000 (Hsp27): A review *J Natl Cancer Inst.* 1993;85:1558–1570.
5. Sung YY, et al. The induction of Hsp70 synthesis by non-lethal heat shock confers thermotolerance and resistance to lethal ammonia stress in the common carp, *Cyprinus carpio* (Linn). *Aquacult Res* 2014; (In-press) 2.
6. Iwama GK, et al. Heat shock protein expression in fish. *Rev Fish Biol Fisher.* 1998;8:35–56.
7. Welch, 1993. W.J. Welch. How cells respond to stress. *Sci Am.* 1993;269:56–64.
8. Bienz M and Pelham HR. Mechanisms of heat shock gene activation in higher eukaryotes. *Adv Genet.* 1987;24:31–72.
9. Kelly PD, et al. Genetic linkage mapping of zebrafish genes and ESTs *Genome Res.* 2000;10:558–567.
10. Mommsen TP, et al. Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. *Rev Fish Biol Fisher.* 1999;9:211–268.
11. Walter L, et al. Comparative analysis of the three major histocompatibility complex-linked heat shock protein 70 (Hsp70) genes of the rat. *Immunogenetics.* 1994;40:325–330.
12. Wendelaar Bonga SE. The stress response in fish. *Physiol Rev.* 1997;77:591–625.
13. Kothary RK, et al. The heat-shock phenomenon in cultured cells of rainbow trout: hsp70 mRNA synthesis and turnover. *Biochim Biophys Acta.* 1984;783:137–143.
14. Lele Z, et al. Hsp47 and hsp70 gene expression is differentially regulated in a stress- and tissue-specific manner in zebrafish embryos. *Dev Genet.* 1997;21:123–133.
15. Molina et al. Cloning and expression analysis of an inducible HSP70 gene from tilapia fish. *FEBS Lett.* 2000;474:5–10.
16. Lim EH and Brenner S. Short-range linkage relationships, genomic organization and sequence comparisons of a cluster of five hsp70 genes in *Fugu rubripes*. *Cell Mol Life Sci.* 1999;55:668–678.
17. Zafarullah M, et al. Molecular cloning and characterization of a constitutively expressed heat-shock-cognate hsc71 gene from rainbow trout. *Eur J Biochem.* 1992;204:893–990.
18. Santacruz H, et al. Molecular characterization of a heat shock cognate cDNA of zebrafish, hsc70, and developmental expression of the corresponding transcripts. *Dev Genet.* 1997;21:223–233.
19. Park JH, et al. Genomic cloning of the Hsc71 gene in the hermaphroditic teleost *Rivulus marmoratus* and analysis of its expression in skeletal muscle: Identification of a novel muscle-preferred regulatory element. *Nucleic Acids Res.* 2001;29:3041–3050.
20. Yin Z, et al. Identification of differentially expressed genes in Con A-activated carp (*Cyprinus carpio* L.) leucocytes. *Comp Biochem Physiol B Biochem Mol Biol.* 1999;124:41–50.
21. Currie S and Tufts BL. Synthesis of stress protein 70 (Hsp70) in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J Exp Biol.* 1997;200:607–614.
22. Pratt WB and Welsh MJ. Chaperone functions of the heat shock proteins associated with steroid receptors. *Semin Cell Biol.* 1994;5:83–93.

23. Pottinger TG. The effect of stress and exogenous cortisol on receptor-like binding of cortisol in the hepatic of rainbow trout, *Onchorynchus mykiss*. *Gen Comp Endocrinol*. 1990;78:194–203.
24. Pearson DS, et al. 1996. Cloning and characterization of a cDNA encoding the collagen-binding stress proteins Hsp47 in zebrafish. *DNA Cell Biol*. 1996;15:263–271.
25. Pearl LH and Prodromou C. Structure and *in vivo* function of hsp90. *Curr Opin Struct Biol*. 2000;10:46–51.
26. Airaksinen S, et al. Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. *J Exp Biol*. 1998;201:2543–2551.
27. Kothary RK and Candido EPM. Induction of a novel set of polypeptides by heat shock or sodium arsenite in cultured cells of rainbow trout, *Salmo gairdneri*. *Can J Biochem*. 1982;60:347–355.
28. Halloran MC, et al. Laser-induced gene expression in specific cells of transgenic zebrafish. *Development*. 2000;127:1953–1960.
29. Misra S, et al. Analysis of stress-induced gene expression in fish cell lines exposed to heavy metals and heat shock. *Biochim Biophys Acta*. 1989;1007:325–333.
30. Bachelet M, et al. Macrophages and heat shock proteins. *Res Immunol*. 1998;149:727–732.
31. Koban M, et al. Induction of heat shock proteins synthesis in teleost hepatocytes: Effects of acclimation temperature. *Physiol Zool*. 1987;60:290–296.
32. Pratt WB. The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. *Biol Chem*. 1993;268:21455–21458.
33. Pan F, et al. Cloning and characterization of salmon hsp90 cDNA: Upregulation by thermal and hyperosmotic stress. *J Exp Zool*. 2000;287:199–212.
34. Smith TR, et al. Hsp70 and a 54 kDa protein (Osp54) are induced in salmon (*Salmo salar*) in response to hyperosmotic stress. *J Exp Zool*. 1999;284:286–298.
35. Janz DM, et al. Elevated ovarian follicular apoptosis and heat shock protein 70 expression in white sucker exposed to bleached kraft pulp mill effluent. *Toxicol Appl Pharm*. 1997;147:391–398.
36. Sanders BM. Stress proteins in aquatic organisms: An environmental perspective. *Crit Rev Toxicol*. 1993;23:49–75.
37. Cho WJ, et al. A novel 90 kDa stress protein induced in fish cells by fish rhabdovirus infection. *Biochem Biophys Res Commun*. 1997;233:316–319.
38. Forsyth RB, et al. Stress protein expression in coho salmon with bacterial kidney disease. *J Aquat Anim Health*. 1997;9:18–25.
39. Polla BS, et al. Differential induction of stress proteins and functional effects of heat shock in human phagocyte. *Inflammation*. 1995;19:368–378.
40. Sung YY, et al. Enhancement of Hsp70 synthesis protects Common Carp *Cyprinus carpio* L. against lethal ammonia toxicity. *J Fish Dis*. 2012;35:563-568.
41. Sung YY, et al. Non-lethal heat shock protects gnotobiotic *Artemia franciscana* larvae against virulent *Vibrios*. *Fish Shellfish Immunol*. 2007;22:318-326.
42. Sung YY, et al. Exposure of gnotobiotic *Artemia franciscana* larvae to abiotic stress promotes heat shock protein 70 synthesis and enhances resistance to pathogenic *Vibrio campbellii*. *Cell Stress Chaperones*. 2008;13:59-66.
43. de la Vega E, et al. Short-term hyperthermic treatment of *Penaeus monodon* increases expression of heat shock protein 70 (HSP70) and reduces replication of gill associated virus (GAV). *Aquaculture*. 2006;253:1-4.
44. Roberts RJ, et al. Heat Shock Proteins (Chaperones) in Fishes and Shellfishes and their Potential Role in Health and Welfare: A Review. *J Fish Dis*. 2010;33:789-801.
45. Baruah K, et al. Priming the prophenoloxidase system of *Artemia franciscana* by heat shock proteins protects against *Vibrio campbellii* challenge. *Fish Shellfish Immunol*. 2011;31:134-141.
46. Sung YY, et al. Ingestion of bacteria over-producing DnaK attenuates *Vibrio* infection of gnotobiotic *Artemia franciscana* larvae. *Cell Stress Chaperones*. 2009a;14:603-609.
47. Sung YY, et al. Feeding *Artemia franciscana* (Kellogg) larvae with bacterial heat shock protein, protects from *Vibrio campbellii* (Baumann) infection. *J Fish Dis*. 2009b;32:675-685.
48. Loc NH, et al. Non-lethal heat shock increased Hsp70 and immune protein transcripts but not *Vibrio* tolerance in the White leg shrimp. *PLoS ONE*. 2013;8:1-7.
49. Ryckaert J, et al. Heat shock proteins protect platyfish (*Xiphophorus maculatus*) from *Yersinia ruckeri* induced mortality. *Fish Shellfish Immunol*. 2010;28:228-231.

50. Johnson JD and Fleshner M. Releasing signals, secretory pathways, and immune function of endogenous extracellular heat shock protein 72. *J Leukoc Biol.* 2006;79:425-434.
51. Singh V and Aballay A. Heat-shock and gene activation of HSF-1 enhance immunity to bacteria. *Cell cycle.* 2006;21:2443-2446.
52. Mizzen LA and Welch WJ. Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. *J Cell Biol.* 1988;106:1105-1116.
53. Jaattela M and Wissing D. Heat shock proteins protect cells from monocyte cytotoxicity: possible mechanism of self-protection. *J Exp Med.* 1993;177:231-236.
54. Barbe MF, et al. Hyperthermia protects against light damage in the rat retina. *Science.* 1988;241:1817-1820.
55. Hume SP and Marigold JCL. Transient, heat-induced thermal resistance in the small intestine of mouse. *Radiat Res.* 1980;82:526-535.
56. Riabowol KT, et al. Heat shock is lethal to fibroblasts microinjected with antibodies against hsp70. *Science.* 1988;242:433-436.
57. Hall DM, et al. Caloric restriction improves thermotolerance and reduces hyperthermia-induced cellular damage in old rats. *FASEB J.* 2000;14:78-86.
58. Mizzen LA and Welch WJ. Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. *J Cell Biol.* 1988;106:1105-1116.
59. Ryan AJ, et al. Synthesis of 70K stress protein by human leukocytes: effect of exercise in the heat. *J Appl Physiol.* 1991;70:466-471.
60. Ananthan J, et al. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science.* 1986;232:522-524.
61. Krebs R and Bettencourt B. Evolution of thermotolerance and variation in the heat shock protein. *Am Zool.* 1999;39:910-919.
62. Lambert GP, et al. Hyperthermia-induced intestinal permeability and the role of oxidative and nitrosative stress. *J Appl Physiol.* 2002;92:1750-1761.
63. Bakau B and Horwich AL. The Hsp70 and Hsp60 chaperone machines? *Cell.* 1998;92:351-366.
64. Davies KJA, et al. Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun.* 1982;107:1198-1205.