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STRESS, HEAT SHOCK PROTEINS & BIOTECHNOLOGICAL INTERVENTIONS

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Fish are exposed to stressors in nature, as well as in artificial conditions such as in aquaculture, or in the laboratory. The increasing contamination of bodies of natural freshwater and marine ecosystem around the world by anthropogenic substances is one category of environmental stressor. Various stressors, such as grading, transportation, and vaccination, are necessary components of modern intensive fish culture. The response of the fish to such stressors involves all levels of organization, from the cell, to the individual organism, to the structure of the population. In as much as the responses of the fish to a stressor is the essence of maintaining homeostasis, it is not surprising that fish respond to a variety of stressors in a generalized way at all these levels of organization. **Stress** is most often associated with a negative perspective. This is natural as the word and concept in common use is generally associated with a system that is severely challenged, and often fatigued. Experimental biologists are all involved in the practice of systematically imposing some perturbation and measuring a response. However, the stress response is a vitally important normal response of all living organisms. Spanning the range from the induction of certain genes and proteins to a behavioural response, the stress response allows us to avoid or cope with challenges to homeostasis. This underscores the importance of clarity in defining this important response. There are many definitions of stress. For this lecture note, **stress will be defined as the response of the cell, or organism, to any demand placed on it such that it causes an extension of a physiological state beyond the normal resting state**. While this definition also encompasses the stress response, for this review the animal's response to stressors may be broadly categorized into those that are either adaptive, which allows homeostatic recovery, or maladaptive and compromise performance.

While maintaining homeostasis is thought to be a key process for coping with stress, recent studies indicate that changes in physiological set points also as an important aspect of the adaptive response, especially as a coping mechanism for stressors that are chronic in nature. This concept of allostasis might explain to a large extent some of the changes in gene expression and protein profile that have been observed in animals acclimatized to altered conditions for long periods, such as temperature and oxygen conditions beyond their normal range; the presence of xenobiotics; and/or restrictions in diet or nutrients. The molecular and biochemical strategies may be part of the normal life of animals, responding to habitat changes that are either diurnal (e.g. heat and osmotic stress for 'tide-pool sculpins' – an intertidal fish, *Oligocottus maculosus*) or seasonal (long-term fasting in anadromous Arctic charr, *Salvelinus alpinus*). A few candidate genes and proteins that are involved in the adaptive strategy to cope with extreme environments (by no means are they exclusive) in the piscine system include antifreeze proteins (sub-zero temperatures), heat shock proteins (HSPs in response to various stressors including high temperature , hypoxia-inducible factor (HIF) and glucocorticoid receptor (GR) regulation (extended fasting).

The cellular stress response – A focus on Heat Shock Proteins (HSPs)

The generalized stress response at the cellular level is characterized by a family of proteins referred to as the **'heat shock proteins' (HSPs)**. The HSPs are highly conserved cellular proteins that have been observed in all organisms, including fish. Extensive studies on model species have revealed three major families of **HSPs: HSP90 (85-90 kDa), HSP70 (68-73 kDa),** and **low molecular weight HSPs (16-24 kDa)**. In the unstressed cell, there is a constitutive production of these proteins that are required in various aspects of cellular homeostasis. In stressed cells, denaturing proteins may enter one of two pathways: either degradation by a cellular protease or refolding facilitated by molecular chaperones. Because heat-shock

proteins (Hsps) act as molecular chaperones and stabilize thermally denaturing proteins, the induction of Hsps is thought to have adaptive value for organisms faced with heat stress. Not surprisingly, Hsp expression patterns have been shown to have ecologically significant ramifications for many organisms, and these patterns are directly related to thermo-tolerance at the organismal level. The most commonly occurring 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. HSP90 is active in supporting various components of cell signalling, including the cytoskeleton, enzymes, and steroid hormone receptors. Typically, HSPs function as oligomers, if not as complexes of several different chaperones, co-chaperones, and/or nucleotide exchange factors. Interaction with chaperones is variously responsible for (*a*) maintaining HSPs' partner proteins in a folding-competent, folded, or unfolded state; (*b*) organellar localization, import, and/or export; (*c*) minimizing the aggregation of non-native proteins; and (*d*) targeting non-native or aggregated proteins for degradation and removal from the cell. Presumably, the last two functions are most important in coping with environmental stress. Not all HSPs are stress-inducible, but those that are respond to a variety of stresses, including extremes of temperature, cellular energy depletion, and extreme concentrations of ions, other osmolytes, gases, and various toxic substances. Activation of various intracellular signaling pathways results in HSP expression. 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. Collectively, the apparent simplicity governing HSP function (i.e. keeping proteins functional) reinforces their importance in the most basic of cellular processes, such as cell division and growth.

All known stresses, if sufficiently intense, induce HSP expression. Accordingly, HSPs are equally well termed stress proteins, and their expression is termed the stress response. A common aspect of these inducing stresses is that they result in proteins having non-native conformations which are consistent with the function of HSPs as molecular chaperones. The possible functions of HSPs in various aspects of fish physiology, including development and aging, stress physiology and endocrinology, immunology, environmental physiology, and acclimation and stress tolerance has been reviewed recently. To a certain degree, all of the studies provided evidence for some functional role of HSPs in many aspects of the physiology of fish. Nonetheless, many of those experiments measured HSP levels in organisms following exposure to certain conditions without elucidating or further exploring the mechanisms underlying their findings. There is little understanding about the mechanisms underlying the relationships between the cellular stress response and the physiology of the fish, and it is our opinion that this area is worthy of attention and further research. It is possible that high-throughput genomic and proteomic technologies, accompanied by appropriate bioinformatics, will enable a more comprehensive profiling of the responses of the cell to stressors. An unbiased description of the protein changes that characterize the generalized response to stressors would contribute to a vital foundation upon which future experiments could be based.

In fish, the induction of various HSP families has been reported in cell lines, primary cultures of cells, as well as in various tissues from whole animals. While the majority of these studies have focused on the various effects of heat shock, there is increasing interest in the physiological and protective role of HSPs following exposure of fish to various environmental stressors. For example, increased levels of various HSPs have been measured in tissues of fish exposed to bacterial pathogens and environmental contaminants, such as heavy metals, industrial effluents, pesticides, and polycyclic aromatic hydrocarbons. It is noteworthy that while many indicators of fish stress, such as plasma cortisol concentration, are altered by handling and sampling procedures. It has been demonstrated that handling stress does not alter levels of hepatic HSP70 in rainbow trout (*Oncorhynchus mykiss*).

A complete understanding of the mechanisms underlying the sensing of a stressor and the regulation of HSPs is far from clear. Studies on HSP70, the most widely studied HSP, have demonstrated that the regulation of HSP70 gene expression occurs mainly at the transcriptional level. Analysis of HSP genes and a comparison of heat shock regulatory elements from a variety of organisms led to the identification of a

palindromic **'heat shock element' (HSE),** CNNGAANNTTCNNG. It has been demonstrated that HSP induction results primarily from the binding of an activated **heat shock transcription factor 1 (HSF1)** to HSE upstream of HSP genes. Since most of the HSP genes do not contain introns, the mRNA is rapidly translated into nascent proteins within minutes following exposure to a stressor. Slowly, genomic sequences for HSP70 are being elucidated in fish, including rainbow trout, medaka (*Oryzias latipes*), zebrafish (*Danio rerio*) and tilapia (*Oreochromis mossambicus*). Heat stress-related increases in mRNA levels have been documented in all these examples. While most studies have focused on the HSP70 response to stress, very few studies have examined the temporal profile of other HSPs, including HSP90, HSP60 and HSP30 in fish. These HSPs are involved in various aspects of cellular function, including cell signalling, cytoskeletal rearrangements and apoptosis, characterizing the role of these HSPs will provide further insights into the mechanism(s) involved in the regulation of homeostasis in unstressed and stressed cells.

While research on heat shock proteins in fish has been conducted for several decades, there has been increasing activity in this field in recent years. Most studies on HSPs in fish have been *in vitro* and/or utilized those proteins as non-specific biomarkers of environmental stress. Before HSPs are used routinely in environmental and health risk assessments, researchers need to elucidate in more detail the functional significance of HSPs in fish physiology, and understand their importance in natural fish populations. There has been recent work examining the relationship between the stress response and the functional importance of HSP70 in fish by using intertidal fishes, the tide-pool sculpin (*Oligocottus maculosus*) and fluffy sculpin (*Oligocottus snyderi*), as model species. While both sculpins live in the intertidal zone along the west coast of North America, it is known that the tidepool sculpin has a higher thermal tolerance than the fluffy sculpin. It was shown that the cellular stress response could be different depending on the stressor as well as the species. The tidepool sculpin induced a clear cellular HSP70 response to thermal, osmotic and hypoxic challenges. The degree to which HSP70 was induced was dependent on the type of stressor as well as the magnitude of the stressor. Levels of HSP70 increased with an increase in the magnitude of a particular stressor; however, once the thermal, osmotic or hypoxic challenges became too severe there was no induction of HSP70 in response to these stressors. The tidepool sculpin and fluffy sculpin showed different patterns of the cellular HSP70 response to a similar heat stress, i.e., the tidepool sculpin had a small increase in the cellular HSP70 level only at 28^{o}C, while the fluffy sculpin had much larger increases in HSP70 levels at lower temperatures. These results indicate that the functional importance of HSP70, as well as the magnitude of a stressor which induces the cellular HSP70 response, is different among species and might have been finely tuned within species during the adaptation to the species-specific habitat. Even though the tidepool and fluffy sculpins are evolutionarily closely related, and their habitats overlap, they show different HSP70 response to heat stress. In addition to the differences that we have observed in the cellular stress response of these fish species, differences in the cellular stress response of a particular fish species inhabiting different geographical locations as well as seasonal variations in the HSP response within a species and between species of fish have been reported. Therefore, in order to use the HSP level as a realistic stress indicator in various fish species, it is essential to understand the relationship between whole-body stress tolerance of fish and the cellular stress response, so that when increased levels of HSP are observed in a particular natural population of fish we can assess whether this is an indicator of stress or an adaptation to tolerate stress.

Experiments on intertidal mussel (*Mytilus californianus*) revealed that the increased chaperone need is induced by exaggerated gill protein denaturation in response to elevated body temperatures. From an organismal perspective, the fish HSF studies highlighted a constantly adjustable regulatory mechanism that was capable of altering HSP gene expression to match existing thermal conditions. The regulatory loop or ''thermostat'' is thus capable of responding to the prevailing chaperone needs, dictated by the levels of thermally denatured and unstable proteins, in the cells of eurytherms via thermal sensitivity of the controlling transcriptional factor, HSF. Thus, the ability to control HSP synthesis that is commensurate with protein degradation rates is a process that matches chaperone pools with chaperoning requirements in eurythermic cells.

Several stressors that can clearly elicit a physiological stress response are also able to affect the cellular stress response in fish. However, the HSP responses seem to vary considerably according to tissue, family of HSP, organism, developmental stage and stressor. In addition to the differences that have been observed in the cellular stress response of fish species with overlapping habitats, differences in the cellular stress response of a particular fish species inhabiting different geographical locations have been reported. Furthermore, seasonal variations in the HSP response within a species and between species of fish have been shown. It is also important to consider that some fish species may not show a heat shock response. Hofmann *et al*. (2000) and Hofmann (2005) showed that HSP70 is not induced by temperature stress in the Antarctic fish *Trematomus bernacchi*. Their findings suggested that the HSP genes in *T. bernacchii* are constitutively expressed and that their transcription is regulated by an HSF1 that is active even at nearly ambient environmental temperatures. The constant expression in *T. bernacchii* of what are stress-inducible genes in other species may reflect an elevated need for protein chaperoning in this cold-adapted fish. Alternatively, an alteration may have occurred in the transcriptional apparatus controlling HSP genes (including but not necessarily limited to the DNA-binding activity of HSF1) that resulted in the constitutive expression of HSPs. If this is the case, the constant production of HSPs must be relatively non-deleterious. Certainly, the cold and generally uncontaminated waters of the near-shore Antarctic ecosystem may have rendered the inability to induce HSPs above a constant level selectively neutral over evolutionary time. Thus, generalizations about the HSP response cannot be made unequivocally, and more knowledge is needed in order to know when one can use a specific HSP family as an indicator of thermal stress in fish. The complexity of the available data makes it difficult to conclude that HSPs are general indicators of the stress response in fish. If HSPs are to be used as indicators of the stress response, it will probably have to be done in a stressor- and species-specific manner. Finally, there is a need to determine the HSP response during periods of chronic stress. Even in terms of the well-established physiological responses, little information is available for persistent stressors. There is also a need to know how fish respond when exposed simultaneously to multiple stressors or to sequential stressors. Further research that will elucidate the relationship between the cellular and physiological stress responses is needed. Our state of understanding of the cellular stress response in fish precludes the simple use of HSPs as indicators of stress in fish.

Possible impact of Global Warming on HSPs

- Global warming is a slow process; HSPs can evolve more efficiently and offer better protection to the individuals or they may fail to offer sustained protection with the continuous increase of temperature.
- Some individuals will have over-expression/mutant forms of HSPs and through natural selection, only these individuals may survive ultimately. Through selective breeding programmes such individuals of cultivable species can be propagated for the coming years.
- HSP70 is the most common protein; gradually these may be replaced by other forms of molecular chaperons.

Biotechnological interventions and future directions/perspectives:

Most characters of economic and evolutionary importance are controlled by many loci and are inherited quantitatively (quantitative trait loci, QTL). The identification of molecular markers tightly linked to QTL allows the direct determination of the effect off QTL on fitness traits (i.e. additivity, epistasis), the number of regions involved and the magnitude of their effects. Temperature tolerance is an important trait from both an economic and an evolutionary perspective in fishes, particularly among cultivable species such as finfishes especially coldwater species, crustaceans and bivalves. Elevated temperatures may negatively affect fitness components, including parameters of growth, development, and reproduction. The polygenic basis of upper temperature tolerance (UTT) has recently been demonstrated in selected lines of rainbow trout and Arctic charr by the detection of two significant QTL by their association with the alleles at two microsatellite loci.

In recent years, interest has grown regarding the cyto-protective and cross-protective role of HSPs in bivalves, often exposed and highly resistant to a variety of environmental stress stimuli. For example, the possibility that a mild thermal stress could enhance the survival of the oyster population subject to mass mortality has been explored. Some bivalves, mainly oysters and mussels, are used as sentinel organisms for environmental bio-monitoring, and the usefulness of HSP70 bio-synthesis as a biomarker of stress has been suggested although still under debate. Natural populations are constantly exposed to challenging environments and it is necessary for the organism to buffer this environmental variation to maintain the cellular homeostasis and high performance across environments. The stress response and heat shock proteins are important for this buffering in relation to stress resistance and adaptation to the environment under some conditions. Intraspecific variation in thermal tolerance and heat shock protein gene expression has been reported in several fishes such as *Fundulus heteroclitus*. Raising thermo-tolerant individuals of cultivable species having increased levels of HSP expression/isoforms of HSPs through selective breeding to withstand the elevated levels of temperature will be of utmost importance in the global warming scenario. Studies of mutant lines might be very powerful tools to target investigations on the effect of specific genes. Microarray/anti-body based/ immuno-detection methods can be of use in identifying the isoforms of HSPs. Investigations on the profile and expression pattern of HSP genes and stress response of the temperature tolerant and cyst-forming crustaceans such as *Triops* (*Apus*) sp., *Artemia* spp; cold-surviving insects; aestivating fishes such as *Channa* spp; and extremophils will throw more light on the protective and repair mechanisms at cellular level and HSP mediated stress tolerance. New technological developments make it possible to investigate the role of genes coding for HSPs (and other candidate genes) in greater detail. A combination of genomics and proteonomics will further elucidate the effects of stress on expression patterns at the DNA, RNA and protein levels and the effect on metabolism. In order to make solid ecological and evolutionary interpretations relevant stress resistance traits could be tested in selection experiments and natural populations with accurate climatic information. By combining physiological and phenotypic studies of laboratory-selected and naturally-adapted populations, we expect to gain a much more detailed understanding of HSP regulation and expression and the role of this response for natural populations.

Suggested Reading:

Boutet, I., Tanguy, A., Rousseau, S., Auffret, M. and Moraga, D. (2003). Molecular identification and expression of heat shock cognate 70 (*hsc70*) and heat shock protein 70 (*hsp70*) genes in the Pacific oyster *Crassostrea gigas*. Cell Stress & Chaperones, 8(1):76 – 85.

Buckley, B. A., Owen, M. – E. and Hofmann, G. E. (2001). Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. The Journal of Experimental Biology, 204: 3571 – 3579.

Buckley, B. A., Place, S. P. and Hofmann, G. E. (2004). Regulation of heat shock genes in isolated hepatocytes from an Antractic fish, *Trematomus bernacchii*. The Journal of Experimental Biology, 207: 3649 – 3656.

Fangue, N. A., Hofmeister, M. and Schulte, P. M. (2006). Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*. The Journal of Experimental Biology, 209: 2859 – 2872.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol, 61: 243 – 282.

Hofmann, G. E. (2005). Patterns of Hsp gene expression in ectothermic marine organisms on small to large biogeographic scales. Integr. Comp. Biol., 45: 247 – 255.

Hofmann, G. E., Buckley, B. A., Airaksinen, S., Keen, J. E. and Somero, G. N. (2000). Heat-Shock protein expression is absent in the Antarctic fish *Trematomus bernacchii* (Family Nototheniidae). The Journal of Experimental Biology, 203: 2331 – 2339.

Iwama, G. K., Afonso, L. O. B., Todgham, A., Ackerman, P. and Nakano, K. (2004). Are hsps suitable for indicating stressed states in fish? The Journal of Experimental Biology, 207: 15 – 19.

Jackson, T. R., Ferguson, M. M., Danzmann, R. G., Fishback, A. G., Ihssen, P. E., O'Connells, M. and T. J. Crease (1998). Identification of two QTL influencing upper temperature tolerance in three rainbow trout (*Oncorhynchus mykiss*) half-sib families. Heredity, 80: 143 – 151.

Piano, A., Valbonesi, P. and Fabbri, E. (2004). Expression of cytoprotective proteins, heat shock protein 70 and metallothioneins, in tissues of *Ostrea edulis* exposed to heat and heavy metals. Cell Stress & Chaperones, 9(2): 134 – 142.

Somorjai, I. M. L., Danzmann, R. G. and Ferguson, M. M. (2003). Distribution of temperature tolerance quantitative trait loci in Arctic Charr (*Salvelinus alpinus*) and inferred homologies in rainbow trout (*Oncorhynchus mykiss*). Genetics, 165: 1443 – 1456.

Sorensen, J. G. and Loeschcke, V. (2007). Studying stress responses in the post-genomic era: its ecological and evolutionary role. J. Biosci., 32(3): 447 – 456.