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Commentary

Are hsps suitable for indicating stressed states in fish?

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Summary

In response to most stressors, fish will elicit a generalized physiological stress response, which involves the activation of the hypothalamic–pituitary–interrenal axis (HPI). As in other vertebrates, this generalized stress response comprises physiological responses that are common to a wide range of environmental, physical and biological stressors. Recently, several families of heat shock proteins (hsps) have been proposed as indicators of a generalized stress response at the cellular level. Recent findings that hsp levels, in various fish tissues, respond to a wide range of stressors have supported the use of these proteins as indicators of stressed states in fish. However, the cellular stress response can vary, for example, according to tissue, hsp family and type of stressor. This brief overview of these responses in fish asks the question of whether changes in levels and families of hsps can be used as a suitable indicator of stressed states in fish. By casting this question in the context of the well-established generalized physiological stress response in fish, we argue that the use of hsps as indicators of stressed states in fish in general is premature.

Key words: cortisol, fish, heat shock protein, stress, stressor.

Introduction

Fish, like other vertebrates, respond to a stressor by eliciting a generalized physiological stress response, which is characterized by an increase in stress hormones and consequent changes that help maintain the animal's normal or homeostatic state (Iwama et al., 1999; Barton, 2002). This generalized response has been considered to be adaptive and represents the natural capacity of the fish to respond to stress. This response includes, for example, increases in plasma cortisol, catecholamines and glucose levels, increases in branchial blood flow and increases in muscular activity (Barton and Iwama, 1991). However, pathological states, even death, can result if the magnitude or duration of the stressor overwhelms the animal's ability to compensate for the negative influences of the stressor. Currently, we have considerable knowledge about the different aspects of the physiological stress response in fish, and, as stated by Barton (2002), the magnitude of this response can be influenced by the stressor, as well as genetic, developmental and environmental factors.

Fish also respond at the cellular level to stressors. This response comprises a suite of protein changes that includes the increased synthesis of heat shock proteins (hsps; Iwama et al., 1998). Our understanding of the cellular stress response in fish has also increased substantially (Basu et al., 2002). In addition to the variables mentioned above, the cellular stress response can vary according to tissue and hsp family. Several studies have attempted to establish a relationship between the physiological and cellular stress responses, but there exist apparent inconsistencies between these two levels of response. Thus, a fish that may present a physiological response to a stressor may not show any change in cellular hsp profile.

The definition and discussion of stress often defaults to taking on a negative perspective. The connotation of the word in common use is generally negative. As experimental biologists, we are all involved in the practice of imposing some perturbation and measuring a biological response. The word or concept of 'stress' is commonly invoked when inexplicable or unexpected, and negative, results are obtained. This underscores the importance of clarity in defining this important response. Thus, indicators of the generalized stress response in fish are worthy of discussion.

Our goal in this brief review is to bring to light several factors that need be taken into account to use hsps as an indicator of a general state of stress in fish. Indeed, none of the current indicators of stress, including the stress hormones, are 100% suitable in reflecting stressed states in fish. We will argue that the use of hsps as indicators of generalized stress response is still premature.

Stressors and the generalized stress response

Fish are exposed to stressors both in the wild and in artificial conditions such as in the laboratory or in aquaculture. The intensity and duration of exposure to the stressor, to a large extent, may determine whether the animal is capable of coping with the stressor. The categories of environmental, physical and biological stressors help to group the diverse possible stressors into a few themes (see Iwama et al., 1999). Environmental stressors mainly include adverse chemical conditions of the water. Although pollutants are common environmental stressors, extreme conditions or changes in water quality parameters such as dissolved oxygen, ammonia, hardness, pH, gas content and partial pressures, and temperature can stress fish. High water concentrations of metals such as copper, cadmium, zinc and iron can also cause stress and death in fish. Contaminants such as arsenic, chlorine, cyanide, various phenols and polychlorinated biphenyls are potent stressors to all fish. Other potential environmental stressors include insecticides, herbicides, fungicides and defoliants. Industrial, domestic and agricultural sources certainly add much of these contaminants to the environment, which affects fish at all life stages. Physical stressors include those that involve handling, crowding, confinement, transport or other forms of physical disturbance. Chasing fish to exhaustion or holding fish in a net in air for a period of time (30 s–1 min) have been used as standard protocols to stress fish in physiological studies. Angling also stresses fish in this way. Biological stressors can be manifested in dominance hierarchies, which can develop between individuals within confines such as experimental tanks or possibly in natural environments. Disease pathogens can also be considered as biological stressors.

The physiological stress response

The physiological responses of fish to stressors have been broadly categorized into the primary, secondary and tertiary responses. The initial primary response represents the recognition of a real (physical and chemical) or perceived (presence of predators) stressor by the central nervous system (Barton, 2002). Most stressors induce a neuroendocrine/endocrine response, which is characterized by a rapid release of stress hormones [catecholamines (CATS) and cortisol] into the circulation (Gamperl et al., 1994). CATS are released from the chromaffin tissue in the head kidney of teleosts and also from the endings of adrenergic nerves (see Randall and Perry, 1992). Cortisol is released from the interrenal tissue, located in the head kidney, in response to several pituitary hormones but most potently to the adrenocorticotrophic hormone (ACTH; see Balm et al., 1994). ACTH may also stimulate the release of the CAT epinephrine, and chronically increased levels of cortisol may affect CAT storage and release in trout (Reid et al., 1996). As both the chromaffin and the interrenal tissues in fish lie in close proximity, a paracrine effect for these stress hormones may exist (Reid et al., 1996). The physiological actions of both hormones are dependent on appropriate receptors on target tissues. The secondary response comprises the wide range of changes that are caused, to a large extent, by the stress hormones (Vijayan et al., 1994) in the blood, organs and tissues of the animal (Barton et al., 2002). The tertiary response represents whole-animal and population level changes associated with stress. If the fish is unable to acclimate or adapt to the stress, whole-animal changes may occur as a result of energy repartitioning by diverting energy substrates to cope with the increased energy demand associated with stress. Thus, chronic exposure to stressors, depending on the intensity and duration, can lead to decreases in growth, disease resistance, reproductive success, smolting, swimming performance and other characteristics of the whole animal or population. At a population level, decreased recruitment and productivity may alter community species abundance and diversity.

The susceptibility of fish to different stressors also has genetic components. There are differences in the stress responses among species (see Vijayan and Moon, 1994) and differences among stocks of the same species in their tolerance to applied stressors (see Iwama et al., 1999).

The cellular stress response

The cell, naturally, responds to stressors. A dominant aspect of the changes in protein profile as part of the cellular stress response is the changes in the concentration of different classes of hsps. Hsps are a family of highly conserved cellular proteins that have been observed in all organisms (Feder and Hofmann, 1999), including fish (Iwama et al., 1998). Extensive studies on model species have revealed several hsp families (Forreiter and Nover, 1998), which are named based on the molecular mass (kDa) of the protein. Of these, three major families of hsps – hsp90 (85–90 kDa), hsp70 (68–73 kDa) and low-molecular-mass hsps (16–24 kDa) – have been studied extensively. In the unstressed cell, there is a constitutive production of these proteins, which are required in various aspects of protein metabolism to maintain cellular homeostasis (see Fink and Goto, 1998). 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 the cytoskeleton, enzymes and steroid hormone receptors. The low-molecular-mass hsps have diverse functions and it has been proposed that they function as molecular chaperones, preventing irreversible protein aggregation (Derham and Harding, 1999).

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 extensively studied hsp, have demonstrated that the regulation of *hsp70* gene expression occurs mainly at the transcriptional level (Fink and Goto, 1998). 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 (Bienz and Pelham, 1987). It has been

demonstrated that hsp induction results primarily from the binding of an activated heat shock transcription factor (hsf) to an hse upstream of *hsp* genes (Morimoto et al., 1992). Recently, it has been shown in zebrafish (*Danio rerio*) that the transcriptional regulation of *hsp* genes, in response to heat shock, is also mediated by an hsf (Rabergh et al., 2000). Since most of the *hsp* genes do not contain introns, the mRNA is rapidly translated into nascent proteins within minutes of exposure to a stressor. Genomic sequences for *hsp70* are being elucidated in fish, including rainbow trout (*Oncorhynchus mykiss*; Kothary et al., 1984), medaka (*Oryzias latipes*; Arai et al., 1995), zebrafish (Lele et al., 1997), pufferfish (*Fugu rubripes*; Lim and Brenner, 1999) and tilapia *(Oreochromis mossambicus*; Molina et al., 2000).

Heat shock proteins as indicators of stress

A recent review paper explored the functions of hsps in various aspects of fish physiology, including development and aging, stress physiology and endocrinology, immunology, environmental physiology, acclimation and stress tolerance (Basu et al., 2002). These studies demonstrated that hsps might play a role 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. Taken together, these studies revealed the complexity in studying the regulation and function of hsps in fish.

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 we could base future experiments.

The induction of various hsp families in fish has been reported in cell lines, primary cultures of cells, as well as in various tissues from whole animals (Iwama et al., 1999). 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.

There have been several efforts to validate the use of the hsp response as an indicator of stressed states in fish. Indeed, it has been shown that several forms of environmental stressors can induce the hsp response in fish. For example, increased levels of various hsps have been measured in tissues of fish exposed to industrial effluents (Vijayan et al., 1998), polycyclic aromatic hydrocarbons (Vijayan et al., 1998), several metals such as copper, zinc and mercury (Sanders et al., 1995; Williams et al., 1996: Duffy et al., 1999), pesticides (Hassanein et al., 1999) and arsenite (Grosvik and Goksoyr, 1996). These studies and others revealed that the use of hsp as an indicator of stressed states in fish is a very complex issue. The hsp response can vary according to tissue (Rabergh et al., 2000; Smith et al., 1999), distinct hsp families (Smith et al., 1999) and stressor (Airaksinen et al., 2003), and the sensitivity of hsp expression can also vary with the species (Basu et al., 2001; Nakano and Iwama, 2002), developmental stage (Santacruz et al., 1997; Lele et al., 1997; Martin et al., 2001) and season (Fader et al., 1994).

When studying stress in aquatic organisms it is very important to establish whether experimental procedures such as handling, sampling and other physical stressors are affecting the hsp response. While handling and sampling procedures can affect common indicators of the physiological stress response in fish, such as plasma cortisol levels, it has already been demonstrated in rainbow trout that handling stress does not alter levels of hepatic hsp70 (Vijayan et al., 1997), and levels of muscle, gill, heart and hepatic hsp60 (Washburn et al., 2002). Recently, Zarate and Bradley (2003) showed that common forms of hatchery-related stressors (exposure to anesthesia, formalin, hypoxia, hyperoxia, capture stress, crowding, feed deprivation and cold stress) did not alter levels of gill hsp30, hsp70 and hsp90 in Atlantic salmon (*Salmo salar*). While these studies demonstrated that common stressors did not elicit an hsp response and therefore do not interfere with its use as an indicator, they also showed that hsp may not be a sensitive indicator of stressed states when physical stressors are applied in aquaculture operations.

There are a few studies that relate the physiological and cellular stress responses *in vivo*. In mammals, it is known that hsps are involved in the immune response (Young, 1990; Breloer et al., 2001). Two studies have demonstrated increased levels of hsp70 in various tissues in fish exposed to pathogens (Forsyth et al., 1997; Ackerman and Iwama, 2001). The later study revealed that rainbow trout infected with a bacterial pathogen (*Vibrio anguilarum*) increased levels of hsp70 in hepatic and head kidney tissues prior to clinical signs of the disease. This study also showed that the peak in hepatic hsp70 levels corresponded to that of plasma cortisol levels, which occurred 5 days after the challenge. However, head kidney hsp70 levels increased significantly on the fourth day after challenge, when plasma cortisol levels were similar to the control group.

Such interests in stress research at the organismal level have taken us to the intertidal zone, where fish cope with changes to their environment, the tidepool, that are unpredictable from one low tide to the next. We have been working extensively on the functional importance of hsp70 in thermal tolerance using two intertidal fishes, the tidepool sculpin (*Oligocottus maculosus*) and fluffy sculpin (*Oligocottus snyderi)*, as model species. While both sculpins inhabit the intertidal zone along the west coast of North America, the tidepool sculpin has a higher thermal tolerance than the fluffy sculpin. As in research by Hightower et al. (1999) on the desert pupfish (*Cyprinodon macularius*), we have observed that the levels of constitutive hsp70 and the scope for increase in hsp correlate with the ability of the tidepool sculpins to cope with these environmental changes (Nakano and Iwama, 2002). The tidepool sculpin, which is exposed to a wider range of

fluctuation in water temperature in upper tidepools, had higher constitutive liver hsp70 levels that were only slightly influenced by changes in water temperature. However, the fluffy sculpin, which prefers lower tidepools with smaller changes in water conditions, showed a larger change in liver hsp70 levels in response to thermal stress at lower temperatures. We suggested that the less thermally sensitive sculpin might enhance its thermal tolerance by having a large constitutive pool of hsp70. Therefore, in order to use the hsp level as a realistic stress indicator in various fish species, it is essential to understand such relationships between the organismal stress tolerance of fish and the cellular stress response so that a better understanding of the functional significance of hsps in natural populations of fish can be obtained.

In addition to the differences that we have 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 by Norris et al. (1995). Furthermore, seasonal variations in the hsp response within a species and between species of fish have been shown by Dietz and Somero (1992) and Fader et al. (1994). It is also important to consider that some fish species may not show a heat shock response. Hofmann et al. (2000) showed that hsp70 is not induced by temperature stress in the Antarctic fish *Trematomus bernacchi*. 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 stress in fish.

A good indicator of cellular mechanisms should also enable the cellular response to be linked to effects at higher levels of biological organization (Lewis et al., 1999). A few recent studies have explored the possible direct relationship between the physiological and cellular stress responses in fish. While we are far from understanding this relationship, these studies have demonstrated that steroid hormones, including cortisol, may have a direct influence on the cellular stress response. Stress levels of plasma cortisol attenuated the heat stress-induced increases of gill hsp30 in cutthroat trout (*Oncorhynchus clarki clarki*; Ackerman et al., 2000), liver and gill hsp70 in rainbow trout, gill hsp70 in tilapia (Basu et al., 2001), hsp90 mRNA in primary cultures of rainbow trout hepatocytes (Sathiyaa et al., 2001) and hsp70 in primary cultures of rainbow trout hepatocytes (Boone and Vijayan, 2002). Recently Basu et al. (2003) showed a functional and structural link between hsp70 and the glucocorticoid receptor in rainbow trout. They also showed that the glucocorticoid receptor heterocomplex contains hsp70 and that the association between hsp70 and the glucocorticoid receptor can be altered according to the stressor.

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. The complexity of the available data makes it difficult to conclude that hsps are general indicators of the stress response in fish. We suggest that 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 (Schreck, 2000). 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.

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