

Heat Shock Proteins and Disease Control in Aquatic Organisms

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Abstract

Four families of heat shock proteins (Hsps), including the small heat shock proteins (sHsps), Hsp70, Hsp90 and Hsp60, are synthesized under normal physiological conditions and in response to stress. sHsps protect proteins from irreversible denaturation independently of ATP. The remaining ATP-responsive Hsps fold nascent proteins, shield proteins from irreversible denaturation during stress and aid protein refolding. Several observations indicate that Hsps contribute to disease resistance in aquatic organisms, the first being that these proteins are produced in finfish, shellfish and bivalves upon infection with viral and bacterial pathogens. Induction of Hsp synthesis by heat shock and incubation with chemicals such as Pro-Tex[®] boosts resistance to pathogens, as does administration of exogenous Hsps to host organisms. The extent of Hsp accumulation and the increase in disease tolerance are generally correlated with one another. Not only do Hsps protect against pathogens by functioning as molecular chaperones, but they are thought to mediate humoral and cellular innate immune responses. Hsp70 is highly immunogenic and serves as a ligand for Toll-like receptors. Hsps elicit cytokine production and they deliver peptides to antigen presenting cells via major histocompatibility complexes (MHC). Vaccines have been produced for use in aquaculture by employing Hsps, either alone or fused to antigens obtained from pathogens. Hsps offer several advantages over current methods for the treatment of disease in commercially important organisms and they are being increasingly exploited as their roles in protein chaperoning and immune modulation are better understood.

Keywords: Heat shock proteins; Molecular chaperone; Innate immunity; Disease; Vaccine; Aquatic organisms; Aquaculture

Introduction

Families of heat shock proteins (Hsps), otherwise known as stress proteins or molecular chaperones, consist of conserved molecules found in all organisms [1,2]. The expression of genes encoding Hsps is either constitutive or induced by stress and their products are essential for cell survival [3,4]. Under normal conditions Hsps mediate nascent protein folding and assembly, translocate proteins through membranes into organelles such as mitochondria, and assist in the degradation of structurally aberrant proteins. Hsps, often when functioning cooperatively with one another, prevent the irreversible denaturation of proteins exposed to physiological stressors such as heat, toxins and disease, thereby facilitating protein refolding and protecting cells from damage.

The Hsp70 family is the best studied group of Hsps in aquatic organisms, with interest centred on thermotolerance [5-7]. Hsp70 accretion is important in cross-protection where an organism acquires enhanced tolerance to a specific stress following an initial transient, but different, stress [2]. Hsp70 serves as a potential bio-indicator of environmental perturbation because it is induced in aquatic organisms during exposure to temperature change, salinity variation, handling, toxins and other stresses [8,9]. Results from such monitoring must however, be interpreted carefully because Hsp gene expression and protein accumulation in test organisms respond to many environmental variables [10]. The Hsp reaction to stress was originally considered to be of short duration, but Hsps are now thought to have a longer term role by modulating the immune system [9]. As part of immune management, Hsp70 is released into extracellular compartments and influences the major histocompatibility complex (MHC)-dependent uptake of peptides by antigen-presenting cells, while at the same time functioning as an endogenous "danger signal", alerting the immune system to cell and tissue injury [11,12]. Hsp70 binds pathogen-associated molecular pattern (PAMP) molecules and modulates PAMP-induced Toll-like receptor (TLR) signalling [1,13],

activities crucial for stimulation of innate immunity and elimination of pathogens.

Modulation of innate and adaptive immune systems by Hsp70 is under study in organisms used for aquaculture [2]. Endogenous Hsp70 increases significantly subsequent to bacterial and viral infection of fin-fish and shrimp, protecting proteins by way of their chaperone activity during the stress of infection and suggesting linkage with the immune response [9,14,15]. Control of disease caused by vibriosis in the crustacean *Artemia franciscana* is achieved by employing non-lethal heat shock to boost endogenous Hsp70 [16,17] and by feeding the organism with bacteria enriched in DnaK, the prokaryotic equivalent of Hsp70 [18,19]. Platyfish are protected against *Yersinia ruckeri* by injecting them with bacterial Hsps, an effect enhanced by non-lethal heat shock [20]. Application of Hsp stimulants such as Tex-OE[®], a patented extract of the prickly pear cactus *Opuntia ficus indica* that non-traumatically enhances stress protein synthesis in fish and shrimp, is useful against several bacterial and viral diseases [9]. Because microbial Hsp60 (GroEL) and Hsp70 (DnaK) are frequently major pathogen-derived antigens that invoke high antibody response, they have the potential to function as highly specific potent vaccines against harmful biotic agents [21,22].

The structures and mechanisms of action of four major Hsp

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families produced by aquatic organisms are presented in this review. The synthesis of Hsps upon exposure to pathogens and the use of Hsps for disease control during aquaculture are considered. Exploiting Hsps for prevention and treatment of infections in commercially cultured aquatic organisms is important because it offers an alternative to the utilization of antibiotics and therapeutic drugs, methods with serious consequences for the environment and consumers, especially when used indiscriminately.

The Structure and Function of Hsps

Hsp families, named according to molecular mass (kDa), amino acid sequence and function, include the small heat shock proteins (sHsps), Hsp60, Hsp70 and Hsp90. The synthesis of Hsps, both those that are expressed constitutively [heat shock cognates (HsCs)] and more commonly those that are not, are induced upon protein denaturation caused by thermal shock, heavy metals, free radicals, desiccation, microbial infection and other stressors. Hsps are molecular chaperones, aiding nascent polypeptide folding and oligomerization, protecting proteins from irreversible denaturation, re-folding or degrading damaged proteins, translocating proteins into membrane-bound cell compartments and contributing to disease resistance. The Hsp family of primary interest for disease control during aquaculture is Hsp70 but the sHsps, Hsp90 and Hsp60, as well as the co-chaperone Hsp40, appear to ameliorate infection by pathogens. The sHsps provide oligomeric platforms for the ATP-independent binding of structurally perturbed proteins, preventing their irreversible denaturation when cells are stressed. Hsp90, Hsp70 and Hsp60 are stress induced and they have the ability to protect proteins from irreversible denaturation. However, the major function of these chaperone families is to bind and fold nascent proteins through ATP-driven allosteric rearrangement, although the molecular structure and mechanism of action of each chaperone differ. The Hsps function cooperatively by forming intracellular networks of chaperones, co-chaperones and accessory proteins.

sHsp monomers, consisting of a conserved α -crystallin domain flanked by an amino-terminal sequence and a carboxyl-terminal extension [4,23,24], assemble into oligomers [25-27]. The α -crystallin domain contributes to dimerization of monomers and substrate binding, activities that depend on the amino- and carboxyl-terminal regions for greatest efficiency [23,24,27-29]. sHsp oligomers either disassemble or undergo structural rearrangement during stress, increasing surface hydrophobicity and enhancing reaction with substrate proteins [24,30-32]. Proteins released from sHsps when stress passes either refold spontaneously or with the assistance of an ATP-dependent Hsp such as Hsp70 [33,34]. The primary role of sHsps during exposure to stress, including infection, is to protect proteins from irreversible denaturation (Table 1).

Hsp70 mediates the ATP-dependent folding of nascent proteins and the refolding of partially denatured proteins. Hsp70 has a conserved amino-terminal ATP binding/hydrolysis domain (NBD) connected by a hydrophobic flexible linker to a variable, carboxyl-terminal substrate binding domain (SBD) capped by a lid structure of unknown function [35,36]. Interaction with substrate and ATP, followed by nucleotide hydrolysis, promotes allosteric changes in the NBD and SBD, strengthening attachment of substrate to the chaperone and promoting folding [36-38]. ATP then replaces ADP, an action supported by co-chaperone nucleotide exchange factors such as Bag1 [39,40], and the substrate is released from Hsp70. Members of the J-domain-containing Hsp40 co-chaperone family shuttle substrate proteins to Hsp70 and improve ATP hydrolysis [40-42]. Intracellular accumulation of Hsp70

protects against protein damage upon viral and bacterial challenge. Moreover, the mechanistic properties of Hsp70 permit binding, transport and release of peptides/proteins that stimulate the immune response and Hsp70 is thought to have this function [11].

Hsp90, an ATP-dependent dimer produced abundantly by cells under normal physiological conditions or induced by stress, is composed of monomers with three domains. The amino-terminal domain houses a nucleotide binding and hydrolysis site, the middle region interacts with substrates and co-chaperones, and the carboxyl-terminus, possessing a MEEVD motif for binding co-chaperones with tetratricopeptide repeats, governs constitutive dimerization [43]. The Hsp90 dimer assumes an open, substrate recognition configuration when monomers are connected only by the carboxyl-termini. The hydrolysis of ATP compacts Hsp90 structure and amino-terminals dimerize, followed by substrate folding and release. Hsp90 combines with many different substrates including those undergoing denaturation [44,45]. Additionally, Hsp90 regulates cell activity through interaction with kinases and steroid hormone receptors [46] and by degrading proteins [47]. Hsp90 forms complexes with proteins late in folding some of which are associated with Hsp70/Hsp40 and are presented to Hsp90 by the co-chaperone Hsp70/Hsp90 organizing protein (Hop) [47-49]. Hop inhibits Hsp90 ATPase and keeps this Hsp in the open state [47,50]. Other co-chaperones such as p23 and Aha1 fine-tune Hsp90 interaction with chaperones and substrates [49].

Hsp60 (chaperonin, TRiC, CCT), the most structurally complex Hsp, is composed of two rings positioned back-to-back, each constructed with eight to nine different but related ATP-hydrolyzing monomers composed of three domains [51,52]. The apical domain reacts with substrate and it is topped by a built-in lid that confines client proteins within the ring cavities where folding occurs [53,54]. The intermediate region connects the Hsp60 apical and equatorial domains, the latter possessing inter- and intra-ring contacts and the ATP hydrolysis site [55,56]. Substrates often bind Hsp60 late in folding and they are released upon ATP hydrolysis and dissociation of ADP + Pi. Hsp60 associates with numerous different substrates via hydrophobic, polar and charged amino acid residues and the cytoskeleton proteins actin and tubulin depend on Hsp60 for correct folding [52,56-58]. Hsp60 has the ability to refold proteins denatured during infection and it may interact with peptides and proteins involved in the invertebrate immune response.

Aquatic Organisms Respond to Disease by Altering Hsp Synthesis

A relationship between Hsps and fish disease is implied by the strong induction of Hsp90 during an *in vitro* challenge of the Chinook salmon embryo cell line CHSE-214 with infectious hematopoietic necrosis virus (IHNV) [8,59,60]. In other examples of virus induced Hsp synthesis in fish Hsp90 increases when the orange-spotted grouper *Epinephelus coioides* encounters nodavirus [61] and exposure to UV-inactivated turbot rhabdovirus (SMRV) up-regulates the expression of three Hsp40 genes in embryonic cell lines from the olive flounder *Paralichthys olivaceus* with marked stimulation of PoHsp40A4 and weaker production of PoHsp40B6 and PoHsp40B11 [62]. Infection of Coho salmon with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease, boosts Hsp70 in liver and kidney [63]. Hsp70 is augmented in rainbow trout after acute *Vibrio anguillarum* challenge [64], as is the case for sea bream, where hepatic Hsp70 peaks 36 h post-infection with live *V. alginolyticus*, although Hsp60 and Hsp90 are unchanged [65]. The mechanisms regulating piscine Hsp synthesis

| Hsp Family ^a | Hsp Structure and Function | References |
|-------------------------------|--|--------------------|
| sHsps | Multimeric complexes composed of monomers containing an amino-terminal region, an α -crystallin domain and a carboxyl-extension; the domains <u>cooperate in oligomerization and substrate binding</u> . Prevent stress-induced irreversible protein denaturation; inhibit apoptosis; ATP independent. | [4,23,24,26,27,32] |
| Hsp70 | Monomeric protein with a conserved ATP binding /hydrolysis site in the amino-terminus, a linker region <u>and a variable carboxyl substrate-binding domain</u> . Binds and folds nascent and denaturing proteins; protects against stresses such as bacterial infection. | [35,36,38,40] |
| Hsp90 | Dimeric protein; each monomer has an amino-terminal domain that hydrolyzes ATP, a substrate-binding middle domain and a carboxyl-domain that <u>interacts with co-chaperones</u> . Folds nascent and denaturing proteins; interacts with kinases and other regulatory molecules; protein degradation. | [43-46,49] |
| Hsp60 (chaperonin, TRiC, CCT) | Two back-to-back rings of eight to nine subunits each with an apical capped domain that binds substrate, an equatorial region with an ATP hydrolysis site and a linking domain. Binds substrates via hydrophobic, polar and charged residues; required by actin and tubulin for folding. | [51,52,54-56,58] |

^aThe Hsps are described separately but they function as co-operative networks to maintain protein homeostasis within cells, either during normal growth or upon exposure to stress such as heat, salinity, toxins and infection.

Table 1: The structure and function of Hsps involved in diseases of aquatic organisms

during disease are unknown but are likely to be similar to other types of stress where transcription is regulated by heat shock factor (HSF) recognition of up-stream heat shock elements (HSEs), a process promoted by protein denaturation. That is, when cells are damaged as the result of microbial infection, protein perturbation occurs, either within cells or on their surfaces [9]. The resulting Hsps protect against irreversible damage of proteins and/or assist in their repair.

Exposure to viral pathogens up-regulates Hsps in shellfish (Table 2). Hepatopancreas Hsp70 transcripts increase in the Chinese shrimp *Fenneropenaeus chinensis* after exposure to white spot syndrome virus (WSSV) [66], suggesting Hsp70 is a useful indicator of virus infection. WSSV triggers expression of Hsp70 in haemolymph of the mud crab *Scylla serrata* but reduces Hsp90, signifying functional differences between Hsp families in response to viral infection [67]. These studies suggest that Hsps influence the crustacean innate immune system. Quantitative PCR reveals a time-dependent increase in Hsp70 mRNA after challenge of the Japanese blue crab *Portunus trituberculatus* with *V. alginolyticus*, the main causative agent of emulsification disease in the swimming crab [68]. Hsp70 and Hsp90 transcripts are up-regulated in gills of the black tiger shrimp *Penaeus monodon* at 3, 12 and 24 h post-infection with *V. harveyi* [69]. However, Hsp90 is unchanged in hemocytes of *P. monodon* 24 h after infection and slightly reduced at 48 h [70], showing that Hsp90 expression is tissue specific. The *P. monodon* sHsp, Hsp21, is not induced concurrently with Hsp70 and Hsp90 but appears 24 h after bacterial contact. LvHsp60 mRNA is highly expressed in haemocytes, muscle, stomach, heart, hepatopancreas and gill tissue of the white leg shrimp *Litopenaeus vannamei* but less so in the intestine. Quantitative PCR and immunoprobings of western blots reveal respectively that LvHsp60 mRNA and protein are significantly up-regulated in the gills, hepatopancreas and haemocytes of *L. vannamei* after infection with *Staphylococcus aureus* and *V. alginolyticus* [71]. LvHsp70 transcripts are induced in haemocytes and the hepatopancreas of *L. vannamei* after bacterial challenge.

Several studies with bivalves focus on the transient synthesis of haemocyte sHsps in response to *Vibrio* challenge and suggest that they protect proteins undergoing denaturation upon pathogen exposure, although other functions are possible. The sHsp genes VpsHSP-1 and VpsHSP-2 are sequentially up-regulated in haemocytes of the Manila clam *Venerupis philippinarum* following *V. anguillarum* challenge [72].

The quantity of VpsHP-1 transcripts grows by 1.5-fold and 9.9-fold at 6 and 96 h post-infection respectively, whereas VpsHSP-2 mRNA multiplies 8.7-fold 24 h post-infection but declines to 2.2-fold after 96 h. mRNA transcripts encoding Tg-sHSP in haemocytes of the bloody clam *Tegillarca granosa* increase gradually from 1.5 h to 12 h after contact with *V. parahaemolyticus* and then decrease over the next few hours [73]. In yet another example, transcription of the gene encoding Hsp22 up-regulates in haemocytes of the Chikong scallop *Chlamys farreri* exposed to *V. anguillarum*, with maximum expression in 12 h followed by decline to the original level by 48 h [74]. The expression of co-chaperone Hsp40 in *V. philippinarum* haemocytes increases 6-fold relative to controls 24 h after incubation with *V. anguillarum* [75]. Hsp90 and Hsp83 rise in haemocytes of the soft-shell clam *Mya arenaria* 1 h after introduction to *V. splendidus* strain LGP32, followed by down-regulation thereafter [76].

MmeHsc71, a constitutive Hsp70 in the Asiatic hard clam *Meretrix meretrix* goes up 2-fold in the gill and hepatopancreas 24 h after infection by *V. parahaemolyticus* [77]. Hsp70 is expressed in the bay scallop *Argopecten irradians* after *V. anguillarum* challenge, with mRNA reaching maximum levels 8 h post-infection and lasting 16 h [78]. Not all bivalve Hsps are amplified during exposure to pathogenic microbes and as a case in point, mitochondrial Hsp60 is down-regulated in gills when the Mediterranean mussel *Mytilus galloprovincialis* comes in contact with *Cylindrospermopsis raciborskii*, a freshwater filamentous cyanobacterium, and with cylindrospermopsin (CYN) toxin [79]. Differential expression indicates Hsp functional diversity. Hsps may protect cell proteins from irreversible denaturation during disease-induced stress and/or assist in refolding once stress terminates. On a more speculative note, some Hsps may mediate the bivalve immune response.

Increasing Hsps in Aquatic Organisms as an Approach to Disease Management

Heat shock: The intensity of thermal stress required to boost Hsp expression in aquatic organisms varies across species and acclimation temperature. A frequently used protocol to stimulate Hsp expression entails a short non-lethal heat shock followed by incubation for several hours under non-stress conditions [2]. As noted previously, non-lethal heat stress at 37°C for 30 min followed by 6 h of recovery increases

| Host animal | Pathogen challenge | Tissue | Induced Hsp | References |
|---|--|---|--------------------------------------|------------|
| Orange-spotted grouper <i>Epi-nephelus coioides</i> | Naturally infected with nodavirus (concentration not available) | Cell line GF-1 from fin | Hsp90AB | [61] |
| Olive flounder <i>Paralichthys olivaceus</i> | 0.5ml of 1 x 10 ⁹ UV-inactivated SMRV | Embryonic cell lines | PoHsp40A4 PoHsp40B6 PoHsp40B11 | [62] |
| Coho salmon <i>Oncorhynchus kisutch</i> | 0.1ml of 3 x 10 ⁴ cells/ml <i>Renibacterium salmoninarum</i> | Liver and kidney | Hsp70 | [63] |
| Rainbow trout <i>Oncorhynchus mykiss</i> | 1 x 10 ⁵ cfu/fish <i>V. anguillarum</i> | Liver and head kidney | Hsp70 | [64] |
| Sea bream <i>Sparus sarba</i> | 4.9 x 10 ⁴ cfu/fish <i>V. alginolyticus</i> | Hepatic | Hsp70 | [65] |
| Chinese shrimp <i>Fenneropenaeus chinensis</i> | 3 µl of WSSV/shrimp, 6 h | Hepatopancrease | Hsp70 Hsp90 | [66] |
| Black tiger shrimp <i>Penaeus monodon</i> | 100 µl/shrimp of 2 x 10 ⁸ cells/ml heat-killed <i>V. harveyi</i> | Gill | Hsp90 Hsp70 Hsp21 | [69] |
| White leg shrimp <i>Litopenaeus vannamei</i> | 10 µl/shrimp of 1 x 10 ⁷ cells/mL <i>V. alginolyticus</i> 10 µl of 1 x 10 ⁷ cells/mL <i>S. aureus</i> | Gills Hepatopancreas Haemocytes | LvHsp60 LvHsp70 | [71] |
| Mud crab <i>Scylla serrata</i> | 0.2 mL of 1:100 diluted WSSV inoculums/crab | Haemolymph | Hsp70 | [67] |
| Japanese blue crab <i>Portunus trituberculatus</i> | 100 µl/crab of 1 x 10 ⁶ CFU/ml <i>V. alginolyticus</i> , | Gill Haemocytes Hepatopancreas EyestalkW | Hsp70 | [68] |
| Manila clam <i>Venerupis philippinarum</i> | 1 x 10 ⁷ CFU/mL <i>V. anguillarum</i> | Haemocytes | Hsp40 | [72] |
| Bloody clam <i>Tegillarca granosa</i> | 20 µl of 2 x 10 ⁶ cfu/ml <i>V. parahaemolyticus</i> , | Haemocytes | sHsp | [73] |
| Manila clam <i>Venerupis philippinarum</i> | 1 x 10 ⁷ CFU/mL <i>V. anguillarum</i> | Haemocytes | Hsp40 | [75] |
| Asiatic hard clam <i>Meretrix meretrix</i> | 100 µl/clam of 5 x 10 ⁶ cfu/ml <i>V. parahaemolyticus</i> (MM21) | Hepatopancreas Gill | MmeHsc71 | [77] |
| Soft-shell clam <i>Mya arenaria</i> | 1 x 10 ⁶ cells/ml <i>V. splendidus</i> (LGP32) | Haemocytes | Hsp90 Hsp83 | [76] |
| Chikong scallop <i>Chlamys farreri</i> | 50 µl/scallop <i>V. anguillarum</i> , 2.0 x 10 ⁸ cells/ml | Haemocytes | Hsp22 | [74] |
| Bay scallop <i>Argopecten irradians</i> | 50 µl/scallop <i>V. anguillarum</i> , 2.0 x 10 ⁸ cells/ml | Haemocytes | Hsp70 | [78] |

Table 2: Induction of Hsp gene expression in aquatic organisms upon pathogen infection

Hsp70 production and shields gnotobiotic *Artemia* larvae against infection by *V. campbellii* and *V. proteolyticus* [16]. This resistance to bacteria may depend on the protection of proteins by Hsps in stressed cells and the greater tolerance to disease also implies a role for Hsps in shrimp immunity, perhaps through activation of TLRs. FcToll of the Chinese shrimp *Fenneropenaeus chinensis* predominantly expressed in the lymphoid organ, is a member of the insect-type Toll family [13]. FcToll increases following *V. anguillarum* contact and is reduced after white spot syndrome virus (WSSV) infection, suggesting involvement of the host innate immune defence system in protection against pathogenic Vibrios. Interaction between viruses and the TLR-mediated NF-κB pathway has been revealed and, as for insects [80], a TLR/MyD88/Tube/Pelle/TRAF6/NF-κB cascade may exist in the white shrimp for activation of immunity-related genes. Whatever the mechanism, these studies show that heat promotes Hsp70 expression and confers pathogen tolerance on *Artemia* larvae. Understanding the relationship between Hsps, the immune system and pathogen resistance is of importance in devising strategies to enhance disease tolerance in commercially exploited organisms.

Chemical inducers of Hsp synthesis: Several recently identified plant-based compounds shown to enhance Hsp synthesis in humans and animals have potential uses in aquaculture. TEX-OE[®], from the

tropical cactus *Opuntia ficus indica*, is an effective non-stressful inducer of endogenous Hsps [9]. Application of Pro-Tex[®], the soluble variant of TEX-OE[®], either by immersion or as a feed additive accelerates fish and shellfish Hsp expression and boosts stress tolerance. For instance, immersion in Pro-Tex[®] protects fingerlings of the common carp *Cyprinus carpio* L. against acute ammonia stress with survival improving from 50 to 95% after incubation in 5.9 mg/L NH₃, the 1 h LC50 [80]. Remarkably, survival of carp exposed to Pro-Tex[®] increases by 20% over controls during lethal challenge with 14.2 mg/L NH₃. Amplification of Hsp70 in gills and muscle is evident, demonstrating that Pro-Tex[®] accelerates Hsp70 synthesis in carp in addition to preventing death. Immersion of the angel fish *Pterophyllum scalare* (Schultze) in TEX-OE[®] raises Hsp70 and Hsp90 synthesis in liver, muscle and gills, a change correlated with a two-fold improvement in protection against 1.1 mg/L NH₃, the 24 h LC50. Circulating Hsps are detected in TEX-OE[®] stimulated fish during NH₃ stress at levels higher than those normally induced by heat [9]. Histological studies of gill tissue from moribund fish incubated with NH₃ reveal lamellar oedema, necrosis of lamellar epithelial cells and clubbing of lamellar tips, whereas the gills in fish exposed to TEX-OE[®] are structurally normal. These results suggest that Hsp accumulation afforded by TEX-OE[®] safeguards against cell damage and/or promotes wound healing, conclusions similar to those for terrestrial animals [9,82].

The primary use of TEX-OE[®] in aquaculture is currently to reduce the effects of transport stress, particularly for salmon and sea bass [9]. However, TEX-OE[®] augments the disease tolerance of fish. As a case in point, Pro-Tex[®] confers resistance against *V. anguillarum* infection of salmon and the gilthead sea bream *Sparus aurata* L [9], reducing mortality by half when compared to control fish. The mechanisms conferring tolerance to infection have yet to be determined but Hsps, as molecular chaperones, fold nascent and partially denatured proteins and they promote strong innate and adaptive immune responses. It is, therefore, of fundamental and applied significance to elucidate the immunoregulatory effects of bio-active compounds such as TEX-OE[®].

Paeoniflorin, from the herbal plant *Paeonia lactiflora* Pall, enhances Hsp70 expression in cultured mammalian cells and it is an alternative to TEX-OE[®]. Paeoniflorin induces thermotolerance in HeLa cells, an effect intensified by heat shock at 42°C for 2 h [83]. The bioactive compound Celastrol, a quinone methide triterpene from the Chinese herbal medicine *Celastraceae*, increases the synthesis of Hsp70, Hsp40, and Hsp27 in HeLa cells. Celastrol activates HSF1 synergistically with stressors and confers protection against lethal heat shock at 42°C in HeLa cells and the neuroblastoma cell line SH-SY5Y [84]. Treatment with Schisandrin B (Sch B), the most active dibenzocyclooctadiene from the herb *Schisandra chinensis*, raises mouse hepatic Hsp70 in a dose-dependent manner and guards against hepatic cell apoptosis upon injection of D-galactosamine sensitized mice with TNF- α [85]. In a related study, Sch B provoked the synthesis of Hsp25 and Hsp70 in rat heart and shielded against ischemia reperfusion (I-R) injury. Hsp expression and protection against ischemia peak at 48 and 72 h post-injection, respectively [86]. Curcumin (diferuloylmethane), a major component of the turmeric plant *Curcuma longa*, induces Hsp70 expression in K562 cells. Hsp build-up, in addition to influencing anti-inflammatory and anti-proliferative responses, is thought to be effective against chronic myelogenous leukemia [87]. Carvacrol, from the oil of *Origanum* species, brings about Hsp70 expression and promotes T cell recognition of endogenous Hsp70, as evidenced *in vitro* by the activation of an Hsp70-specific T cell hybridoma and *in vivo* by amplified T cell responses to this Hsp [88]. Carvacrol increases the number of spleen and joint CD4+CD25+FoxP3+T cells and suppresses experimental arthritis caused by proteoglycan. All of the compounds just described profoundly affect Hsp expression and/or disease tolerance in humans and other animals and it should be possible to exploit them in novel strategies to control disease of aquatic organisms. Such treatments, if successful, offer a decided advantage over many of the techniques presently used to curb disease in aquaculture.

Administration of exogenous Hsps: Feeding aquatic organisms with Hsp-enriched bacteria is a new approach to control disease in aquaculture. For example, supplying gnotobiotic larvae of *Artemia* with *E. coli* over-producing DnaK, the prokaryotic equivalent of Hsp70, enhances survival approximately two- to three-fold upon challenge with pathogenic *V. campbellii* [18]. 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 controls [19]. Immunoprobings of western blots and quantification by ELISA demonstrate that 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 [19]. Support for an immunological effect is offered by the observation that feeding DnaK-enriched bacteria stimulates the prophenoloxidase (ProPO) cascade system of *Artemia*, a mechanism

important for pathogen melanisation by the innate immune system [89]. Feeding *Artemia* with mmn9 yeast enriched in Hsp70 by heating, boosts *Artemia* tolerance against *V. campbellii* two-fold with effects apparent 36 h after challenge [15]. Heated gas1, a yeast strain with high chitin but low beta-glucan, contributes similar security against *V. campbellii* challenge. Taken together, these studies indicate that increases in disease tolerance occur in concert with the accretion of Hsps produced by either eukaryotic or prokaryotic cells and this may have practical applications in aquaculture.

Ingestion of *E. coli* over-producing *Artemia* Hsp70 shelters brine shrimp against *V. campbellii*, possibly by triggering the innate immune response to produce anti-inflammatory substances and suppress infection [14]. DnaK and *Artemia* Hsp70 exhibit 59.6% similarity in the peptide-binding domain and the protective capacity of these proteins, termed the innate immunity-activation portion, may reside within this molecular domain, a conclusion similar to that made for Hsp70 from dendritic cells [90,91].

Intracoeelomic injection of the recombinant bacterial Hsps, DnaK and GroEL, the latter representing the prokaryotic equivalent of Hsp60, protects the platyfish *Xiphophorus maculatus* against *Yersinia ruckeri* and the effect is amplified by non-lethal heat shock [20]. These data indicate a role for Hsps in increasing resistance against bacterial infections in fish, as seen for *Artemia*.

Hsps and Immunity

An intriguing observation indicating the influence of Hsps on fish and shellfish immunity is that the enhancement of Hsp70 synthesis in *P. monodon* by a short hyperthermic stress coincides with increased resistance against gill associated virus (GAV) [92]. GAV load is reduced for 21-days by heat shock but there is no response to osmotic and hypoxic stress, suggesting the lowered amount of virus is not attributable to holding animals under conditions preventing GAV replication, but from Hsp70 accumulation. A similar phenomenon occurs in *Artemia* where the build-up of Hsp70 in response to non-lethal heat shock protects against pathogenic *V. campbellii* and *V. proteolyticus* [16]. Likewise, a combined hypo- and hyper-thermic stress, followed by recovery at ambient temperature, brings about Hsp70 synthesis and shields *Artemia* larvae against *V. campbellii* [17]. The survival of *Artemia* larvae upon *Vibrio* challenge expands two-fold in each study and the rise in Hsp70 production coincides with increased resistance to bacteria, suggesting this and/or other Hsps protect larvae from infection. Hypothermic and osmotic stresses do not cause Hsp70 accumulation nor do they improve *Artemia* tolerance to Vibrios, indirectly implicating Hsp70 in resistance to pathogenic bacteria [93].

There are several mechanisms by which Hsps, and particularly Hsp70, guard against bacterial infection. Hsps may be required to stabilize cells against injury due to pathogen proliferation, for the proper folding of cell proteins synthesized in response to bacterial pathogens [94], for the storage and re-folding of partially denatured proteins and for stimulation of the innate immune response. As an example of immune stimulation, heat-induced sHsps and Hsp90 trigger nematode *Caenorhabditis elegans* immunity to pathogenic *Pseudomonas aeruginosa* by a process that engages HSF-1 and the DAF-2/DAF-16 pathway [95]. Increased Hsp70 reduces bacterial load in *Artemia* during challenge, an indication of immune system action [17]. Hsp70 is a ligand for Toll like receptors (TLRs) and shrimp may attenuate infection by way of these receptors [2]. Upon binding, Hsps activate TLRs, transferring inflammatory signals to cells of the innate immune system and promoting resistance against disease [96-98]. TLRs

have yet to be characterized in *Artemia* but in another crustacean, the white shrimp, they contain an extracellular domain with 16 leucine-rich repeats (LRRs) flanked by cysteine-rich motifs and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain, the latter with 59.9% and 54.3% identity, respectively, to corresponding proteins in the Western honey bee *Apis mellifera* and fruit fly *Drosophila melanogaster* [99]. Characterization of a novel cDNA from the gills of *P. monodon* yielded a protein with a deduced amino acid sequence 59% similar to an *A. mellifera* Toll-related protein [100]. Collectively, these findings suggest that Hsps stimulate the immune system in *Artemia* and other shrimp, thereby promoting pathogen recognition and destruction.

Like other invertebrates, shrimp and bivalves lack the adaptive immune system and their ability to eliminate pathogens depends on the cooperation of innate cellular and humoral mechanisms. The haemocytes of shellfish play a key role in phagocytosis, the major cellular immune response against pathogens [101-103]. Phagocytosis functions with other components of the immune system such as prophenoloxidase (ProPO) [14] and antimicrobial peptides [104]. Large amounts of Hsp70 are released from the haemocytes of *L. vannamei* following infection by *V. alginolyticus* [71], results similar to those for the Pearl Oyster *Pinctada fucata*, a marine bivalve [105]. The up-regulation of Hsps in haemocytes upon bacterial and viral exposure links Hsps with the immune system but it is unclear if Hsp70 from shrimp and bivalve haemocytes stimulates immunity. However, Hsps are thought to maintain phagocytic cells [106] either by repairing damage or safeguarding against autolysis and apoptosis due to auto-oxidation brought about by internal defence systems [8]. Preserving haemocytes is of primary importance to ensure the degradation of infectious pathogens [101,102].

As for invertebrates, the role of Hsps in the fish immune system is poorly defined although several studies are available on Hsp gene expression following bacterial and viral contact. Use of the peripheral blood lymphocyte (PBL)/lipopolysaccharide (LPS) model from the grass carp *Ctenopharyngodon idella* has aided research on fish immunity and shed light on Hsp70 function [107]. In reaction to LPS, an endotoxin of the outer membrane of Gram-negative bacteria, the expression of grass carp genes encoding Hsp70/Hsc70 is enhanced in twelve immune-related tissues including PBLs, head kidney, kidney, spleen, intestines, gill and thymus. The abundance of Hsp70/Hsc70 in PBLs indicates selective modulation of gene expression and the detection of extracellular Hsp70 implies that release of this Hsp contributes to immunity. Extracellular Hsps are postulated to mediate production of cell surface peptides, thus helping the immune

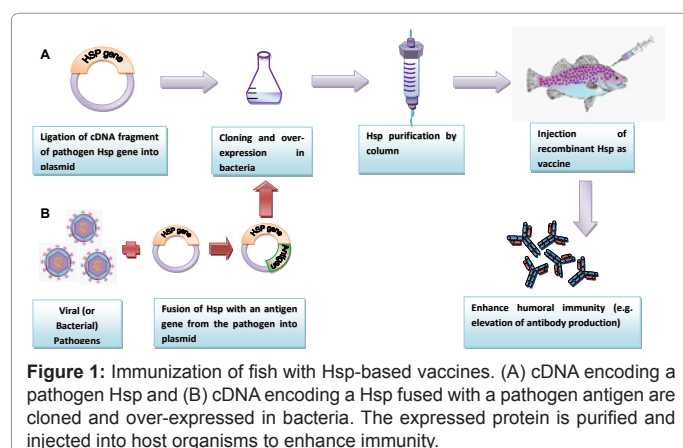
system recognize distressed cells [65,108,109]. Hsp70 communicates inflammatory “danger signals” to immune cells such as monocytes, dendrites, neutrophils and macrophages [108,110,111] via stimulation of TLR2 and TLR4 [96,112,113]. The secretion of inflammatory cytokines, nitric oxide (NO) synthase, NO, tumor necrosis factor-alpha (TNF- α , Interleukin1-beta (IL-1 β and IL-6 from macrophages and neutrophils is intensified by Hsps [114]. During adaptive immunity Hsps may play an integral part in antigen presentation via assembly of major histocompatibility complex (MHC) – peptide complexes, activating T cells to destroy or co-ordinate the killing of pathogens and infected, malfunctioning cells [11,98]. Class I and class II MHC are membrane-bound glycoproteins responsible for presenting processed antigens to CD8⁺ and CD4⁺ T lymphocytes. Teleost fish MHCs are equivalent to their mammalian counterparts in exon–intron organization and polymorphism of their genes and in protein structure [115]. Understanding the role of extracellular Hsp70 released from fish lymphocytes and the functions of other Hsps in immune regulation is likely to reveal strategies for the protection of fish and shellfish, this of particular use in aquaculture where infection and disease are major obstacles to increasing productivity.

Hsp-Based Vaccines Restrict Infection by Pathogens

Vaccines raised against bacteria and viruses for delivery by immersion, injection and orally, prevent fish disease and they offer important alternatives to antibiotic use by providing long-lasting protection with little or no adverse environmental effect [116]. Attenuated pathogens are commonly used in vaccine production for immunization of fish [117], but interest has grown in the use of Hsps for this purpose. Pathogen derived Hsps are dominant antigens in many microbial infections and they have been used successfully to generate vaccines against bacterial fish pathogens [2,117]. To obtain Hsps for immunization, cDNAs encoding pathogen Hsps are cloned, over-expressed in bacteria and purified after which they are introduced into host organisms (Figure 1).

Injection with Hsp60 and Hsp70 from *Piscirickettsia salmonis* protects Atlantic salmon *Salmo salar* against *P. salmonis* infection, boosting survival by 89.6% at 50 days post-challenge when compared to non-immunized controls [118]. Characterization of a novel immunogenic agent termed ChaPs synthesized by *P. salmonis* obtained from infected salmonid fish, reveals a prokaryotic Hsp, supporting the idea that bacterial Hsps are suitable for development of vaccines against salmonid rickettsial septicemia (SRS) [21], a disease responsible for massive mortalities of salmon worldwide. Moreover, vaccination with purified DnaJ, the prokaryotic homologue of Hsp40, from *Edwardsiella tarda* shields the Japanese flounder *Paralichthys olivaceus* against this Gram negative pathogen, increasing survival by 62% over non-immunized controls [22].

Flavobacterium psychrophilum Hsp60 and Hsp70 are highly immunogenic [119], but vaccination with these proteins alone, or in combination, does not safeguard the rainbow trout *Oncorhynchus mykiss* against infection. Antibody titres to Hsps remain low even 4 weeks post-immunization [117,120]. However, a fusion protein composed of Hsp70 and a protein or peptide from a pathogen is highly immunogenic, apparently because the Hsp70 functions as an adjuvant. As one example, fusing *Mycobacterium* Hsp70 with p24, an HIV protein, induces higher production of antibodies to p24 and more interferon than does Hsp70 or p24 when acting alone [117,121]. A novel vaccine raised against a fusion protein composed of Infectious Salmon Anemia Virus (ISAV) protein subunits and fish Hsps is being tested for use in aquaculture [122]. Employing Hsps for immunization



offers a seemingly effective approach for disease control with potential applications in many different commercially important aquatic organisms while reducing the environmental impact of aquaculture operations.

Conclusions

Aquatic organisms respond to pathogen infection by the production of sHsps, Hsp70, Hsp90 and Hsp60, all of which function as molecular chaperones and protect cells during stress. That is, Hsps modulate the folding of nascent proteins, prevent irreversible protein denaturation and either refold or assist in the elimination of damaged proteins. Finfish, shellfish and bivalves synthesize Hsps in response to bacterial and viral infections indicating a role for these proteins in disease resistance. Increasing Hsps in aquatic organisms by heat shock, chemical application and feeding exogenous Hsps also enhances resistance to infection. The level of tolerance correlates with the amount of accumulated Hsp. In addition to protecting proteins Hsps are thought to improve resistance to pathogens by stimulating humoral and cellular aspects of host innate immunity, perhaps best explaining how the feeding of exogenous Hsps contributes to disease tolerance. Further studies with *Artemia* are of particular interest in the context of feeding Hsps to host animals as a protective strategy. Hsps, either in isolation from other proteins or fused to pathogen-derived antigens are effective vaccines for the prevention of disease in aquatic organisms. Vaccine targets include Hsps from pathogens which are produced in abundance upon infection and pathogen protein/peptides fused to Hsps. Clearly, there is a strong case for exploring Hsps as agents of disease resistance and an excellent possibility that their application will present a significant advance in preventing diseases of aquatic organisms used for aquaculture.

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