ISJ 5: 135-161, 2008 ISSN 1824-307X

Review

HSP expression in bivalves

E Fabbri, P Valbonesi, S Franzellitti

Interdepartment Centre for Environmental Science Research, University of Bologna, campus of Ravenna, Italy

Accepted September 26, 2008

Abstract

One of the molecular responses which mostly contribute to the physiological plasticity of bivalves is the heat shock response mediated by heat shock proteins (HSP). Variations of HSP response were observed under environmental conditions, correlated with differences in environmental temperature and degree of heterogeneity across geographic thermal gradients and through time. Laboratory experiments characterized the expressions of different protein isoforms and coding genes, which are induced by heat as a prototypical stimulus. Nevertheless, other physical and chemical factors significantly induce HSP gene and protein expressions in bivalves, that can be different depending on tissues and the nature of the insult. Multiple alignments of the deduced amino acid sequences indicated that the bivalve HSP70 proteins share common structural and evolutionary features with the mammalian HSP70, while some appear to be exclusive. The rate at which new findings are made regarding the bivalve HSP response is still increasing. However, some major questions remain unanswered. Among them, the possibility that the bivalve HSP response is related to cell signalling pathways and acts as a component of the acute systemic response to stress is also discussed in this review.

Key words: bivalves; HSP; HSP expression; HSP phylogeny; HSP sequence; stress response

Introduction

Living systems have evolved a variety of strategies to respond to external or internal environmental challenges. While these responses are often behavioural or metabolic, a powerful mechanism widely employed to maintain cellular homeostasis under stress is the adjustment of gene expression. Due to their immobility, sessile organisms in particular rely on this physiological plasticity to adapt to environmental insults and colonize rapidly fluctuating habitats. Bivalves living in intertidal zones are one of the best examples of ectotherms surviving highly stressful conditions, facing exposure to natural changes in temperature, salinity, and oxygen availability exacerbated by the extensive presence of pollutants and anthropogenic disturbances in general (Hofmann, 1999). These same organisms living at their physiological stress limit will be affected by the continuous rise in temperature predicted under climate change scenarios, and only those endowed with sufficient defence mechanisms will be able to survive.

The aim of this review is to outline the current state of our knowledge of one of the molecular responses which most contribute to the physiological plasticity of bivalves, i.e., the heat shock response mediated by heat shock proteins (HSP), examined at the level of gene and protein expressions. We also discuss structural features of the *HSP70* and *HSP90* multigene families in bivalves, and their phylogenetic relationships. Traditionally the heat shock response has been considered an intracellular phenomenon with little or no association with intra/extracellular signalling. In this review we want to draw attention to the fact that the HSP response is related to cell signalling pathways, and as it is a component of the acute systemic response to stress, it is integrated with the physiological stress response. These relationships are presently under active investigation in mammals, and initial findings suggest that they may also apply to bivalves (Lacoste *et al.,* 2001a).

Corresponding author:
Elena Fabbri
Interdepartment Centre for Environmental Science Research
University of Bologna, campus of Ravenna
via S. Alberto 163, 48100 Ravenna, Italy
E-mail: elena.fabbri@unibo.it

The terminology used is in accordance with the following criteria: capital letters are used to refer to whole HSP or the whole family (e.g., HSP70). Non-capital letters are used to refer to a specific protein from a family (e.g., Hsp70, Hsp72, etc.). Italics are used for genes (i.e., *Hsp70, Hsp72*, etc.).

The integrated stress response

The definition of stress and stressors has a long and controversial history. In this review, however, we will adopt the definition used by Wendelaar Bonga (1997) that stress is a condition in which dynamic equilibrium of animal organisms, called homeostasis, is threatened or disturbed by intrinsic or extrinsic stimuli, commonly defined as stressors. These responses typically involve all levels of animal organization and are referred to as the "integrated stress response" (Wendelaar Bonga, 1997). In this light, stress acquires a less negative connotation, in so far as it indicates all the forces or stimuli in the environment, internal or external, that can induce changes and adaptations in the organism to help it better fit its environment. However, if the stress or the stress factors persist without physiological adaptation, illness or even death may occur. Many hormones are involved in the mammalian integrated stress response, but the dominant role of catecholamines (CA) and glucocorticoids in this response is generally recognized (Wendelaar Bonga, 1997). These hormones are the primary messengers of the two major routes through which the brain coordinates the stress response: the brain-sympathetic-adrenal medulla axis and the brain-pituitary-adrenal axis. Their main functions involve stimulation of oxygen uptake and transfer, mobilization of energy substrates, reallocation of energy away from growth and reproduction.

Past studies on molluscs revealed remarkable structural, functional and biochemical parallelisms with vertebrates. Numerous studies actually identified in mussels and other molluscs neuroendocrine and nervous system functions analogous to the hypothalamic-pituitary system in vertebrates (Stefano *et al*., 2002). In general the elements at the basis of the response and their triggering are similar to those of vertebrates, but take place in a different and simpler scenario (Ottaviani and Franceschi, 1996). Most mechanisms are represented in one single multifunctional cell, the hemocyte (Malagoli *et al.*, 2000). As the cell component of bivalve hemolymph, this cell is involved in several functions, including respiration and nutrition, immune response, and stress response (Ottaviani and Franceschi, 1996). CA together with β-endorphin, α-MSH, and ACTH are the most important mediators of stress response in bivalves, and some molecules similar to cortisol were identified (Ottaviani *et al*., 1998a, 1999). Although the mechanisms controlling the CA release in invertebrates have not been fully elucidated, the production of CRH by nerve cells probably induces the release of ACTH-like molecules, which in turn trigger the release of CA, mainly noradrenaline and dopamine. In oysters, the release of CA starts from neurosecretory cells which resemble the vertebrate chromaffin cells (Lacoste *et al*., 2001b). There is clear evidence that, under stress conditions, the CRH-ACTH modulated axis also determines the activation of bivalve hemocytes, which possess CRH and ACTH receptors (Ottaviani *et al*., 1998b; Malagoli *et al*., 2000). It has also been suggested that that the Platelet Derived Growth Factor (PDGF) and Transforming Growth Factor (TGF-β) exert a direct control on CA release, thus playing an important role in stress response in these organisms (Ottaviani *et al.,* 1998a, 2001)

Relationship between integrated and cell response to stress

In mammals, circulating levels of prolactin and glucocorticoids are increased by thermal stress and are associated with the modification of intracellular heat shock by enhancing expression of *HSP* genes (Vijayan *et al*., 2003). Prostaglandins are also associated with thermal stress and are known to induce HSP synthesis (Collier *et al*., 2006). Mechanical disturbances and changes in temperature or salinity were shown to increase circulating levels of noradrenaline and dopamine in oysters. The response was rapid and transient, according to the duration of the stress exposure (Lacoste *et al*., 2001c). The CA response to acute stimulation took place in a few minutes, raising the circulating levels of noradrenaline and dopamine (Lacoste *et al*., 2001c). Further experiments clearly demonstrated that treatments with adrenergic compounds induced the *Hsp70* gene promoter in oyster hemocytes, indicating that the integrated response to stress is related to the heat shock response elaborated by cells. Specifically, the response was stimulated through α-adrenoceptor mediated pathways, involving PLC and PKC activation (Lacoste *et al*., 2001a). Treatments with CA also induced high viability in hemocytes exposed to severe heat stress, indicating that α-adrenergic stimulation leads to cell thermotolerance through HSP overexpression (Lacoste *et al*., 2001a). Although this relationship has not been further elucidated, it represents one of the most interesting issues to be investigated in this field.

The cell response to stress

Cellular exposure to stress factors induces a number of anomalies in cellular function, including a general inhibition of protein synthesis, alterations in protein structure and function, cytoskeleton rearrangements, shifts in metabolism, alterations in cell membrane dynamics and fluidity, etc. These anomalies trigger major changes in gene transcription and protein synthesis, known as the cell response to stress mediated by HSP, metallothioneins, antioxidant enzymes, etc. The timing and success of these changes ultimately determines cell survival and acclimation or cell death. Therefore, cells have developed strategies that allow the identification of, and the reaction to, stress conditions. Although in oysters it has been demonstrated that HSP is induced by CA (Lacoste *et al.*, 2001a) these proteins are also naturally induced at stress levels lower than those required

for activation of the integrated stress response. The overexpression of HSP in the absence of an integrated stress response involving the whole organism reflects the fact that the expression of these proteins can be regulated at the cellular or tissue level, not only at the organism level.

As a general pattern, a transcription factor family known as heat shock factor (HSF) is the first responder during the onset of elevated cell temperature. The physiological importance of HSF is exemplified by its evolutionary conservation, from unicellular organisms to mammals (Pirkkala *et al*., 2001). Amongst other HSF, HSF1 is primarily responsible for induction of *HSP* gene expression after heat shock (Pirkkala *et al*., 2001). The current model of HSF1 transcriptional activity indicates that nonstressed cells contain folded HSF1 monomers bound to HSP within the cytoplasm. Upon heat stress, the HSP dissociate from HSF1 monomers, which then unfold and bind to two other HSF1 monomers to form a trimer which then translocates to the nucleus. Once in the nucleus, the trimer binds promoters containing heat shock elements (HSE) to trigger heat shock gene transcription. Although HSF1 was traditionally associated with regulation of HSP, recent evidence now links it to regulation of mammalian carbohydrate metabolism, transport, cytoskeleton, and ubiquitination during heat shock (Page *et al*., 2006).

The heat shock proteins

HSP are evolutionarily ancient and highly conserved intracellular molecular chaperones constituting several multigene superfamilies *(*Barral *et al*., 2004*).* They are present in all the different subcellular compartments of all cell types from prokaryotes to eukaryotes. The initial nomenclature for HSP was based on the apparent molecular weight of each single protein (i.e., Hsp70, 72, 73, etc.) and they were grouped according to their nearest size (e.g., the HSP70 family). Some proteins are referred to by different names, as they were thought to be different when they were discovered and, for historical reasons, these names have been preserved (see Vos *et al*., 2008 for the most recent classification of human HSP).

HSP can also be grouped into constitutive and inducible isoforms. Proteins reported as HSC (heat shock cognate) represent the constitutive forms; these are always expressed under physiological conditions and serve as molecular chaperones. The inducible forms, properly referred to as HSP, are synthesised by cells under stressful conditions and display a cytoprotective role. Further proteins constitutively present but also induced by stress have been reported in most bivalves (Piano *et al*., 2002).

Molecular chaperones appear to be essential for protein folding or trafficking, and for regulated proteolysis in cells (Hendrick and Hartl, 1995; Fink, 1999). Most of the information we have on chaperone functioning relies on studies on bacteria, where proteins are on average smaller and less complex than the corresponding proteins of eukaryotes (Hartl and Hayer-Hartl, 2002). However main research on human chaperones was performed, also due to the correlation between protein misfolding and major diseases (Barral *et al.*, 2004). To become functionally active, newly synthesized proteins must fold into unique three-dimensional conformations, based on the information encoded in their amino acid sequences. Although many proteins can fold to their native state spontaneously *in vitro*, the involvement of high efficiency molecular chaperones is required *in vivo*. A further role of molecular chaperones is to prevent protein misfolding and aggregation, anomalous reactions that would otherwise impair cell functioning. The properties of the peptide bond confer a high degree of conformational flexibility to the protein backbone, while the amino acid side chains allow a large number of mostly non-covalent interactions. Thus the protein chain can theoretically adopt an enormous number of different conformations, and generally only one of these corresponds to its native state, sufficiently stable and biologically active under physiological conditions. These non-native states often expose hydrophobic residues and tend to self-associate into disordered aggregates, driven by hydrophobic forces and interchain hydrogen bonding. Chaperones promote the re-folding process through cycles of substrate binding and release, generally regulated by ATPase activity and various co-factors. More recent reports showed that in addition to protein folding and refolding, chaperones are also involved in several other physiological processes in mammals, including signal transduction, apoptosis, immune response, etc. (van Noort, 2008).

Members of the HSP70 family are the most highly conserved molecular chaperones. HSP70 found in prokaryotic and eukaryotic cells are able to recognize exposed hydrophobic amino acid side chains within an accessible polypeptide backbone, features that are generally found in non-native proteins. All HSP70 functions are performed through an ATP-regulated cycle of substrate binding and release in the presence of different cofactors, including DnaJ-proteins or the eukaryotic equivalent HSP40.

Biochemical studies of HSC70 fragments generated using recombinant DNA technology have led to mapping and characterization of the domains. The 44-kDa fragment (amino acid residues 1-386) from the N-terminus has been characterized by X-ray crystallography and contains the ATPase domain. The 18-kDa fragment contains the peptide-binding domain that binds unfolded and folded peptides (Wang *et al.*, 1993). The 44- and 18-kDa fragments are the same as those making up HSP70. The 10 kDa C-terminal of HSP70 differs by 26 amino acid residues relative to HSC70 and is 6 amino acids shorter (Leung and Hightower, 1997).

Members of the 90 kDa heat-shock protein (HSP90) family act downstream of the HSP70/HSP40-chaperone system, and play an important role in conformational protein regulation and cell signalling. They are highly conserved, essential proteins found in all organisms from bacteria to humans. HSP90 are the most abundant chaperones in the cell, representing 1 - 2% of cellular proteins, making it one of the most abundant proteins even in the absence of stress. HSP90

act at the core of a network of protein complexes, with over a dozen known cofactors, some of which link it to other multi-protein complexes such as the ubiquitin proteasome system and the HSP70 system. Unlike HSP70, HSP90 has several identified specific interactions, for example, with cytoskeleton elements, signal transduction proteins including steroid hormone receptors, and protein kinases (Fink, 1999). Moreover, it plays a regulatory role in inducing conformational changes in folded, native-like substrate proteins, leading to their activation or stabilization (Wandinger *et al.*, 2008)

HSP60 are large, oligomeric, ring-shaped proteins known as chaperonins, present in all biological compartments with the exception of the endoplasmic reticulum. This family includes two groups of proteins on the basis of sequence homology: GroEL-like proteins, present in bacteria, mitochondria and chloroplasts, and the TCP-1 (CCT or TRiC) family, found in Archaea and the eukaryotic cytosol. HSP60 play an essential role in all cells, assisting a large variety of newly synthesized and newly translocated proteins to reach their native forms by binding them and facilitating their folding. The folding-active state is reached by conformational changes, induced by the action of ATP binding and, for the organellar/bacterial chaperonins, also by the binding of a lid-like co-chaperonin, (cpn10) (GroES in *E. coli*) (Bukau and Horwich, 1998).

Other HSP distributed among organisms are the small heat shock proteins (sHSP). This family consists of 12 to 43 kDa proteins, which assemble into large multimeric structures, present in the cytosol, nucleus, and mitochondria. These proteins act as ATP-independent chaperones and many of them are produced only under stress conditions. One of the best studied sHSP is Hsp27 (also denoted HspB1, Hsp28, and Hsp25 in murine cells). Hsp27 protect cells against oxidative stress by counteracting the accumulation of proteolysis-resistant large aggregates of oxidized proteins (lipofuscin) which interfere with proteasome activity and are extremely deleterious to the cell. Hsp27 is an early target for phosphorylation, which occurs rapidly following exposure to various stresses, but also in unstressed cells upon stimulation by serum or a variety of mitogens, cytokines, and inducers of differentiation (Fink, 1999).

The heat shock proteins in bivalves

Aquatic environments are often highly dynamic and provide a wide variety of stress factors for individuals living there. Bivalves may be subject to a variety of sources of stress, including temperature and salinity fluctuations, oxygen availability, different quantities and quality of food, presence of predators or competitors, presence of toxic natural compounds and contaminants. Moreover, they are always in contact with microbes and their immune system is alerted to avoid an accumulation of invading pathogens. Aquaculture introduces additional forms of stress, including mechanical stress during handling and transportation (Lacoste *et al*., 2001c).

The heat shock response entails the rapid synthesis of HSP, which have been ubiquitously detected in all bivalves studied so far. The absence of HSP response was reported in an Antarctic fish (Hofmann *et al*., 2000) and an Antarctic ciliate (La Terza *et al.*, 2001); however, the Antarctic clam *Laternula elliptica* does rely on HSP to counteract the effects of thermal stress (Park *et al*., 2007). Although the name “HSP” derives from stress due to temperature elevation (Ritossa, 1962), a number of factors have actually been shown to induce this response in bivalves and will be discussed below. Nevertheless, HSP are also constitutively expressed and play a role in bivalves under physiological conditions.

HSP70 is the best investigated family, and most of the following discussion will focus on these proteins. Mitochondrial HSP60 induction was proposed as an early warning of adverse physiological effects in bivalves, as it is enhanced in mussels (Sanders *et al*., 1992; Sanders and Martin, 1993; Snyder *et al*., 2001), oysters (Ivanina *et al*., 2008a), and clams (Franco *et al.,* 2006) exposed to stress stimuli. Dowling *et al*. (2006) observed no effect of pesticides on HSP60 expression in gill, mantle and digestive gland of *Ruditapes decussatus*, shedding some doubts as to the role of HSP60 as biomarkers of stress.

In bivalves, HSP90 have received less attention than other HSP. It is true that the behaviour of HSP90 expression is often similar to that of HSP70 in response to cadmium exposure (Choi *et al*., 2008), prolonged heat stress (Snyder *et al.,* 2001; Anestis *et al*., 2007), or low electromagnetic fields (Malagoli *et al*., 2004), but a tissue specific and species-specific expression of HSP90 in bivalves is underlined by several authors (Lyons *et al*., 2003; Dowling *et al.,* 2006; Ivanina *et al.,* 2008a).

Other HSP have been poorly studied or not studied at all in bivalves. However, they will be considered in this review, where data are available.

The *HSP70* multigene family in bivalves

Along with the extensive characterization of the HSP70 response at the functional level, the bivalve *HSP70* multigene family was also investigated at the gene level, and the availability of nucleotide and deduced amino acid sequences has increased noticeably during the last few years (Table 1).

Marine mussels and oysters are amongst the bivalves most often used to study the heat shock response, and studies on *HSP70* gene regulation in these organisms provide important insights into the molecular basis of the response to environmental challenges. Sequences encoding multiple HSP70 isoforms are reported mainly for the Mediterranean mussel *Mytilus galloprovincialis*, and for the Ostreidae *Ostrea edulis* and *Crassostrea gigas* (Table 1). For these species, different full-length sequences encoding both HSC70 and HSP70 proteins are available, and detailed characterizations of their functional features and gene expression profiles have been carried out (Boutet *et al.*, 2003a, b; Piano *et al.*, 2004, 2005; Franzellitti and Fabbri, 2005, 2006; Kourtidis *et al.*, 2006; Cellura *et al*., 2006,

Table 1 The bivalve HSP70 protein sequences available in GenBank

Species (Abbreviation)	Gene	Genebank Ac Numb	Reference
Crassostrea ariakensis (Cariak)	Hsc70	AAO41703	unpublished
Crassostrea columbiensis (Ccol)	Hsp70	ABC02062	unpublished
Crassostrea gigas (Cgig)	Hsc71	BAD15287	unpublished
	Hsc72	AAD31042	(Gourdon *et al*., 2000)
	Hsp70	CAC83009	(Boutet *et al*., 2003b)
	Hsp70	BAD15286	unpublished
	Grp78	BAD15288	(Yokoyama *et al*., 2006)
	Grp94	BAF63637	unpublished
	Hsp68	BAD15285	unpublished
	Hsc70	CAC83683	(Boutet *et al*., 2003b)
Crassostrea virginica (Cvir)	Hsp70	CAB89802	(Rathinam *et al*., 2000)
Ostrea edulis (Oed)	Hsc70	CAC83684	(Boutet *et al*., 2003a)
	Hsp70	AAM46635	(Piano *et al*., 2005)
	Hsp70	AAM46634	(Piano *et al*., 2005)
	Hsp70	CAC83010	(Boutet *et al*., 2003a)
Saccostrea palmula (Sacp)	Hsp70	ABC02063	unpublished
Mytilus galloprovincialis (Mg)	Hsc71	CAH04109	(Kourtidis *et al*., 2006)
	Hsc70	CAH04110	(Kourtidis *et al*., 2006; Kourtidis and Scouras, 2005)
	Hsc70	ABA61049	(Franzellitti and Fabbri, 2005)
	Hsp70	AAW52766	(Cellura *et al*., 2006)
	Hsp70	ABA61046	(Franzellitti and Fabbri, 2005)
	Hsp70	ABA61047	(Franzellitti and Fabbri, 2005)
	Hsp70b	BAD99027	(Toyohara *et al*., 2005)
	Hsp70a	BAD99026	(Toyohara *et al*., 2005)
	Hsp70	CAH04108	(Kourtidis *et al*., 2006)
	Hsp70	CAE51348	(Kourtidis *et al*., 2006)
	Hsp70	CAH04106	(Kourtidis *et al*., 2006)
	Hsp70	CAH04107	(Kourtidis *et al*., 2006)
Mytilus edulis (Med)	Hsc70	AAD48065	(Luedeking and Koehler, 2002)
Bathymodiolus azoricus (Baz)	Hsp70	CAJ40877	unpublished
Perna viridis (Pvir)	Hsp71	ABJ98722	unpublished
	Hsc71	ABQ11278	unpublished
Diplodon chilensis (Dpic)	Hsp70	ABW06851	unpublished
Chlamys farreri (Cfar)	Hsp70	AAO38780	unpublished
Chlamys farreri (Cfar)	Hsp70	ABE77386	unpublished
Argopecten irradians (Airr)	Hsp70	AAS17723	(Song *et al*., 2006)
Mizuhopecten yessoensis (Myess)	Hsp70	AAS17724	(Song *et al*., 2006)
Pinctada fucata (Pfuct)	Hsp70	ABJ97378	unpublished
Pteria penguin (Ppeng)	Hsp70	ABJ97377	unpublished
Laternula elliptica (Lellipt)	Hsp70a	CAL25331	(Clark *et al*., 2008)
	Hsp70b	CAL25333	(Clark *et al*., 2008)
	Hsc70	CAL25332	(Clark *et al*., 2008)
	Grp78	CAL25334	(Clark *et al*., 2008)
	Hsp70	ABM92345	(Park *et al*., 2007)
Venerupis decussates (Vdec)	Hsp70	ACB38005	unpublished
Dreissena polymorpha (Dpol)	Hsp70	ABP88104	unpublished

2007). Other full-length or partial sequences have been obtained for *Crassostrea virginica, Crassostrea ariakensis*, *Crassostrea columbiensis*, and *Saccostrea palmula* (Table 1). The scallops *Argopecten irradians* and *Chlamys farreri* have received recent attention due their extensive employment in aquaculture (Song *et al*., 2006). Other bivalves have been studied as representatives of peculiar environments; for example an *HSP70* cDNA sequence was recently obtained for the Antarctic clam *L. elliptica* (Park *et al*., 2007).

A cDNA sequence encoding the 78 kDa glucose regulated (GRP78) protein was cloned from *C. gigas* (Yokoyama *et al.*, 2006). A further partial sequence from *C. gigas* putatively encoding a 94 kDa GRP is also available (unpublished, Table 1). To our knowledge these are the only representatives of the GRP members of the HSP70 family obtained from a bivalve species.

The multiple alignment of the full-length HSP70 amino acid sequences of bivalves with their human homologues (Fig. 1) indicates that they share common structural and evolutionary features (Piano *et al*., 2005; Kourtidis *et al*., 2006). In particular, they display the canonical conserved domain structure of the *HSP70* consisting of: i) a cleavable signal sequence at the N-terminus, which characterizes the GRP78 proteins (Yokoyama *et al*., 2006); ii) an ATPase domain; iii) a peptide binding domain; iv) a G/P-rich C-terminal domain region, which enables the proteins to bind co-chaperones and other HSP (Daugaard *et al.,* 2007), and contains intracellular localization signal sequences (Fig. 1). Further important structural features, such as the presence of three HSP70 family signatures IDLGTTYS, IFDLGGGTFDVSIL, and VVLVGGDTRIPKIQK (Gupta and Singh, 1994), were also highly conserved (Fig. 1).

Nevertheless, some structural features appear to be exclusive to the bivalve HSP70. The HSC70 from *C. gigas* and *O. edulis* possess an extra NQSQ tetrapeptide within the ATPase domain (Fig. 2). This sequence is also found in other oyster HSC70 (Kourtidis *et al.*, 2006), and appears to be unique for Ostreidae. It should be noted that the NQSQ tetrapeptide encodes a putative glycosylation domain (Laursen *et al*., 1997), so that its correlation with peculiar functional and expression features of the Ostreidae HSC70 deserves further investigation.

The inducible *HSP70* gene products from mussel and oyster possess an extra serine (S) residue within the conserved ATPase domain (Fig. 2). As previously reported (Kourtidis *et al.*, 2006), this serine residue is shared by the inducible HSP70 of all invertebrate species studied so far. However, no specific role has yet been ascribed to this residue.

As shown in Fig. 3, the *O. edulis* HSC70 display a large amino acid deletion of about 60 residues encompassing the end of the peptide-binding domain and a part of the C-terminal domain. A similar deletion was also found in other Ostreidae HSP70 (Fig. 3, Kourtidis *et al.*, 2006), which also share a reduced molecular weight. This deletion could be responsible for a change in the functional role of these 65 kDa-proteins. In fact, two cognate variants in humans (Hsc54; Tsukahara *et al*., 2000) and rats (Hsc49; Yamada *et al*., 1999) bearing a similar deletion were found to act as negative competitors towards the normal HSC70.

As previously reported in mammals (Demand *et al*., 1998; Fuertes *et al*., 2004), a low homology within the C-terminal domain is also observed in mollusc HSP70, particularly between HSP70 and HSC70 (Piano *et al*., 2005; Fig. 3). This domain is thought to be involved in the interaction with co-chaperones of the DNAJ class and in the regulation of the substrate binding kinetics and affinity (Fuertes *et al.*, 2004). Interestingly, in this domain the bivalve HSC70 possess a large insertion compared with the inducible HSP70. Less extensive sequence variations are also identified in the same region when comparing mammalian HSP70 and HSC70. They might be partially responsible for the functional differences between inducible and constitutive HSP70 (Fuertes *et al*., 2004). This could also be true for the bivalve proteins, since the HSC70 sequence insertion contains two repeats of the tetrapeptide GGMP (Fig. 3), an important element mediating cofactor binding to the HSP molecule (Demand *et al*., 1998). Since no extensive biochemical characterization of the bivalve *HSP70* and *HSC70* genes is available, at present we do not know the extent to which such a structural variation affects their expression profiles as hypothesized in vertebrates (Demand *et al.*, 1998; Fuertes *et al*., 2004).

The C-terminal region contains a consensus motif that enables HSP70 to be localized into specific cellular compartments where these proteins perform different functions, including protein folding (endoplasmic reticulum), translocation to organelles (mitochondria), and stress response (cytosol/nucleus) (De Maio, 1999). The localization motif specific for the cytosolic proteins is GP(T/K)(V/I)EE(V/M)D (Boorstein *et al.*, 1994; Demand *et al.*, 1998). This sequence is found in the bivalve HSP70 sequences analyzed thus far, indicating that most of the *HSP70* gene products obtained for bivalves encode cytosolic proteins. Only one full-length protein sequence was obtained for an endoplasmic reticulum (ER) HSP70 protein, i.e., the GRP78 protein from *C. gigas* (Table 1); no records are currently available for the mitochondrial HSP70. The oyster GRP78 sequence displayed the characteristic ER localization motif Lys-Asp-Glu-Leu (KDEL), and shared a high sequence homology with GRP78 homologues from fish and other vertebrates (Fig. 1; Yokoyama *et al.,* 2006).

The phylogenetic analysis of several molluscan, mammalian, and fish cytosolic HSP70 reveals that the bivalve genes can be divided into two groups (Fig. 4), one containing the inducible (*BIVALVE HSP70*), and the other containing the cognate genes (*BIVALVE HSC70*). The branching pattern is in agreement with the generally accepted molluscan classification, differentiating Bivalvia from Gastropoda (Kourtidis *et al.*, 2006).

Some HSP70 sequences obtained from *C. gigas* (GenBank Ac. Numb CAC83009; Boutet *et al*., 2003b), *O. edulis* (GenBank Ac. Numb CAC83010; Boutet *et al*., 2003a), *M. galloprovincialis* (GenBank

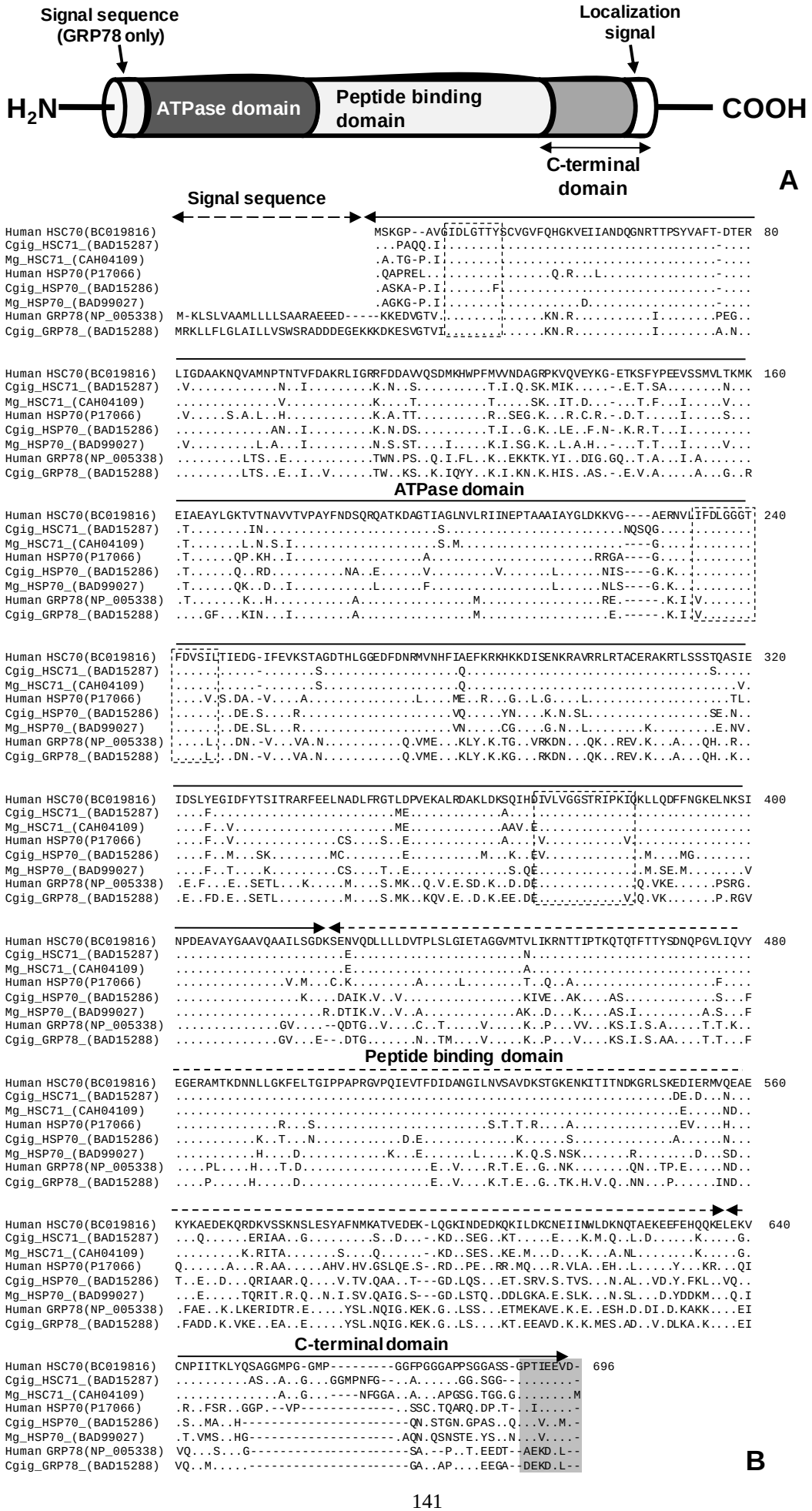
Signal sequence
(GRP78 only)
Localization
signal
H2N
ATPase domain
Peptide binding
domain
COOH
C-terminal
domain
A
Signal sequence
ATPase domain
Peptide binding domain
C-terminal domain
B

Fig. 1 Predicted amino acid sequences of the bivalve HSP70 family proteins and their domain structures. (**A**) Cartoon showing a linear representation of the general domain structure for the Hsp70 protein family. (**B**) Multiple alignment of deduced amino acid sequences of bivalve HSP70, HSC70, and GRP78 with the human homologoues showing the position of the conserved domains. Sequences from *C. gigas* (Cgig) and *M. galloprovincialis* (Mg) are used as representatives for the bivalve HSP70 and HSC70 sequences. The GRP78 sequence from *C. gigas* is the representative for the endoplasmic reticulum HSP70 of bivalves (Yokoyama *et al*., 2006). GenBank accession numbers are given in brackets. Identical amino acid residues are indicated by dots. Gaps (indicated by dashes) were added to improve the alignment. Dashed squares indicate the three HSP70 family signatures IDLGTTYS, IFDLGGGTFDVSIL, and VVLVGGDTRIPKIQK. A grey square indicates the C-terminal localization signal. The alignment was performed with the MEGA4 software (www.megasoftware.net).

Ac. Numb AAW52766; Kourtidis *et al.*, 2006), and from the Pectinidae *C. farreri (*unpublished GenBank Ac. Numb AAO38780 and ABE77386), *A. irradians* (GenBank Ac. Numb AAS17723; Song *et al.*, 2006), and *Mizuhopecten yessoensis (*GenBank Ac. Numb AAS17724; Song *et al.*, 2006), were originally referred to as inducible HSP70 proteins, although according to the phylogenetic analysis they belong to the BIVALVE HSC70 cluster (Fig. 4). This finding is further supported by the occurrence of structural characteristics of the cognate gene products in all the above protein sequences (Piano *et al.*, 2005; Kourtidis *et al.*, 2006; Figs 2, 3). However, since no data regarding their expression pattern are available, we cannot exclude a different functional classification. Also the *Hsp68* gene product from *C. gigas* (Table 1) has an uncertain phylogenetic position. This sequence is highly differentiated, and lacks the C-terminal EEVD tetrapeptide. We can speculate that it represents a pseudogene or is truncated because of sequencing errors.

The phylogenetic analysis also indicated that bivalve *HSP70* and *HSC70* gene sequences are more closely related to other bivalve *HSP70* and *HSC70*, respectively, than to each other. This is a common feature of the *HSP70* multigene family, and similar inter-specific homology between *HSP70/HSC70* members was also observed in fish and mammals (Ohta, 1994; Yamashita *et al.*, 2004). It is consistent with the occurrence of *HSP70* gene duplication events during evolution, suggesting that inducible and cognate genes undergo divergent evolution. This can explain why inducible and cognate genes, apart from having different expression patterns, also perform related but different functions. In fact, divergent evolution predominates when different functions that have to be maintained are acquired (Ohta and Nei, 1994).

The presence of multiple copies of the heat-inducible *HSP70* gene products (Table 1) is in agreement with the occurrence of gene duplication events, indicating that the evolutionary model proposed for vertebrates (Yamashita *et al*., 2004) could also be applied for the bivalve *HSP70.* In fact, a recent phylogenetic reconstruction of *HSP70* evolution in bivalves indicated the occurrence of multiple duplication events in the *HSP70* family, and also suggested a possible scenario for the *HSP70* evolution in which three successive duplication events occurred (Kourtidis *et al.*, 2006).

The *HSP90* gene family in bivalves

Apart from the *HSP70* family, there is very little information currently available on gene identification in bivalves for other HSP subfamilies. However, some progress has been made very recently on the cloning and characterization of the 90 kDa members of the family. To our knowledge, three full-length protein sequences are currently available, from the Pacific oyster *C. gigas* (Choi *et al.*, 2008), the scallops *C. farreri* (Gao *et al*., 2007) and *A. irradians* (Gao *et al*., 2008).

The multiple alignment indicates that these bivalve HSP90 display a high degree of sequence homology with human HSP90, and contain conserved structural features typical of these proteins. In particular, they possess five signal peptides that are referred to as signatures for the HSP90 family (Gupta, 1995; Gao *et al.*, 2007), and the MEEVD consensus sequence at the C-terminus, which characterizes the cytosolic HSP90 proteins (Fig. 5).

Two different cytosolic *HSP90* genes have been identified in vertebrates, i.e., *Hsp*90-α and *Hsp*90-β; they are different in so far as the Hsp90-β isoform lacks the glutamine-rich sequence (QTQDQ) at the N-terminus, a site of phosphorylation by a dsDNA-dependent kinase (Lees-Miller and Anderson, 1989). All three bivalve sequences obtained so far lack this QTQDQ sequence (Fig. 5), so that they display a greater similarity with the vertebrate Hsp90-β. Until now only genes encoding the Hsp90-β isoform have been reported in invertebrates, except for *Anopheles albimanus*, which contains both isogenes (Benedict *et al.,* 1996); however, sequencing information is too scarce to exclude the occurrence of a *Hsp90*-α gene in bivalves.

The bivalve HSP response to thermal stress

Field observations

Variations of HSP expression in nature are correlated with differences in average environmental temperature and degree of environmental heterogeneity across large- and small-scale geographic thermal gradients and through time (Hofmann and Somero, 1995; Roberts *et al*., 1997; Chapple *et al*., 1998; Dahlhoff and Rank, 2000; Dahlhoff *et al*., 2001; Helmuth and Hofmann, 2001). The observed variations encompass a suite of traits including expression of different HSP isoforms, i.e., ubiquitin,

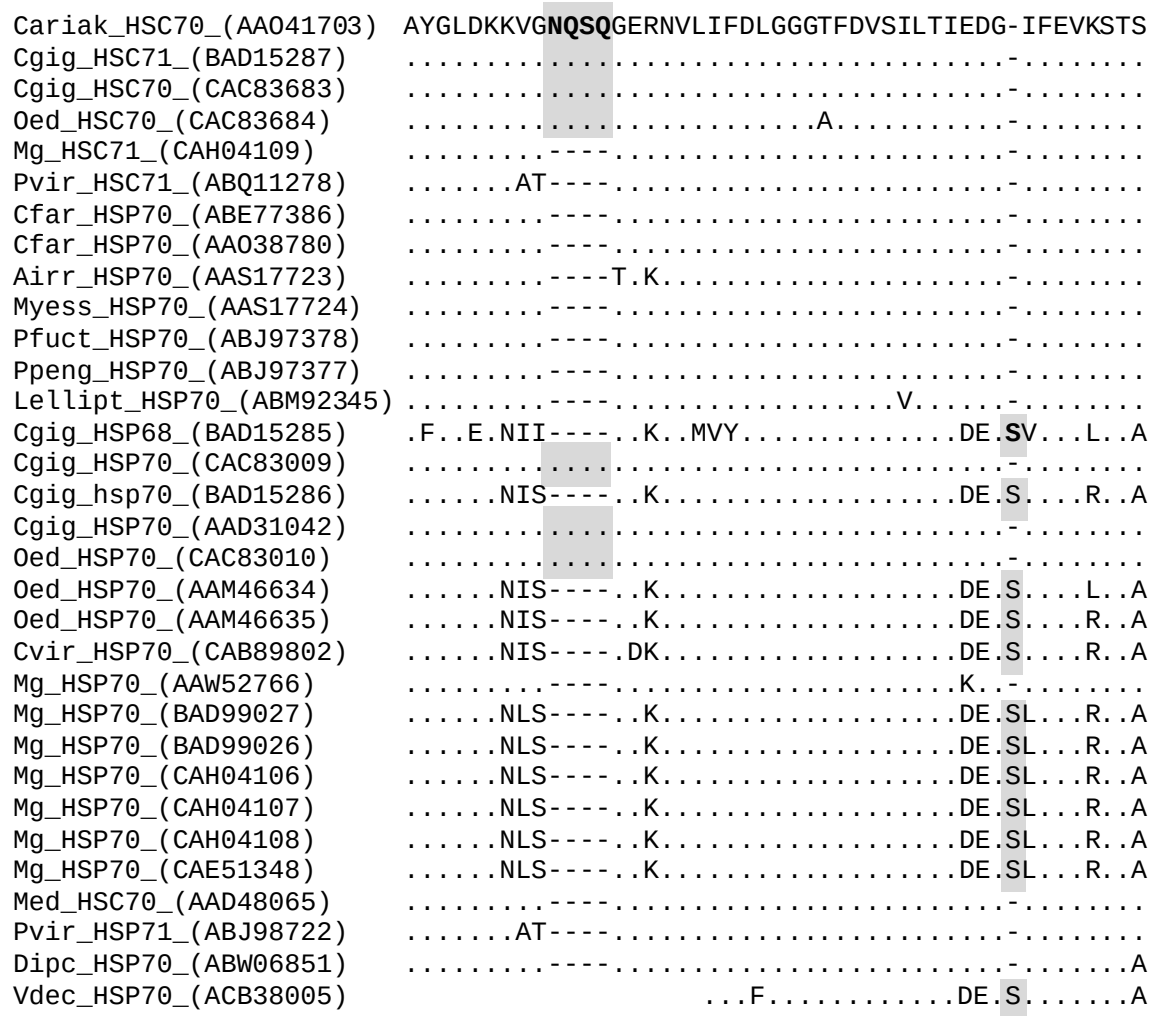

Fig 2 Multiple sequence alignment of several bivalve HSC70 and HSP70 proteins in the variable region of the ATPase domain. Grey squares indicated the insertion of the glycosilation (NQSQ) motif in the Ostreidae, and of the serine (S) residue in the bivalve inducible HSP70 gene products. Bivalve specie abbreviations are given in Table 1. GenBank accession numbers are given in brackets. Identical amino acid residues are indicated by dots. Gaps (indicated by dashes) were added to improve the alignment. The alignment was performed with the MEGA4 software (www.megasoftware.net).

constitutive, or inducible isoforms, variations in endogenous levels of HSP, and different threshold temperature at which the *HSP* gene expression is activated. One concept at the basis of this physiological feature is that the function of most HSP requires ATP, and so are costly for organisms. Considering the energetic costs associated with HSP activity, natural selection appears to have worked towards optimizing the cost/benefit ratio of stress proteins, so that variations in HSP production occur as a function of environmental conditions. However, seasonal acclimatization involves other physiological adaptations, including the different utilization of metabolic pathways, modification of protein turnover, change in membrane composition, etc., so that HSP seasonality appears as only one of the multiple aspects of a complex strategy that allows life and reproduction in a particular habitat (Hofmann and Somero, 1996; Chapple *et al*., 1998; Feder and Hofmann, 1999).

A number of fundamental experiments carried out in the past (Hofmann and Somero, 1996; Chapple *et al*., 1998; Feder and Hofmann, 1999; Hofmann, 1999) provided some crucial evidence that is still at the basis of our understanding of HSP expression. There were observed to be seasonal differences in the endogenous HSP70 levels, which were higher in summer than in winter-acclimatized *Mytilus trossulus* (Hofmann and Somero, 1995). Similarly, in *Mytilus californianus* collected from the field, HSP70 levels were higher in summer than in winter (Roberts *et al*., 1997). In the same intertidal mussels, the threshold temperature of HSP induction showed seasonal differences, rising to higher temperatures in summer. Amongst intertidal mussels, differences in latitude distribution were correlated with

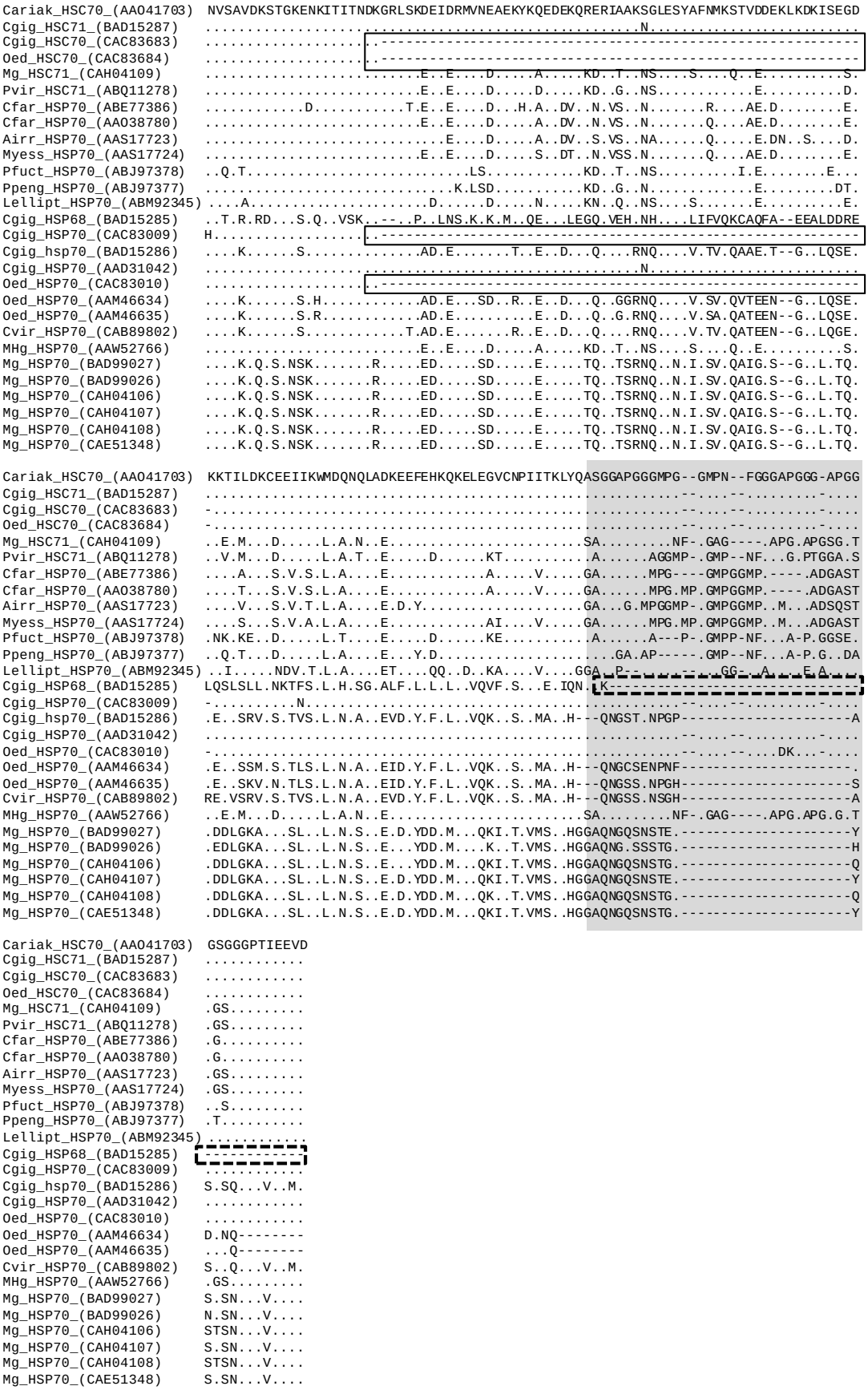

Fig 3 Multiple sequence alignment of several bivalve HSC70 and HSP70 proteins in the variable region of the C-terminal domain. A grey square indicates the characteristic GG[A,M]P repeats characterizing the HSC70 gene products. A solid-line square indicates the 60 amino acid deletion showed by four Ostreidae gene products. A dashed-line square indicates the truncated C-terminal region of the *C. gigas* HSP68 gene product. Bivalve species abbreviations are given in Table 1. GenBank accession numbers are given in brackets. Identical amino acid residues are indicated by dots. Gaps (indicated by dashes) were added to improve the alignment. The alignment was performed with the MEGA4 software (www.megasoftware.net).

interspecies difference in thermal sensitivity. *M. trossulus*, the northern species along the Pacific coast of USA, appeared more sensitive to heat stress than the congener *M. galloprovincialis*, living at a southern latitude (Hofmann and Somero, 1996). Both species were acclimated at 13 °C and analysed for some parameters related to thermal sensitivity. Higher levels of HSP70 were found in *M. trossulus* than in *M. galloprovincialis*, indicating that a greater degree of protein denaturation took place in the former. Moreover, higher levels of ubiquitinated proteins were measured in *M. trossulus*, indicating a higher protein degradation, which was consistent with the lower protein stability of organisms living at more northern latitudes (Hofmann and Somero, 1996). *C. gigas* showed significant increases in constitutively expressed HSP70 in summer as compared to winter. Consistently, increases in thermal limits and threshold for stress-inducible isoforms were observed (Hamdoun *et al.*, 2003)

The expression of HSP is under the control of specific transcription factors (Scharf *et al*., 1998), and there is evidence that this regulation differs among organisms, according to their life history and adaptation capacity (Hofmann and Somero, 1995). In nature, organisms continuously experience changes in the physico-chemical conditions of the environment, on a day or seasonal scale. A well-studied case is that of intertidal molluscs, which undergo regimes of immersion and emersion and are exposed to a series of abiotic stresses. During midday low tide, mussels may experience an increase of up to 25 °C in body temperature within 8 h accompanied by a strong induction of HSP70 synthesis (Hofmann and Somero, 1995). The HSP70 synthesis in *M. trossulus* was compared with that in *M. galloprovincialis*, a species which is phylogenetically and ecologically similar, although living at a lower latitude and higher temperatures. A comparison of the intensity of the HSP70 expression following thermal stress in individuals from these two species, either collected from the natural environment or maintained in aquaria, showed that it was significantly higher in *M. trossulus* (about 8 times the basal level) than in *M. galloprovincialis* (1.5 times). Moreover, the northern species showed an increase of different HSP70 isoforms and greater levels of damaged proteins as a consequence of similar temperature changes (Hofmann, 1999). Interestingly, HSP expression differed for mussels living at different heights in the intertidal zone; specimens of *M. californianus* occurring in locations farther up the shore displayed higher HSP70 levels than mussels collected from the lower portion of the mussel bed (Roberts *et al*., 1997). These considerations are supported by further results, including the very recent observations on *M. californianus* analysed along a vertical stress gradient, from the low intertidal low-stress zone to the high intertidal high-stress zone (Petes *et al*., 2008). High-edge mussels developed an HSP response modulated by acute stress during a single tide, and chronic stress from repeated exposure at low tide. Furthermore, the total HSP70 produced was always significantly higher in the high-edge mussels. A negative correlation was found in these mussels between HSP synthesis and reproductive potential. Maintenance of HSP response is costly for organisms, and this may negatively influence a diversified energy allocation.

According to the above studies, the temperature of acclimatization or acclimation influenced the set-point of the “cellular thermometer” and changed the temperature required to induce HSP synthesis in mussels. Therefore the hypothesis proposed by several authors (Hofmann and Somero, 1995, 1996; Chapple *et al*., 1998; Hofmann, 1999; Buckley *et al*., 2001) that environmentally-induced protein damage plays a role in setting the limits of species distribution is rather convincing.

As Antarctic animal species have evolved under a cold and thermally stable environment, it seemed plausible that the inability of heat shock to induce the HSP response was due to the loss of the regulation pathway in the *HSP* gene expression during their evolutionary history (Hofmann *et al*., 2000; La Terza *et al*., 2001). However, the Antarctic clam *L. elliptica* showed the constitutive expression of *HSP70*-mRNA levels in gills and digestive glands at their living temperature of 1 °C, and levels were enhanced by exposure to 10 °C. The transcript levels were significantly higher than in controls after 6 h, increasing further up to 12 - 24 h, and declining thereafter (Park *et al*., 2007). Gills were more responsive to stress than digestive glands, developing a faster and more marked response. This ability would appear to be of great importance as we move towards global warming scenarios, when temperature will be a major factor affecting the growth and survival of Antarctic species.

Interesting persective is provided by studies on deep-sea mussels. *Bathymodiolus childressi* is adapted to a cold, thermally stable environment, it is exposed to a variety of stressors, including hydrocarbons, low oxygen levels, high salinity and hydrogen sulphide. Interestingly, this species possesses a high thermal tolerance, although it does not express an inducible Hsp70 protein. High constitutive levels of HSP70 are indeed present that probably remediate protein damage from the above

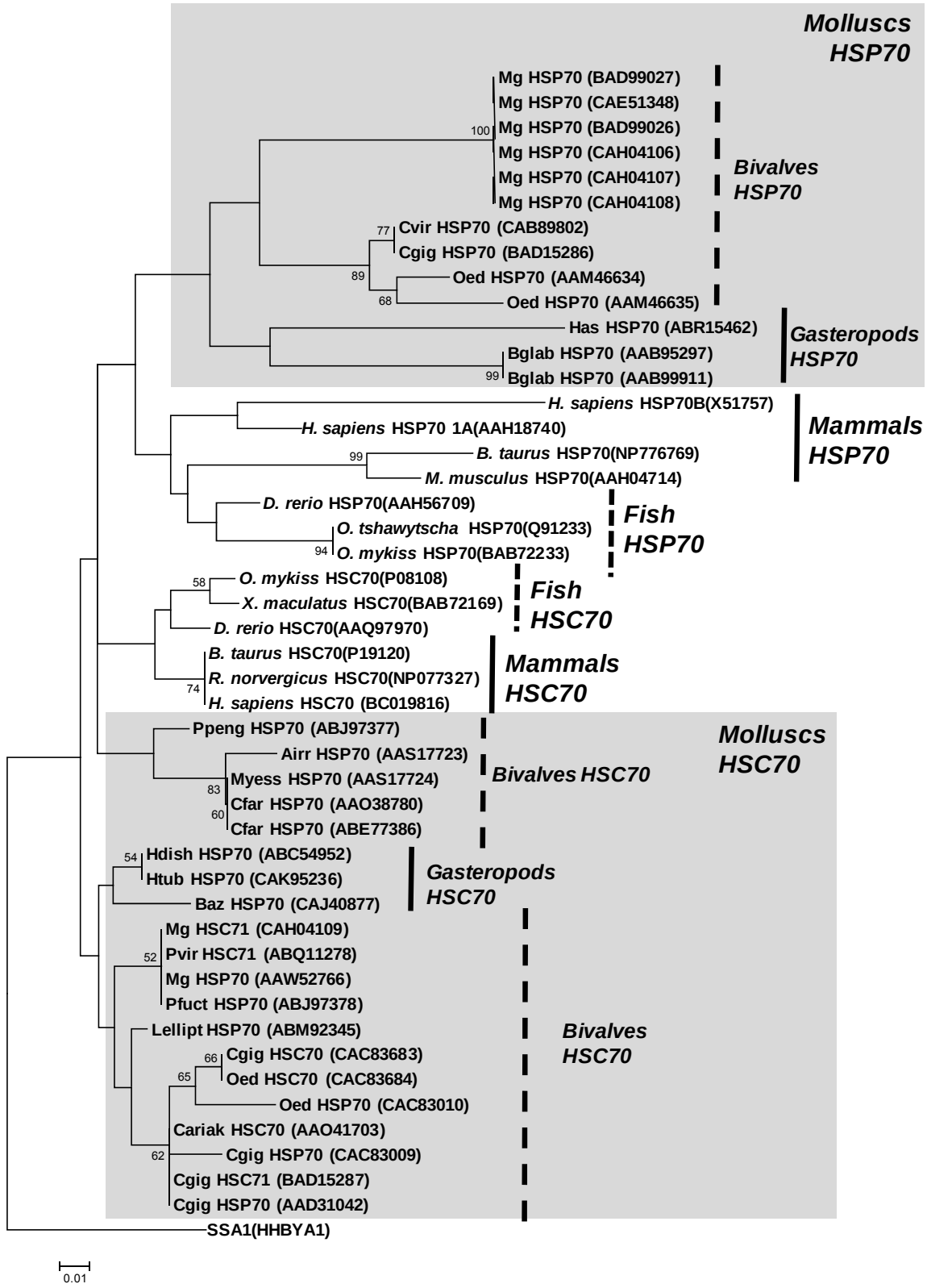

Fig. 4 Phylogenetic relationship among HSP70 deduced amino acid sequences of mammals, fish, and bivalves. The tree was constructed by the Neighbour Joining algorithm using the MEGA 4 software (www.megasoftware.net). Bootstrap confidence values for the sequence groupings are indicated in the tree (*n = 1000 replicates*). SSA1 from *S. cerevisae* was used as outgroup. Bivalve species abbreviations are given in Table 1. Gasteropod specie abbreviations: Bglab, *Biomphalaria glabrata*; Has, *Haliotis asinina*; Hdish, *H. discus hannai*; Htub, *H. tubulata*. GenBank accession numbers are given in brackets.

stressors and confer tolerance against thermal stress (Berger and Young, 2006). Differently, *Bathymodiolus azoricus* inhabits the harsh hydrothermal vent environment, being thus exposed to elevated temperature and pressure, heavy metals, sulphide and radionuclides. The expression patterns of the HSP70 levels in *B. azoricus* show tissue-specific and cellular localization differences. A constitutive Hsp70 was detected in mantle and in gills. Two additional HSP70 isoforms were found in this latter tissue, one of which was stress-inducible, pointing to a crucial role played by these stress proteins throughout the life of the vent mussel in its naturally hostile environment (Pruski and Dixon, 2007).

Laboratory observations

All bivalves studied to date showed HSP70 overexpression in response to thermal stress, whether studied in the natural environment or in laboratory conditions. Within the wide range of Ostreidae distribution, water temperature may occasionally exceed 40 °C, evoking a strong synthesis of inducible HSP, as demonstrated in haemocytes of *C. virginica* (Tirard *et al*., 1995). Gills of *C. gigas* acclimated at 12 °C and exposed to 37 °C for 1 h showed an increase in the constitutive isoforms Hsp72 and 77, together with the induction of a newly synthetized 69 kDa isoform (Clegg *et al*., 1998).

In *O. edulis* we recognized two HSP isoforms of about 72 and 77 kDa in both gills and mantle of animals maintained in control conditions, while expression of a 69 kDa isoform increased after 1 h of exposure to heat shock at different temperatures. The Hsp69 protein was newly induced after exposure at temperatures ≥32 °C. The maximum expression measured after 3 h of post-stress recovery was detected at 35 °C, while individuals exposed to 38 °C showed low, if any, expression of Hsp69 (Piano *et al*., 2002). Further experiments showed that in oysters exposed for 1 h to 38 °C the *Hsp69* mRNA transcription actually was performed, but after a striking delay, as it was significant after 24 h of post stress recovery (Piano *et al*., 2004). We argued that the high temperature partially compromises the biochemical machinery at the basis of the HSP response, and that this delayed response may be related to the high mortality of *O. edulis* observed at this temperature (Piano *et al*., 2002). The maximum expression of Hsp69 caused by 1 h of heat shock at 35 °C was developed after 24 and 48 h of post-stress recovery in the gills and mantle, respectively, and the protein was still clearly detectable in both tissues 7 days after the heat challenge, persisting in the gills for up to 14 days. High levels of HSP70 were also expressed in tissues of *C. gigas* for up to 14 days after HS, during which period the animals were able to tolerate an otherwise lethal heat shock (Clegg *et al*., 1998). Expression of Hsp72 and Hsp77 in the gills and mantle of *O. edulis* remained easily detectable at the end of the 14 day period. This confirms the constitutive role of these proteins. The Hsp69 expression was never detected in the digestive glands of oysters exposed to thermal stress.

The expression of Hsp69 in gills and mantle of *C. gigas* was induced at temperatures ≥38 °C, which interestingly corresponded to the apparent half-lethal temperature stated for *O. edulis,* and densitometric analysis indicated that the maximum was reached at 40 °C in both tissues. In agreement with previous reports (Clegg *et al*., 1998), 44 °C was found to be the minimum lethal temperature for *C. gigas*.

According to the models for the transcriptional activation of *HSP* genes, denatured proteins are the trigger for the enhancement of HSP synthesis, and it has been suggested that induction of the HSP response mirrors the thermal stability of cell proteins (Dietz and Somero, 1992). In this context, the biochemical machinery of *O. edulis* might have a higher susceptibility to heat than that of *C. gigas*. An alternative suggestion is that heat directly activates a single transcriptional factor, HSF1, which trimerizes and binds to specific regions in the promoter of *HSP* genes (Zhong *et al*., 1998). In this case, the hypothesis is that the two oysters might differ as to the thermal sensitivity of the promoter or the HSF. As a matter of fact, *C. gigas* is more resistant to stress stimuli than are other oyster species (Tirard *et al*., 1995), and one could speculate that a higher threshold of stress sensitivity contributes to the ability of *C. gigas* to colonize new habitats in competition with autochthonous species.

The expression of newly synthesised HSP70 isoforms, like the Hsp69 in oysters, is not a common feature in bivalves exposed to thermal stress. The clams *Tapes philippinarum* and *Scapharca inaequivalvis* exposed to thermal stress for 1 h in the range of 30 - 40 °C did not show the expression of merely inducible isoforms, although HSP proteins already present in control conditions were significantly over-expressed by heat (Piano *et al*., 2004). Moreover, reports from different laboratories on different species indicate that also mussels exposed to thermal stress display a strong overexpression of apparently two HSP70 isoforms already present in control conditions without the synthesis of new isoforms (Hofmann and Somero, 1995, 1996; Piano *et al*., 2004). However, due to a better resolution or more probably to the different specificity of the antibody used, Snyder *et al*. (2001) reported three distinct HSP70 bands of 67, 70, and 74 kDa. in digestive glands of the mussel *M. galloprovincialis.* The Hsp67 was poorly expressed and not over-expressed by heat. After animal exposure to 28 °C for 1 h, Hsp70 and Hsp74 declined within 2 h, whereas they increased after 15 h of post stress recovery at 15 °C. This feature may explain why no increase of HSP70 was shown in the digestive glands of mussels exposed to heat shock and allowed to recover for up to 6 h (Piano *et al*., 2004). Table 2 summarizes the patterns of HSP70 bands detected by western blotting in several stress conditions, analysed in different organisms and tissues through different antibodies. The occurrence of stress-inducible *HSP70* mRNA or the related proteins under unstressed conditions is consistent with the ability of mussels and some clams to thrive in transitional environments, where significant fluctuations in physical and chemical parameters may

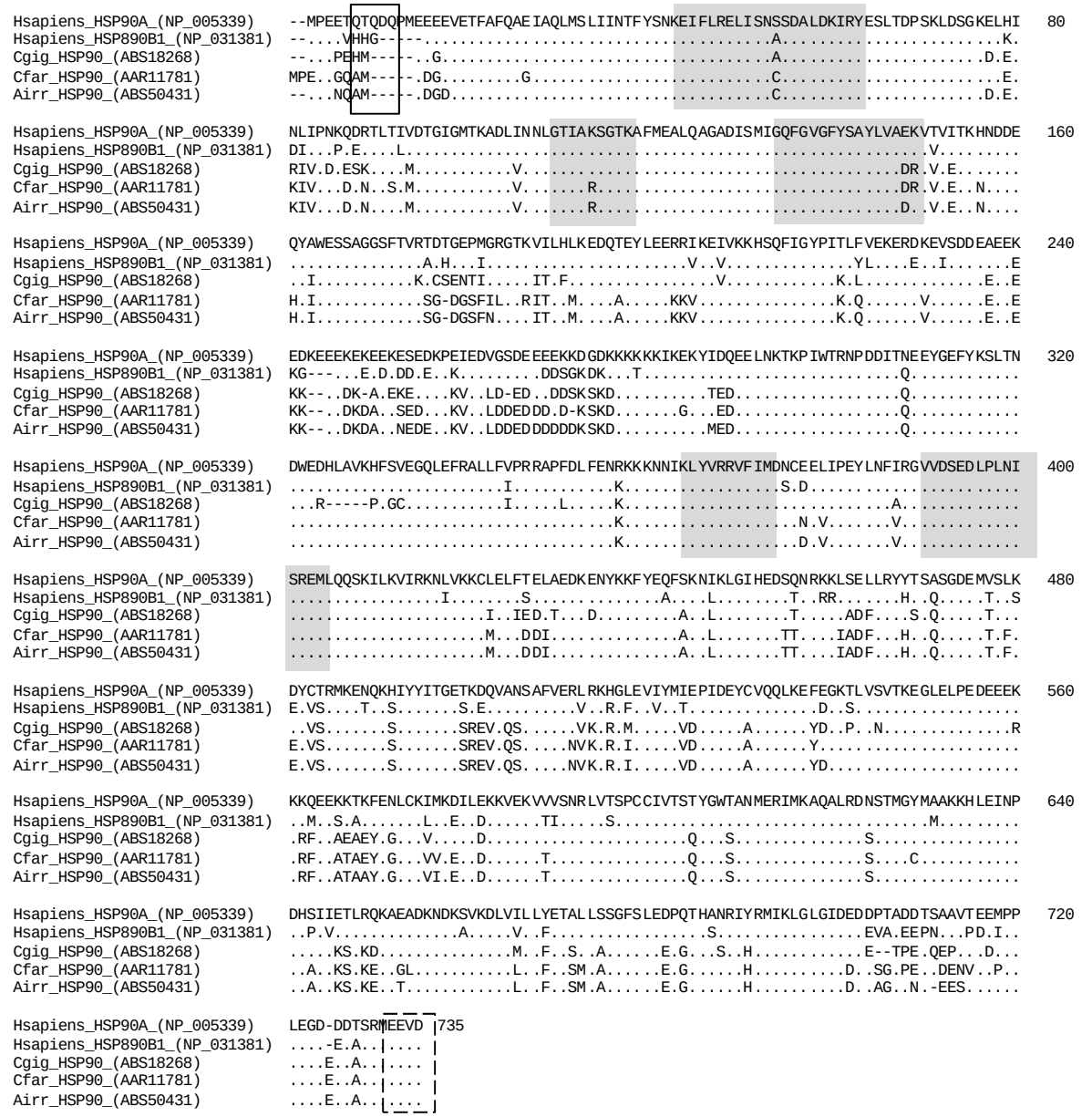

Fig. 5 Multiple alignment of deduced amino acid sequences of bivalve HSP90 with the human homologues (HSP90α and HSP90β) showing the position of conserved domains. The HSP90 sequences available for the bivalves were from *C. gigas* (Cgig), *C. farreri* (Cfar) and *A. irradians* (Airr). GenBank accession numbers are given in brackets. Identical amino acid residues are indicated by dots. Gaps (indicated by dashes) were added to improve the alignment. A solid-line square indicates the glutamine-rich QTQDQ consensus sequence; a grey square indicates the HSP90 family signatures. A dashed-line square indicates the C-terminal localization signal. The alignment was performed with the MEGA4 software (www.megasoftware.net).

occur. To minimize the effects of these environmental stressors, these animals may elaborate a molecular strategy where inducible HSP70 isoforms are physiologically expressed at low levels, their synthesis promptly increasing as the animal experiences adverse environmental change. We believe that the relatively rapid induction of the heat shock response by means of the inducible *HSP70* gene product is another component of the molecular adaptation of mussels to transitional environments, providing an effective tool to cope with rapidly changing environmental conditions. This hypothesis is corroborated by the occurrence of analogous mechanisms in several mussel species (Minier *et al*., 2000; Buckley *et al.,* 2001) and aquatic vertebrates exposed to fluctuating environments, including the teleosts *Fundulus heteroclitus* (Koban *et al*., 1991) and *Sparus sarba* (Deane and Woo, 2005).

Further and more detailed observations have recently been made of the HSP response in different tissues of animals exposed to thermal stress. In the

Table 2 An overview of HSP70 detected in different bivalve species by western blotting

Species	Tissue	HSP70	Primary Antibody	Immunogen	Reference
M. galloprovincialis	M, PAM	2 bands	mouse mAb clone BRM-22 (H-5147) Sigma-Aldrich	bovine brain HSP70	(Anestis *et al.,* 2007)
M. trossulus	G	2 bands	rat mAb clone 7.10 (MA3-001) Affinity Bioreagents	human HSP70 (437-479 AA)	(Buckley *et al.,* 2001)
M. edulis	G, M	3 bands	mouse mAb clone 5A5 (MA3-007) Affinity Bioreagents	human HSP70 (122-264 AA)	(Chapple *et al*., 1997, 1998)
	PAM	2 bands			
P. perna	G	2 bands	rabbit pAb (SPA-811) Stressgen	human HSP70	(Franco *et al.,* 2006)
M. galloprovincialis	F	No bands	mouse mAb clone N27F3-4 Calbiochem	human HSP70/HSC70 (from HeLa cells)	(Gonzalez-Riopedre *et al.,* 2007)
	G	1 band			
	H	2 bands			
	PAM	1 band			
	M	1 band			
M. galloprovincialis	G	2 bands	mouse mAb clone BRM-22 (H-5147) Sigma-Aldrich	bovine brain HSP70	(Hamer *et al.,* 2004)
M. trossulus	G	Up to 4 bands	rat mAb (clone 7.10) Dr Susan Lindquist	HSP70/HSC70	(Hofmann and Somero, 1995)
M. trossulus	G	Up to 3 bands	rat mAb clone 7.10 (MA3-001) Affinity Bioreagents	human HSP70 (437-479 AA)	(Hofmann and Somero, 1996)
M. galloprovincialis	G	Up to 3 bands			
M. galloprovincialis	G, M	1 band	rabbit pAb Cell Signalling Technology	human HSP70	(Kefaloyianni *et al.,* 2005)
M. galloprovincialis	H	1 band	goat pAb SantaCruz	human HSP70 (C-terminus)	(Malagoli *et al.* 2004, 2006)
M. galloprovincialis	DG	2 bands	mouse mAb clone BRM-22 (H-5147) Sigma-Aldrich	bovine brain HSP70	(Minier *et al.,* 2000)
M. edulis	G, M	2 bands	rabbit pAb (SPA-812) Stressgen	human HSP70 (HSP72)	(Pruski and Dixon, 2007)
B. azoricus	G	3 bands (1 merely inducible)			
	M	1 band			
M. edulis	G	1 band	mouse mAb clone 3A3 (MA3-006) Affinity Bioreagents	recombinant human HSP70 (over expressed in *E. coli.*)	(Radlowska and Pempkowiak, 2002)
M. californianus	G	Up to 4 bands (1 merely inducible)	rat mAb (clone 7.10) Dr. Susan Lindquist	HSP70/HSC70	(Roberts *et al.,* 1997)
M. edulis	G	3 bands (2 merely inducibles)	rabbit pAb (S. Ullrich, according to Ehrhart *et al.,* 1988)	human HSP70 (23 AA at the C-terminus)	(Sanders and Martin, 1993; Sanders *et al.,* 1994)
	M	1 band			
D. polymorpha	Soft tissues	2 bands	mouse mAb clone BRM-22 (H-5147) Sigma-Aldrich	bovine brain HSP70	(Singer *et al*., 2005)
M. edulis	G	3 bands (1 merely inducible)	mouse mAb clone 5A5 (MA3-007) Affinity Bioreagents	human HSP70 (122-264 AA)	(Smerdon *et al.,* 1995)
M. galloprovincialis	DG	3 bands	mouse mAb (SPA-822) Stressgen	chicken hsp70/hsp90 complex	(Snyder *et al.,* 2001)
M. galloprovincialis	M	3 bands (1 merely inducible)	mouse mAb clone BRM-22 (H-5147) Sigma-Aldrich	bovine brain HSP70	(Toyohara *et al.,* 2005)
C. gigas	G	3 bands (1 merely inducible)	rat mAb clone 7.10 (MA3-001) Affinity Bioreagents	human HSP70 (437-479 AA)	(Clegg *et al.,* 1998)
C. gigas	H	2 bands (1 merely inducible)	mouse mAb clone 3A3 (MA3-006) Affinity Bioreagents	recombinant human HSP70 (over expressed in *E. coli.*)	(Lacoste *et al.,* 2001a)
C. gigas	G	3 bands (1 merely inducible)	rat mAb clone 7.10 (MA3-001) Affinity Bioreagents	human HSP70 (437-479 AA)	(Hamdoun *et al*., 2003)
O. edulis	DG	2 bands	rat mAb clone 7.10 (MA3-001) Affinity Bioreagents	human HSP70 (437-479 AA)	(Piano *et al.,* 2002, 2004)
	G, M	3 bands (1 merely inducible)			
C. gigas	G	3 bands (1 merely inducible)			
C. virginica	H	2 bands	mouse mAb clone 3A3 (MA3-006) Affinity Bioreagents	recombinant human HSP70 (over expressed in *E. coli.*)	(Tirard *et al.,* 1995)
R. decussatus	DG, G, M	1 band	rabbit pAb (386035) Calbiochem	Recombinant human Hsp70 (HSP72)	(Dowling *et al.,* 2006)
S. inaequivalvis	G	1 band	rat mAb clone 7.10 (MA3-001) Affinity Bioreagents	human HSP70 (437-479 AA)	(Piano *et al.,* 2004)
T. philippinarum	G	1 band			

Key abbreviations: DG, digestive gland; F, foot muscle; G, gills; H, hemocytes; M, mantle; PAM, posterior adductor muscle

mussel *M. galloprovincialis,* all the tissues examined developed a different response to heat shock. Considering only the results obtained by Western blotting, since these were more comparable to other studies where mammalian anti-HSP antibodies were used, Gonzalez-Riopedre *et al*. (2007) observed that mussels acclimated at 20 °C displayed different proteins in different tissues: Hsp70 was detected in the mantle, in the adductor posterior muscle and in the hemocytes; Hsp60 was present only in the mantle and in the gills. The posterior muscle displayed only Hsp70, while neither Hsp70 nor Hsp60 was present in basal conditions in the foot muscle. However, Hsp70 was no longer detected in the mantle from individuals exposed for 5 or 45 min to 45 °C, while levels of Hsp60 remained stable. In the adductor posterior muscle Hsp70 was overexpressed after 5 min, and was not detected after 45 min of heat shock at 45 °C. In the foot muscle, neither Hsp70 nor Hsp60 levels were expressed after heat shock. Hsp70 was never detected in the gills, while Hsp60 was expressed in a significant manner. Interestingly, both Hsp70 and Hsp60 were present in haemocytes withdrawn from stressed animals, although apparently only Hsp60 expression was affected by heat; in contrast, haemocytes withdrawn from unstressed animals and cultured for three days at 20 °C did not show basal levels of either Hsp70 or Hsp60. The two protein isoforms remained absent after 5 min exposure, whereas they were detected after 45 min exposure at 45 °C. A different HSP response in the different tissues was highlighted by different laboratories; for example, we observed (see above) that oyster mantle and gills developed a strong HSP response to thermal stress, while no effect was seen in the digestive glands (Piano *et al*., 2004). However, if we compare the above results with previous findings on mussels, some main discrepancies appear, in particular regarding the Hsp70 expression in gills. In fact, Hsp70 constitutive expression has been documented in gills of all mussel species studied so far (e.g. Hofmann, 1999; Piano *et al*., 2004), significantly increasing after heat shock.

The disappearance of both Hsp70 and Hsp60 isoforms in the mantle of individuals exposed to 45 °C for 5 or 45 min does not fully agree with previous data either. Although such factors as seasonality, feeding, reproductive states etc. may interfere with the protein expression, in the light of previous observations it is worth noting that Gonzales-Riopedre *et al*. (2007) assessed the HSP response immediately after heat shock, which lasted 5 or 45 min. In other laboratory experiments, a period of post-stress recovery was adopted to let the organisms develop the response (Piano *et al*., 2002, 2004), or the exposure period lasted longer. Some hours of treatment and/or of post-stress recovery had in fact been considered necessary in previous experiments to allow the response to develop completely (e.g., Hofmann and Somero, 1995; Piano *et al*., 2002; Franzellitti and Fabbri, 2005). The delayed protein expression is also supported by data on gene expression, which show that mRNA transcripts reach maximum levels after 3 h of post-stress recovery (Franzellitti and Fabbri, 2005), declining thereafter. Similar observations were made on other bivalves, including oysters and clams (Piano *et al*., 2004; Park *et al*., 2007).

Interestingly in this connection is also the transient reduction of *HSP70* transcript levels observed at 1 h of recovery in *M. galloprovincialis* (Franzellitti and Fabbri, 2005) as well as in *O. edulis* exposed to 35 °C for 1 h (Piano *et al*., 2004). This feature is consistent with the peculiar regulation of RNA metabolism during the heat shock response (Yost *et al.*, 1990) and with the onset of several and probably time-delayed mechanisms underlying *HSP70* gene expression control (De Maio, 1999). In general, the different responses obtained in the same mussel tissues may be partly attributable to the different times allowed for the HSP response to develop. The disappearance of HSP after 45 min exposure to 45 °C appears to relate to the phenomenon observed in oysters (Piano *et al*., 2002), where the animals' exposure to 38 °C, a temperature lethal for most organisms, led to a strong reduction of Hsp70 expression levels. Concurrently, the mRNA transcripts were absent and were once more expressed only after 24 h after the heat shock (Piano *et al*., 2004).

Mussel hemocyte cells withdrawn from stressed mussels displayed basal levels of HSP and a significant HSP response to heat (Gonzales-Riopedre *et al.,* 2007), apparently involving different protein isoforms. Only one study focused on hemocytes withdrawn from control mussels, cultured for three days at 20 °C and subsequently exposed to thermal stress (Gonzales-Riopedre *et al.,* 2007). While these hemocytes did not display Hsp60 or Hsp70 at 20 °C or after 5 min of exposure at 45 °C, protein bands were detected after 45 min exposure at 45 °C. On the basis of this single report, we can speculate that cultured cells lose the physiological modulation performed by neuroendocrine factors, which may influence the HSP pattern both in basal as well as in stressed conditions (Lacoste *et al*., 2001a), thus leading to substantial differences between the two cell populations.

In general, in line with what is reported by Hofmann (1999), new evidence confirms that HSP overexpression is a ubiquitous molecular mechanism for coping with stress, but animals show individual responses with different thresholds of sensitivity and tissue specificity. Some of these differences have been analysed in the same individuals exposed to different conditions, and account for physiological differences in thermal stress response.

The bivalve HSP response to non-thermal stress

Although the term HSP specifically refers to heat shock, the accumulation of these proteins is not increased only by heat. A large body of evidence indicates that there is a variety of stimuli that result in an increase in their concentrations. The question arises of how different harmful stimuli can provoke such a similar effect. A first explanation was provided by Hightower (1991), who noticed that many factors responsible for the HSP response acted *in vitro* as protein denaturating agents, i.e.,

substances altering the tridimensional configuration of proteins, thus provoking the loss of biological functionality. Different chemical substances directly or indirectly affect cell proteins, e.g., by oxidation of thiol groups and disulfide-bonds, thus destabilizing the protein structure. This occurs for example in the case of heavy metals, free radicals, and pesticides (Feder and Hofmann, 1999; Gonzales-Riopedre *et al*., 2007; Farcy *et al*., 2007). Organic compounds including alcohols, phenols, and solvents in general can also affect protein integrity by interacting with the hydrophobic domains, normally located within the hydrophobic core (Ait-Aissa *et al*., 2000). Further evidence showed that radiation must be included in the list of HSP inducers (Malagoli *et al*., 2004). Because of the great diversity in toxic factors, it can be hypothesised that the HSP response is triggered by multiple mechanisms; however, proteotoxicity remains at the moment the sole common factor at the cellular level. We discuss here some of the numerous observations on HSP response induced by non-thermal factors in bivalves.

Effect of non essential metals

Of the substances able to induce HSP overexpression, heavy metals have attracted major attention. Chemicals may have toxic effects at the cell and tissue level and, above a certain threshold, also elicit an integrated stress response. Considerable experimental evidence obtained *in vitro* and *in vivo* testifies to the ability of cadmium to increase HSP levels in humans (Polla *et al*., 1995; Valbonesi *et al*., 2008), rats (Curtis *et al*., 1996), fish (Hansen *et al*., 2007), sponges (Schroder *et al*., 1999), *Drosophila* (Courgeon *et al*., 1984) etc. No role for Cd in the metabolism of living organisms is yet known, and the metal is extremely hazardous to animal life. Recent studies implicated Cd in the elevated metabolic demand and concomitant impairment of ATP production, reduced aerobic capacity and oxidative stress in oysters (Cherkasov *et al*., 2007; Ivanina *et al*., 2008a). The short term effect of Cd exposure induced a dose-dependent increase of metallothioneins, Hsp60, and Hsp70, but not Hsp90 synthesis, in *C. virginica* (Ivanina *et al*., 2008a). Within 4 h of exposure to 10 - 2000 µM Cd, a great difference was observed between the tissues analysed, with the HSP expression much greater in gills than in digestive glands. Interestingly, this was inversely related to the metallothionein induction, which was greater in oyster digestive glands than in gills (Ivanina *et al*., 2008a). When other evidence on the activation of antioxidant systems was also taken into account, it was concluded that the HSP response is a secondary line of cellular defence significantly activated in gills, when inducible metallothioneins and GSH appear to be insufficient to fully prevent damage due to Cd exposure (Ivanina *et al*., 2008a). However, longer animal exposures to 4 µM Cd indicated that metallothioneins (at 3, 7 and 15 days) and HSP70 (at 3 and 15 days) can be simultaneously immunolocalized in both gills and digestive glands of *C. gigas* (Moraga *et al.*, 2005). In agreement with previous reports (Boutet *et al*., 2003b) it emerged clearly that HSP70 levels decreased at 7 days. Several hypotheses have been made regarding the inhibition of HSP expression by metals or degradation temporarily exceeding the synthesis rate. Further considerations emerging from data on constitutive and inducible *HSP70* gene expression in mussels exposed to Hg^{2+} or Cr^{6+} (Franzellitti and Fabbri, 2005) will be discussed below. Cd also induced HSP70 overexpression in *O. edulis* exposed to the metal (100 - 500 µg/l) for 7 days (Piano *et al*., 2004). At these experimental conditions, Cd elicited significant increases of both metallothioneins and HSP70 in gills and digestive glands. The response to heat shock in oysters (Clegg *et al*., 1998; Piano *et al*., 2002) led to a strong induction of Hsp69. Interestingly, Cd induced the expression of Hsp69 in gills and also in digestive glands of *O. edulis* where Hsp69 was not induced by heat (Piano *et al*., 2004). This would suggest that the *Hsp69* mRNA transcription is differently regulated by the two stress factors at least in digestive glands.

Cd affects the expression of HSP90, as was well demonstrated in *C. gigas* (Choi *et al*., 2008). *HSP90* mRNA levels increased dose- and time-dependently, up to a 40 fold expression, after 7 days of treatment at 0.1 ppm Cd. Expression was significantly reduced after 11 days of exposure, probably due to a decrease in metabolic capacity of the organisms or to oxidative stress generated by prolonged exposure to Cd. The response was similar in gills and digestive glands, and indicated that, in bivalves, HSP90 may be induced to maintain homeostasis and protect the cells against xenobiotics. In general few studies are available on the HSP90 response to chemical stressors, and point to a substantial similarity to the HSP70 response.

Studies carried out at the gene expression level made it possible to clearly distinguish between differently time-modulated HSP responses to heavy metals, and also to establish a differential HSP70 expression in bivalves exposed to different stress stimuli (Franzellitti and Fabbri, 2005, 2006). Time course experiments showed that the inducible *Hsp70* and the constitutive *Hsc70* gene expressions were differently modulated during exposure to Hg^{2+}. The abundance of *Hsp70* transcript increased during the early response to Hg^{2+} (8 - 24 h) and the basal level was recovered within 6 days, while *Hsc70* expression showed a biphasic response with a reduction at 1 day and an induction at 6 days of treatment. This pattern may be partially related to the peculiar structural, functional, and regulatory features of heat shock genes. In fact, in accordance with their role as stress-responsive genes, *HSP70* genes are typically intron-less (Gunther and Walter, 1994), while the HSC70 coding region is interrupted by several introns. Nevertheless, we also hypothesize that differential expression of the two genes is related to a specific mechanism of short- and long-term cell protection.

The peculiar *HSP70/HSC70* expression profile after prolonged Hg^{2+} exposure was confirmed by data from Cr^{6+} exposure. After a 1 week treatment, induction of *Hsc70* and inhibition of *Hsp70* expression were observed. In more detail, the *MgHsp70* expression increased immediately after a 1 h heat shock or after an 8 h heavy metal

exposure, while *Hsc70* was either unmodified or inhibited. *Hsc70* induction occurred only after a longer-term exposure to Hg^{2+}or Cr^{6+}, at a time when *Hsp70* expression had returned to basal levels.

Franzellitti and Fabbri (2006) also showed that the chemical form of the contamination could dramatically affect the toxic potential of metals and, as a consequence, the magnitude of the cellular response to its exposure. CH_3Hg^+, probably by virtue of its higher hydrophobicity, was more effective in evoking a cytoprotective response than Hg^{2+}. In particular, the response to CH_3Hg^+ exposure was achieved through the strong, stable up-regulation of the constitutive transcript *Hsc70*, while the inducible counterpart *Hsp70* was progressively down-regulated. This is in contrast with the expression profiles following Hg^{2+} exposure described above, and suggests that the higher toxicity of CH_3Hg^+ requires the onset of the chronic, rather than the acute, response pathway.

A low density microarray approach was applied to evaluate alterations of gene expression profiles after mussel exposure to Hg^{2+} (Dondero *et al*., 2006). The set of genes included *Hsp27* and *Hsp70*. The expression of none of them was modified after *M. galloprovincialis* exposure to 750 nM Hg^{2+} for 6 days, although an alteration in *Hsp*27 expression was expected on the basis of mammalian responses (Lavoie *et al*., 1995). As to *HSP70*, the oligoprobes were designed in a region of high homology amongst inducible and constitutive *HSP70*, so that the microarray analysis could not discriminate between the expression of the two genes. These data might reflect the lack of inducible *Hsp70* overexpression at 6 days of exposure to Hg^{2+} observed in *M. galloprovincialis* by Franzellitti and Fabbri (2005); the sum of *Hsp70* and *Hsc70* at this stage brought about a protein expression level similar to control values.

Platinum group elements (PGE) have been released in the last few decades mainly as a consequence of anthropogenic activities. Major sources are hospital releases, because of Pt-based anticancer drugs, and overall car emissions due to the use of PGE in catalytic converters. Soluble and particle-bound PGE are biologically available to living organisms (Ravindra *et al*., 2004). The few data available on the ability of PGE to induce HSP overexpression (Singer *et al*., 2005) are nevertheless interesting. Zebra mussels, *Dreissena polimorpha,* exposed for up to 10 weeks to 500 μg/L Rh, Pd and Pt, bioaccumulated the metals and showed a significant overexpression of 70 kDa proteins. A 19-fold increase compared to the control levels was obtained with Pt and Rh, and a 25-fold increase with Pd. In parallel experiments mussels were exposed to Cd or Pb, and an increase in Hsp70 expression of about 6 and 12 fold respectively was obtained. Although further data on PGE are not available, the clear induction of Hsp70 expression suggests that these metals produced strong proteotoxic effects.

Effect of essential metals

Some data are available on the HSP response after bivalve exposure to essential metals, namely copper and zinc. It is well-known that prolonged exposure to Cu or Zn produces toxic effects and decreases animal fecundity, hatchability and reproduction (Lock and Janssen, 2003), although these metals have a physiological role (Madsen and Gitlin, 2007). *M. edulis* exposed for 7 days to increasing Cu concentrations (30-100 μg/l) showed an increased expression of Hsp60 and Hsp70 in the mantle, and to a greater extent in the gills (Sanders *et al*., 1994). In particular, the highest Cu concentration induced the *ex novo* synthesis of two protein isoforms, with the appearance of three protein bands in the related immunoblotting. Radlowska and Pempkowiak (2002), evaluated on the same organisms the effects of Cd and Cu administered alone or in combination resembling the most common condition in nature. These experiments revealed that mixtures had a synergistic effect on Hsp70 expression with respect to the single contaminants. Moraga *et al*. (2005) demonstrated the greater effect of a combination of Cu and Cd on *C. gigas* Hsp70 immunolabelling. In contrast, the combination of Cd and Zn induced a significant increase of Hsp70 expression in gills and digestive glands of *O. edulis*, an increase which, however, was smaller than the effect of Cd alone used at the same concentration (Piano *et al*., 2004). Zn *per se* did not change the Hsp70 expression. The lower effect of the combination could be ascribed to a lower water filtering rate by the animal exposed to high levels of contaminants, or to competition between the two metals for cellular uptake mechanisms. There are examples of the reduction of Cd assimilation and uptake after *Perna viridis* preexposure to Zn (Blackmore and Wang, 2002). The absence of any effect of Zn on Hsp70 expression has also recently been confirmed in tissues of brown mussels, *Perna perna* (Franco *et al*., 2006). The different parameters analysed clearly indicated that animals exposed to 10, 30 and 100 μM Zn for 48 h were subjected to oxidative stress; in this context, Hsp60 proteins were significantly over-expressed while Hsp70 levels remained unchanged (Franco *et al*., 2006). The overall responses to the essential metals Cu and Zn show once again that different species respond differently and that often mixtures of metals induce synergistic effects. Although the small number of reports does not allow a more detailed discussion, we may posit that other cytoprotective responses are elicited in cells by excesses of Cu and Zn, namely metallothioneins and antioxidant enzymes. Therefore, the different extent of the HSP response may be related to the different induction of the other cytoprotective responses activated.

Effect of organic compounds

Besides heavy metals, other environmental contaminants were examined for their ability to stimulate the HSP response in bivalves. The mussel *M. galloprovincialis* exposed to xenobiotics (phenobarbital, heptaclor and pentachlorophenol) or to a mixture of hydrocarbon degradation products showed increases in HSP expression, although to different extents (Snyder *et al*., 2001). In mussel digestive glands a dose- and time-dependent increase of Hsp67, Hsp70 and Hsp74 isoforms was observed after exposure to degraded oil. *C. gigas*

were exposed to the same mixture of hydrocarbons reported by Snyder (2001), and significant increases in both mRNA and protein levels were observed after 7 days of exposure, reaching a maximum at 15 days, and declining thereafter (Boutet *et al.*, 2004). The effect of oil mixtures on mussel gene expression was recently assessed (Dondero *et al*., 2006). Application of a low-density microarray produced the observation that 8 days of exposure to 0.5 ppm of a crude oil mixture (North Sea Oil) caused a negative modulation of several gene expressions in mussel digestive glands, including that of *HSP70* genes. As only a few isolated studies have been performed on the effects of hydrocarbons on HSP response, with different concentrations and times of exposure, we are not yet ready to draw a picture of the cytoprotective role played by HSP towards these compounds.

Chlorinated organic compounds are lipophilic chemicals widespread in the environment, the best-known of them being DDT. Although banned they are highly persistent, and are therefore still responsible for inducing clear endocrine disruptive effects (Binelli *et al*., 2004). The DDT metabolite DDE (*p,p'*-dichlorodipehnyldichloroethylene) was assessed for its potential effects on stress response and protein carbonylation in the clam *R. decussatus* (Dowling *et al*., 2006). Hsp60 and Hsp70 isoforms were present in gills, mantle and digestive glands of control animals, while Hsp90 was constitutively present only in gills. Neither Hsp60 nor Hsp70 proteins were over-expressed by DDE exposure in gills and mantle. Similarly, DDE did not cause Hsp90 overexpression in gills. Instead, a newly synthesised band of 90 kDa appeared in the mantle. Carbonylation in response to ROS generation after DDE exposure was evident in digestive glands and mantle, while not observed in gills. Thus the lack of HSP overexpression in gills was well related with the lack of a DDE effect on the proteome. The strong induction of Hsp90 in the mantle may reflect the need for cytoprotective proteins in this tissue where ROS were mainly generated. Not in line with these results is the lack of induction or overexpression of HSP in digestive glands. This reflects once more the tissue-specific nature of HSP expression in bivalves often highlighted in this review.

Effect of hypoxia

Quantification of HSP70 revealed an increased protein expression in tissues of *C. gigas* exposed to hypoxia from 0 to 21 days; instead, HSP70 transcription was up regulated after 17 days, but down regulated after 21 days of exposure (David *et al.*, 2005). These inconsistent results let to the conclusion that the HSP response to hypoxia in oysters remains relatively unknown. Clams were vulnerable to moderate hypoxia which caused high mortality (Joyner-Matos *et al*., 2006). However, the stimulus did not trigger any change in sHSP, HSP60 or HSP70.

The response elaborated by clams towards hypoxia or hyperoxia was influenced by seasonality. Exposure to the stressors for more than 24 h was lethal. Clams collected in spring showed an increased stress protein response, decreased levels of lipid peroxidation and increased survival compared to those collected in fall (Joyner-Matos *et al*., 2006). A number of factors besides temperature vary between seasons, including availability of nutrients, reproductive status, and growth cycle. We must consider the possibility that all of them may influence the animal's response to stress factors.

Effect of electromagnetic fields

Low frequency electromagnetic fields (EMF) may affect several physiological functions in cells and tissues (Funk and Monsees, 2006), and elicit the HSP response. According to Goodman and Blank (1998) the control of Hsp70 induction by low frequency electromagnetic fields takes place at the level of gene transcription and is mediated at the level of two peculiar nucleotide sequences (nCTCTn) located on the Hsp70 promoter gene. Haemocytes from mussels exposed for 30 min at fifty hertz EMF at 300 or 400 μT intensity showed a delayed response to chemotactic factors, and at 600 μT their motility was suppressed (Malagoli *et al*., 2003). The different physiological alterations concomitant with this effect included the HSP response, elicited in hemocytes of mussels exposed to EMF (Malagoli *et al*., 2004). While at 300 μT no modification of the HSP pattern was observed, at 400 μT and 600 μT overexpression of both Hsp70 and Hsp90 was elicited, and increased with multiple exposure and with the duration of exposure. The triggering of HSP expression indicates that EMF represent a physical stressor to which the mussel reacts. Given the few studies carried out on this topic, we are far from understanding the action mechanisms through which EMF may induce the HSP response in bivalves. Further evidence of EMF effects on different invertebrate parameters may provide useful clues for understanding the phenomenon in more complex organisms. At present little is known about the action mechanism of EMF in mammals either, and it is the object of major debate.

Effect of pathogens

Various pathogens induce HSP overexpression in infected tissues. Examples are the increase in Hsp70 levels in human macrophages exposed to *Staphylococcus aureus*, erhytrocytes infected by *Escherichia coli* and glial cells attacked by mycobacteria (Kantengwa and Polla, 1993), and Hsp60 increases in neutrophils infected by *S. aureus* (Zheng *et al*., 2004). *C. virginica* infected by *Perkinsus marinus* showed the overexpression of Hsp69 (Encomio and Chu, 2007). Oysters pre-exposed to a sublethal thermal stress (40 °C for 1 h) and subsequently challenged with *P. marinus* improved their survival, indicating that HSP overexpression by heat provides tolerance to a further stress stimulus, namely *P. marinus*.

Stress factor interactions

A large body of evidence indicates that a mild heat shock provides greater resistance to hyperthermia, anoxia, heavy metals, hydrogen peroxide, etc (De Maio, 1999). Oyster specimens (*C. gigas*) cultivated at 12 °C did not survive 1 h exposure to 44 °C, which is therefore the lethal temperature for these organisms (Clegg *et al*.,

1998). However, *C. gigas* exposed for 1 h to 37 °C survived the subsequent treatment of 1 h to 44 °C to which they were subjected one week later. Such a tolerance phenomenon is present in primitive organisms such as bacteria through more complex organisms up to mammals, leading to the hypothesis that it is indeed a primitive mechanism of cellular defence. A mild heat shock protects cell processes such as transcription, splicing, trafficking etc. from further exposure to different type of stress (Yost *et al*., 1990; Parsell and Lindquist, 1993; De Maio, 1995) probably because the HSP levels are already higher, so that the defence mechanisms can be activated faster.

Unfortunately this issue has not received much attention in the latest studies on bivalves, and a comparison amongst species or stressors is difficult to perform. Consistently, the molecular mechanisms at its basis are far from being understood.

HSP response and cell signaling

Interestingly, and in a manner related to the above topics, a difference was observed between the temperature inducing an increase in protein levels and the temperature at which gene expression is triggered. While the threshold temperature for Hsp70 induction varied according to the thermal history of two groups of *M. trossulus*, there were no variations between the endogenous levels of the constitutive Hsp70 isoform and of HSF1. Moreover, the activation temperature of HSF1 found in *M. trossulus* was not identical to the threshold temperature for Hsp70 synthesis in the congeneric *M. californianus*. As described above, transactivation of heat shock genes is mediated by the interaction of HSF1 and HSE (Wu, 1995). HSF1 is normally located in the cytosol, linked to Hsp70/90 and other proteins; it is released by Hsp70 in response to stress and translocates into the nucleus. It appears that HSF1 is released by Hsp70 in response to small increases in temperature, and remains presumably inactive on the promoter until a higher temperature is reached. Consistently, a quantitative change in levels of HSF1 is not necessary to tune the heat-shock response during acclimatization. Rather, the controlling steps underlying the "adjustment of the thermostat" probably occur after HSF1 has bound the promoter. These might involve further cell proteins and also signalling pathways, including the mitogen-activated protein kinases (MAPK) signalling cascade which is responsible for HSF1 phosphorylation events preceding gene transactivation (Buckley *et al*., 2001). Different MAPK may be involved in this mechanism; in fact, a marked increase in the levels of the phosphorylated form of p38-MAPK and c-Jun N-terminal kinase (JNK, also known as Stress Activated Protein Kinase, SAPK) in tissues from *M. galloprovincialis* exposed to temperatures beyond 24 °C has been reported (Anestis *et al*., 2007). The increased phosphorylation of kinases paralleled increased HSP expression, strongly supporting involvement of MAPK signalling cascade in the induction of *HSP* genes in the tissues of *M. galloprovincialis* during thermal stress. In these experiments it was shown that *M. galloprovincialis* cannot survive sea water temperatures beyond 26 °C over extended periods of time, and that the mortality of mussels increased drastically during warming to 30 °C. Consistently with further evidence, it was established that the increase in the mortality of mussels during exposure to temperatures higher than 24 °C might be attributed to a reduced ability to assimilate food and associated energy, before the direct consequences due to heat. However, acclimation up to 28 °C induced overexpression of Hsp70 and Hsp90 and concomitant phosphorylation of SAPK and p38-MAPK. Although the cause-effect relationship between MAPK and HSP has not been proven in bivalves, the involvement of MAPK signalling in HSP expression may account for the regulation of HSP expression by integrated factors, besides protein damage. In agreement with the above evidence are also data reported by Malagoli *et al*. (2004), who observed the parallel induction of p38-MAPK and Hsp70/90 by electromagnetic fields. Moreover, in gills of *M. galloprovincialis*, stimuli that are known to trigger the HSP response also induce activation of MAPK pathway. Cu, Zn and Cd induced p38-MAPK activation with maximum levels reached within 1 h. Hypothermia (4 °C) induced a moderate kinase phosphorylation (maximised at 30·min), whereas hyperthermia (30 °C) induced rapid p38-MAPK phosphorylation that remained considerably above basal levels for at least 2 h (Kefaloyianni *et al*., 2005).

In fact, besides HSP overexpression, which takes place in a few hours, another evolutionarily conserved response to heat shock develops in minutes and leads to activation of the major signalling transduction pathways involving JNK and p38-MAPK (Dorion *et al.,* 1999). The mechanisms of activation and the roles of these pathways during heat shock have been the subject of several studies, with no clear conclusions (Dorion and Landry, 2002). Recent findings revealed that HSP in mammals can regulate both the signalling and the execution of major cell death pathways (reviewed in Jäättelä, 1999; Beere and Green, 2001). Consequently, HSP play a primary role in the resistance to a variety of toxic agents and situations that do not necessarily involve protein denaturation. Overexpression of Hsp70 can inhibit JNK activation by various stimuli, including heat shock, UV light, and H_2O_2 through a mechanism involving the direct binding of Hsp70 to JNK or an Hsp70-mediated protection of a JNK phosphatase from heat denaturation (Park *et al.,* 2001). The inhibition of JNK activity could therefore be a factor of acquired thermotolerance. In contrast, the activation of the p38-MAPK pathway leads to the phosphorylation of Hsp27 (Huot *et al.,* 1995), an event that is generally assumed to be protective. Phosphorylation of Hsp27 is catalyzed by MAPK-2, a serine-protein kinase itself activated by phosphorylation by p38-MAPK (Rouse *et al.,* 1994; Huot *et al.,* 1995).

This recent progress in the field of cellular stress underlines a new concept that cell response to stress stimuli mediated by HSP is not only the consequence of protein damage. It results from the integration of stress and evolutionarily conserved and interconnected physiological mechanisms, i.e., the activation of HSP response and of MAPK

cascades. In fact, HSP regulate MAPK activation and MAPK regulate HSP activation and activities in mammals (Dai *et al.,* 2000). Much remains to be studied in bivalves regarding this issue. Although not dealing with MAPK-HSP interaction, elucidation on the role of MAPK pathways in mussels was recently provided by Kefaloyianni *et al.* (2005), Anestis *et al.* (2007) and by the extensive work of Canesi *et al.* (2005, 2006a, 2006b).

HSP as molecular biomarkers

As detailed above, the induction of several HSP isoforms by environmental contaminants is widely documented. For a number of years HSP have been considered as potential biomarkers to be included in biomonitoring programmes (Nadeau *et al*., 2001; Snyder *et al*., 2001; Radlowska and Pempkowiak, 2002). The implication of these proteins within the main mechanisms of cellular protection would render them good markers of the stress status of an organism. The advantages are that they give information about general conditions of health and provide early warnings of intoxication, before complex functions are compromised. Although in some cases HSP may show a specific pattern of response to selected stimuli, they do not usually provide specific information on the type of stress factor present in the environment. However, this application of HSP requires a certain caution, and their use as biomarkers should be based on previous studies on the biochemical and physiological features of the analysed animal. In fact, HSP are differently expressed in different tissues of the same organism; they are also subject to seasonal and physiological variations e.g. related to temperature, oxygen availability and salinity (Minier *et al*., 2000, Bodin *et al.* 2004, Hamer *et al.* 2004). The main difficulty in using biomarkers in a monitoring program is the interference of natural environmental factors with the biological responses. Although the HSP response is one of the fastest and most sensitive, we know that it is also subject to seasonality.

These drawbacks have led to criticism in some reports of HSP being used as biomarkers (Pyza *et al*., 1997; Bierkens, 2000). We believe that one main obstacle to this use is the methodology needed to assess the HSP response, requiring the use of the Western blotting technique for protein assessment and of the PCR, qPCR or microarray for gene expression profiling. If we agree that biomarkers should be clear and repetitive responses measured through relatively simple and cheap methodologies (Viarengo *et al*., 2007), HSP cannot meet these requirements at the moment. Nevertheless, including HSP assessment in a battery of biomarkers can add useful information and sometimes point the way to further studies.

Future challenges

There was a perception that the major patterns of HSP expression in eukaryotes were becoming so obvious that additional descriptive work was difficult to justify (Feder and Hofmann, 1999). However, the rate at which new findings are being made is still increasing, and major advances have occurred in the last few years. Some major questions remain unanswered, while new aspects have emerged in mammals which have not yet been taken into consideration in bivalves.

Only one detailed study has been addressed to the evaluation of HSP in aging bivalves (Ivanina *et al*., 2008b). The issue deserves more attention, both as an approach to the physiology of aging in bivalves, and also because knowledge of ancestral mechanisms may provide clues for understanding the complex aging phenomenon in humans. The oyster *C. virginica* and the clam *Mercenaria mercenaria* were studied at different ages from 7 months to 4 years. Mitochondrial Hsp60 significantly decreased with age, suggesting an age-related decline in mitochondrial chaperone protection. However, the possibility that other protective mechanisms are increased by senescence in mitochondria was not evaluated. Different trends in the Hsp70 and Hsp90 expressions were developed by the two bivalves at different ages (Ivanina *et al.*, 2008b). Hsp90 levels in *C. virginica* increased progressively, while Hsp70 levels did not change. Hsp70 levels increased with age in *M. mercenaria,* while Hsp90 decreased. This non-uniformity also appeared in non-bivalve organisms from *Drosophila* to humans (Wheeler *et al*., 1995; Colotti *et al*., 2005), so that at present we cannot answer the question of whether chaperone systems compensate for aging-related proteotoxicity or not.

The relationship established in mammals and more recently in mussels (Kefaloyianni *et al*., 2005; Anestis *et al*., 2007) between the MAPK signalling cascade and the induction of *HSP* genes during thermal stress is rather intriguing, since it links the HSP response to intracellular signalling. Moreover, is well known that MAPK cascades are activated by stress factors and also by physiological factors, which could therefore modulate HSP expression. On the other hand, catecholamines induced *HSP* gene expression in bivalves (Lacoste *et al.*, 2001a). From this initial evidence it is clear that the HSP response must be regarded as an integrated phenomenon, with intracellular and systemic components.

Well-related to the above observations is the fact that extracellular HSP70 are emerging as important mediators of intercellular signalling and transport in mammals (Calderwood *et al*., 2007). The release of these proteins from cells is triggered by physical trauma, stress, and exposure to immunological stimuli. Stress protein release occurs both through physiological secretion mechanisms and during cell death by necrosis. After release into the extracellular fluid, HSP enter the bloodstream and possess the ability to act at distant sites in the body, then bind to the surfaces of adjacent cells and initiate signal transduction cascades as well as the transport of antigenic peptides (van Noort, 2008). Many of the effects of extracellular stress proteins are mediated through cell surface receptors located on neurons, immune cells, blood vessels, etc. To the best of our knowledge it appears that the phenomenon of HSP release to the extracellular environment has not yet been tackled in bivalves. Considering the roles played by bivalve haemocytes in stress response, immunity and metabolism,

investigations in this field are strongly recommended.

We are fully aware that the challenges we have chosen to outline here represent just a few of the many topics deserving of wider exploration. However, the emergence of major new roles for HSP points the way forward to a fruitful line of future study. From a broader perspective, apart from providing further basic information, we hope that the new synergistic approaches will help to elucidate the integration between the HSP response and cell signalling at the cellular level, as well as the role of HSP as a cellular component of the integrated stress response.

Acknowledgement

The Authors are grateful to Dr. A Piano, who set up the first studies on HSP in Prof. Fabbri's laboratory during her PhD, and allowed us to continue this work on solid basis of knowledge.

In this review we focused mainly on bivalve HSP. However, it was impossible to acknowledge all the work that has led to our current knowledge on the HSP response. We apologise for any omissions and invite readers to find further information in the excellent reviews and articles cited herein.

Reference

Ait-Aissa S, Porcher J, Arrigo A, Lambre C. Activation of the hsp70 promoter by environmental inorganic and organic chemicals: relationships with cytotoxicity and lipophilicity. Toxicology 145: 147-157, 2000

Anestis A, Lazou A, Portner HO, Michaelidis B. Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293: R911-R921, 2007.

Barral JM, Broadley SA, Schaffar G, Hartl FU. Roles of molecular chaperones in protein misfolding diseases. Semin. Cell Dev. Biol. 15: 17-29, 2004.

Beere HM, Green DR. Stress management - heat shock protein-70 and the regulation of apoptosis. Trends Cell Biol. 11: 6-10, 2001.

Benedict MQ, Levine BJ, Ke ZX, Cockburn AF, Seawright JA. Precise limitation of concerted evolution to ORFs in mosquito Hsp82 genes. Insect Mol. Biol. 5: 73-79, 1996.

Berger MS, Young CM. Physiological response of the cold-seep mussel *Bathymodiolus childressi* to acutely elevated temperature. Mar. Biol. 149: 1397-1402, 2006.

Bierkens JG. Applications and pitfalls of stress-proteins in biomonitoring. Toxicology 153: 61-72, 2000.

Binelli A, Bacchetta R, Mantecca P, Ricciardi F, Provini A, Vailati G. DDT in zebra mussels from Lake Maggiore (N. Italy): level of contamination and endocrine disruptions. Aquat. Toxicol. 69: 175-188, 2004.

Blackmore G, Wang WX. Uptake and efflux of Cd and Zn by the green mussel *Perna viridis* after metal preexposure. Environ. Sci. Technol. 36: 989-995, 2002.

Bodin N, Burgeot T, Stanisiere JY, Bocquene G, Menard D, Minier C, *et al*. Seasonal variations of a battery of biomarkers and physiological indices for the mussel *Mytilus galloprovincialis* transplanted into the northwest Mediterranean Sea. Comp. Biochem. Physiol. 138C: 411-427, 2004.

Boorstein WR, Ziegelhoffer T, Craig EA. Molecular evolution of the HSP70 multigene family. J. Mol. Evol. 38: 1-17, 1994.

Boutet I, Tanguy A, Moraga D. Organization and nucleotide sequence of the European flat oyster *Ostrea edulis* heat shock cognate 70 (hsc70) and heat shock protein 70 (hsp70) genes. Aquat. Toxicol. 65: 221-225, 2003a.

Boutet I, Tanguy A, Rousseau S, Auffret M, Moraga D. 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 Chap. 8: 76-85, 2003b.

Boutet I, Tanguy A, Moraga D. Response of the Pacific oyster *Crassostrea gigas* to hydrocarbon contamination under experimental conditions. Gene 329: 147-157, 2004.

Buckley BA, Owen ME, Hofmann GE. Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus Mytilus) changes as a function of thermal history. J. Exp. Biol. 204: 3571-3579, 2001.

Bukau B, Horwich AL. The Hsp70 and Hsp60 chaperone machines. Cell 92: 351-366, 1998.

Calderwood SK, Mambula SS, Gray PJ Jr., Theriault JR. Extracellular heat shock proteins in cell signaling. FEBS Lett. 581: 3689-3694, 2007.

Canesi L, Betti M, Lorusso LC, Ciacci C, Gallo G. 'In vivo' effects of Bisphenol A in Mytilus hemocytes: modulation of kinase-mediated signalling pathways. Aquat Toxicol. 71: 73-84, 2005.

Canesi L, Betti M, Ciacci C, Lorusso LC, Pruzzo C, Gallo G. Cell signalling in the immune response of mussel hemocytes. Inv. Surv. J. 3: 40-49, 2006a.

Canesi L, Ciacci C, Lorusso LC, Betti M, Guarnieri T, Tavolari S, *et al.* Immunomodulation by 17beta-estradiol in bivalve hemocytes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 291: R664-R673, 2006b.

Cellura C, Toubiana M, Parrinello N, Roch P. HSP70 gene expression in *Mytilus galloprovincialis* hemocytes is triggered by moderate heat shock and *Vibrio anguillarum*, but not by *V. splendidus* or *Micrococcus lysodeikticus*. Dev. Comp. Immunol. 30: 984-997, 2006.

Cellura C, Toubiana M, Parrinello N, Roch P. Specific expression of antimicrobial peptide and HSP70 genes in response to heat-shock and several bacterial challenges in mussels. Fish Shellfish Immunol. 22: 340-350, 2007.

Chapple JP, Smerdon GR, Hawkins AJS. Stress-70 protein induction in *Mytilus edulis*: tissue-specific responses to elevated temperatures reflect relative vulnerability and physiological function. J. Exp. Mar. Biol. Ecol. 217: 225-235, 1997.

Chapple JP, Smerdon GR, Berry RJ, Hawkins AJS. Seasonal changes in stress protein 70 levels reflect thermal tolerance in the marine bivalve *Mytilus edulis* L. J. Exp. Mar. Biol. Ecol. 229: 53-68, 1998.

Cherkasov AS, Overton RAJ, Sokolov EP, Sokolova IM. Temperature-dependent effects of cadmium and purine nucleotides on mitochondrial aconitase from a marine ectotherm, *Crassostrea virginica*: a role of temperature in oxidative stress and allosteric enzyme regulation. J. Exp. Biol. 210: 46-55, 2007.

Choi YK, Jo PG, Choi CY. Cadmium affects the expression of heat shock protein 90 and metallothionein mRNA in the Pacific oyster, *Crassostrea gigas*. Comp. Biochem. Physiol. 147C: 286-292, 2008.

Clark MS, Fraser KP, Peck LS. Antarctic marine molluscs do have an HSP70 heat shock response. Cell Stress Chap. 13: 39-49, 2008.

Clegg JS, Uhlingher KR, Jackson SA, Cherr GN, Rifkin E, Friedman CS. Induced thermotolerance and heat shock protein-70 family in Pacific oyster *Crassostrea gigas*. Mol. Mar. Biol. Biotechnol. 7: 21-30, 1998.

Collier RJ, Stiening CM, Pollard BC, VanBaale MJ, Baumgard LH, Gentry PC, *et al*. Use of gene expression microarrays for evaluating environmental stress tolerance at the cellular level in cattle. J. Anim. Sci. 84: E1-13, 2006.

Colotti C, Cavallini G, Vitale RL, Donati A, Maltinti M, Del Ry S, *et al*. Effects of aging and anti-aging caloric restrictions on carbonyl and heat shock protein levels and expression. Biogerontology 6: 397-406, 2005.

Courgeon AM, Maisonhaute C, Best-Belpomme M. Heat shock proteins are induced by cadmium in Drosophila cells. Exp. Cell Res. 153: 515-521, 1984.

Curtis SL, Nonavinakere VK, Potmis RA, Rasekh HR, Reams R, Early JL. Subacute exposure to cadmium chloride induces HSP-72 in rat liver. Res. Commun. Mol. Pathol. Pharmacol. 94: 221-224, 1996.

Dahlhoff EP, Rank NE. Functional and physiological consequences of genetic variation at phosphoglucose isomerase: heat shock protein expression is related to enzyme genotype in a montane beetle. Proc. Natl. Acad. Sci. USA 97: 10056-10061, 2000.

Dahlhoff EP, Buckley BA, Menge BA. Physiology of the rocky intertidal predator *Nucella ostrina* along an environmental stress gradient. Ecology 82: 2816-2829, 2001.

Dai R, Frejtag W, He B, Zhang Y, Mivechi NF. c-Jun NH_2-terminal kinase targeting and phosphorylation of heat shock factor-1 suppress its transcriptional activity. J. Biol. Chem. 275: 18210-18218, 2000.

David E, Tanguy A, Pichavant K, Moraga D. Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. FEBS J. 272: 5635-5652, 2005.

Daugaard M, Rohde M, Jaattela M. The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS Lett. 581: 3702-3710, 2007.

De Maio A. The heat-shock response. New Horiz. 3: 198-207, 1995.

De Maio A. Heat shock proteins: facts, thoughts, and dreams. Shock 11: 1-12, 1999.

Deane EE, Woo NY. Cloning and characterization of the hsp70 multigene family from silver sea bream: modulated gene expression between warm and cold temperature acclimation. Biochem. Biophys. Res. Commun. 330: 776-783, 2005.

Demand J, Luders J, Hohfeld J. The carboxy-terminal domain of Hsc70 provides binding sites for a distinct set of chaperone cofactors. Mol. Cell Biol. 18: 2023-2028, 1998.

Dietz TJ, Somero GN. The threshold induction temperature of the 90-kDa heat shock protein is subject to acclimatization in eurythermal goby fishes (genus Gillichthys). Proc. Natl. Acad. Sci. USA 89: 3389-3393, 1992.

Dondero F, Piacentini L, Marsano F, Rebelo M, Vergani L, Venier P, *et al*. Gene transcription profiling in pollutant exposed mussels (Mytilus spp.) using a new low-density oligonucleotide microarray. Gene 376: 24-36, 2006.

Dorion S, Berube J, Huot J, Landry J. A short lived protein involved in the heat shock sensing mechanism responsible for stress-activated protein kinase 2 (SAPK2/p38) activation. J. Biol. Chem. 274: 37591-37597, 1999.

Dorion S, Landry J. Activation of the mitogen-activated protein kinase pathways by heat shock. Cell Stress Chap. 7: 200-206, 2002.

Dowling V, Hoarau PC, Romeo M, O'Halloran J, van Pelt F, O'Brien N, *et al*. Protein carbonylation and heat shock response in *Ruditapes decussatus* following p,p'-dichlorodiphenyldichloroethylene (DDE) exposure: a proteomic approach reveals that DDE causes oxidative stress. Aquat. Toxicol. 77: 11-18, 2006.

Encomio VG, Chu FL. Heat shock protein (hsp70) expression and thermal tolerance in sublethally heat-shocked eastern oysters *Crassostrea virginica* infected with the parasite *Perkinsus marinus*. Dis. Aquat. Organ. 76: 251-260, 2007.

Farcy E, Voiseux C, Lebel JM, Fievet B. Seasonal changes in mRNA encoding for cell stress markers in the oyster *Crassostrea gigas* exposed to radioactive discharges in their natural environment. Sci. Total Environ. 374: 328-341, 2007.

Feder ME, Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61: 243-282, 1999.

Fink AL. Chaperone-mediated protein folding. Physiol. Rev. 79: 425-449, 1999.

Franco JL, Trivella DB, Trevisan R, Dinslaken DF, Marques MR, Bainy AC, *et al*. Antioxidant status and stress proteins in the gills of the brown mussel *Perna perna* exposed to zinc. Chem. Biol. Interact. 160: 232-240, 2006.

Franzellitti S, Fabbri E. Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. Biochem. Biophys. Res. Commun. 336: 1157-1163, 2005.

Franzellitti S, Fabbri E. Cytoprotective responses in the Mediterranean mussel exposed to Hg^{2+} and CH_3Hg^+. Biochem. Biophys. Res. Commun. 351: 719-725, 2006.

Fuertes MA, Perez JM, Soto M, Menendez M, Alonso C. Thermodynamic stability of the C-terminal domain of the human inducible heat shock protein 70. Biochim. Biophys. Acta 1699: 45-56, 2004.

Funk RH, Monsees TK. Effects of electromagnetic fields on cells: physiological and therapeutical approaches and molecular mechanisms of interaction. A review. Cells Tissues Organs 182: 59-78, 2006.

Gao Q, Song L, Ni D, Wu L, Zhang H, Chang Y. cDNA cloning and mRNA expression of heat shock protein 90 gene in the haemocytes of Zhikong scallop *Chlamys farreri*. Comp. Biochem. Physiol. 147B: 704-715, 2007.

Gao Q, Zhao J, Song L, Qiu L, Yu Y, Zhang H, *et al*. Molecular cloning, characterization and expression of heat shock protein 90 gene in the haemocytes of bay scallop *Argopecten irradians*. Fish Shellfish Immunol. 24: 379-385, 2008.

Gonzalez-Riopedre M, Novas A, Dobano E, Ramos-Martinez JI, Barcia R. Effect of thermal stress on protein expression in the mussel *Mytilus galloprovincialis* Lmk. Comp. Biochem. Physiol. 147B: 531-540, 2007.

Goodman R, Blank M. Magnetic field stress induces expression of hsp70. Cell Stress Chap. 3: 79-88, 1998.

Gourdon I, Gricourt L, Kellner K, Roch P, Escoubas JM. Characterization of a cDNA encoding a 72 kDa heat shock cognate protein (Hsc72) from the Pacific oyster, *Crassostrea gigas*. DNA Seq. 11: 265-270, 2000.

Gunther E, Walter L. Genetic aspects of the hsp70 multigene family in vertebrates. Experientia 50: 987-1001, 1994.

Gupta RS, Singh B. Phylogenetic analysis of 70 kD heat shock protein sequences suggests a chimeric origin for the eukaryotic cell nucleus. Curr. Biol. 4: 1104-1114, 1994.

Gupta RS. Phylogenetic analysis of the 90 kD heat shock family of protein sequences and an examination of the relationship among animals, plants, and fungi species. Mol. Biol. Evol. 12: 1063-1073, 1995.

Hamer B, Hamer DP, Muller WE, Batel R. Stress-70 proteins in marine mussel *Mytilus galloprovincialis* as biomarkers of environmental pollution: a field study. Environ. Int. 30: 873-882, 2004.

Hamdoun AM, Cheney DP, Cherr GN. Phenotypic plasticity of HSP70 and HSP70 gene expression in the Pacific oyster (*Crassostrea gigas*): implications for thermal limits and induction of thermal tolerance. Biol. Bull. 205: 160-169, 2003.

Hansen BH, Garmo OA, Olsvik PA, Andersen RA. Gill metal binding and stress gene transcription in brown trout (*Salmo trutta*) exposed to metal environments: the effect of pre-exposure in natural populations. Environ. Toxicol. Chem. 26: 944-953, 2007.

Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295: 1852-1858, 2002

Helmuth BS, Hofmann GE. Microhabitats, thermal heterogeneity, and patterns of physiological stress in the rocky intertidal zone. Biol. Bull. 201: 374-384, 2001.

Hendrick JP, Hartl FU. The role of molecular chaperones in protein folding. FASEB J. 9: 1559-1569, 1995.

Hightower LE. Heat shock, stress proteins, chaperones, and proteotoxicity. Cell 66: 191-197, 1991.

Hofmann GE, Somero GN. Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel *Mytilus trossulus*. J. Exp. Biol. 198: 1509-1518, 1995.

Hofmann GE, Somero GN. Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis*: evidence from the heat-shock response and protein ubiquitination. Mar. Biol. 126: 65-75, 1996.

Hofmann GE. Ecologically relevant variation in induction and function of heat shock proteins in marine organisms. Am. Zool. 39: 889-900, 1999.

Hofmann GE, Buckley BA, Airaksinen S, Keen JE, Somero GN. Heat-shock protein expression is absent in the antarctic fish *Trematomus bernacchii* (family Nototheniidae). J. Exp. Biol. 203: 2331-2339, 2000.

Huot J, Lambert H, Lavoie JN, Guimond A, Houle F, Landry J. Characterization of 45-kDa/54-kDa HSP27 kinase, a stress-sensitive kinase which may activate the phosphorylation-dependent protective function of mammalian 27-kDa heat-shock protein HSP27. Eur. J. Biochem. 227: 416-427, 1995.

Ivanina AV, Cherkasov AS, Sokolova IM. Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters, *Crassostrea virginica* Gmelin. J. Exp. Biol. 211: 577-586, 2008a.

Ivanina AV, Sokolova IM, Sukhotin AA. Oxidative stress and expression of chaperones in aging mollusks. Comp. Biochem. Physiol. 150B: 53-61, 2008b.

Jäättelä M. Heat shock proteins as cellular lifeguards. Ann. Med. 31: 261-271, 1999.

Joyner-Matos J, Downs CA, Julian D. Increased expression of stress proteins in the surf clam *Donax variabilis* following hydrogen sulfide exposure. Comp. Biochem. Physiol. 145A: 245-257, 2006.

Kantengwa S, Polla BS. Phagocytosis of *Staphylococcus aureus* induces a selective stress response in human monocytes-macrophages (M phi): modulation by M phi differentiation and by iron. Infect. Immun. 61: 1281-1287, 1993.

Kefaloyianni E, Gourgou E, Ferle V, Kotsakis E, Gaitanaki C, Beis I. Acute thermal stress and various heavy metals induce tissue-specific pro- or anti-apoptotic events via the p38-MAPK

signal transduction pathway in *Mytilus galloprovincialis* (Lam.). J. Exp. Biol. 208: 4427-4436, 2005.

Koban M, Yup AA, Agellon LB, Powers DA. Molecular adaptation to environmental temperature: heat shock response of the eurythermal teleost *Fundulus heteroclitus*. J. Exp. Mar. Biol. Ecol. 1: 1-17, 1991.

Kourtidis A, Scouras ZG. Analysis and characterization of the transcriptional unit of a new *Mytilus galloprovincialis* (Mollusca: Bivalvia) hsp70 gene. DNA Seq. 16: 36-43, 2005.

Kourtidis A, Drosopoulou E, Nikolaidis N, Hatzi VI, Chintiroglou CC, Scouras ZG. Identification of several cytoplasmic HSP70 genes from the Mediterranean mussel (*Mytilus galloprovincialis*) and their long-term evolution in Mollusca and Metazoa. J. Mol. Evol. 62: 446-459, 2006.

La Terza A, Papa G, Miceli C, Luporini P. Divergence between two Antarctic species of the ciliate Euplotes, *E. focardii* and *E. nobilii*, in the expression of heat-shock protein 70 genes. Mol. Ecol. 10: 1061-1067, 2001.

Lacoste A, De Cian MC, Cueff A, Poulet SA. Noradrenaline and alpha-adrenergic signaling induce the hsp70 gene promoter in mollusc immune cells. J. Cell. Sci. 114: 3557-3564, 2001a.

Lacoste A, Malham SK, Cueff A, Jalabert F, Gelebart F, Poulet SA. Evidence for a form of adrenergic response to stress in the mollusc *Crassostrea gigas*. J. Exp. Biol. 204: 1247-1255, 2001b.

Lacoste A, Malham SK, Cueff A, Poulet SA. Stress-induced catecholamine changes in the hemolymph of the oyster *Crassostrea gigas*. Gen. Comp. Endocrinol. 122: 181-188, 2001c.

Laursen JR, di Liu H, Wu XJ, Yoshino TP. Heat-shock response in a molluscan cell line: characterization of the response and cloning of an inducible HSP70 cDNA. J. Invertebr. Pathol. 70: 226-233, 1997.

Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. Mol. Cell. Biol. 15: 505-516, 1995.

Lees-Miller SP, Anderson CW. The human double-stranded DNA-activated protein kinase phosphorylates the 90-kDa heat-shock protein, hsp90 alpha at two NH2-terminal threonine residues. J. Biol. Chem. 264: 17275-17280, 1989.

Leung SM, Hightower LE. A 16-kDa protein functions as a new regulatory protein for Hsc70 molecular chaperone and is identified as a member of the Nm23/nucleoside diphosphate kinase family. J. Biol. Chem. 272: 2607-2614, 1997.

Lock K, Janssen CR. Comparative toxicity of a zinc salt, zinc powder and zinc oxide to *Eisenia fetida*, *Enchytraeus albidus* and *Folsomia candida*. Chemosphere 53: 851-856, 2003.

Luedeking A, Koehler A. Indentification of six mRNA sequences of genes related to multixenobiotic resistance (MXR) and biotransformation in *Mytilis edulis*. Mar. Ecol. Prog. Ser. 238: 115-124, 2002.

Lyons C, Dowling V, Tedengren M, Gardestrom J, Hartl MG, O'Brien N, *et al*. Variability of heat shock proteins and glutathione S-transferase in gill and digestive gland of blue mussel, *Mytilus edulis*. Mar. Environ. Res. 56: 585-597, 2003.

Madsen E, Gitlin JD. Copper and iron disorders of the brain. Annu. Rev. Neurosci. 30: 317-337, 2007.

Malagoli D, Franchini A, Ottaviani E. Synergistic role of cAMP and IP(3) in corticotropin-releasing hormone-induced cell shape changes in invertebrate immunocytes. Peptides 21: 175-182, 2000.

Malagoli D, Gobba F, Ottaviani E. Effects of 50-Hz magnetic fields on the signalling pathways of fMLP-induced shape changes in invertebrate immunocytes: the activation of an alternative "stress pathway". Biochim. Biophys. Acta 1620: 185-190, 2003.

Malagoli D, Lusvardi M, Gobba F, Ottaviani E. 50 Hz magnetic fields activate mussel immunocyte p38 MAP kinase and induce HSP70 and 90. Comp. Biochem. Physiol. 137C: 75-79, 2004.

Malagoli D, Gobba F, Ottaviani E. 50 Hz magnetic fields of constant or fluctuating intensity: effects on immunocyte hsp70 in the mussel *Mytilus galloprovincialis*. Bioelectromagnetics 27: 427-429, 2006.

Minier C, Borghi VV, Moore MN, Porte C. Seasonal variation of MXR and stress proteins in the common mussel, *Mytilus galloprovincialis*. Aquat .Toxicol. 50: 167-176, 2000.

Moraga D, Meistertzheim AL, Tanguy-Royer S, Boutet I, Tanguy A, Donval A. Stress response in Cu^{2+} and Cd^{2+} exposed oysters (*Crassostrea gigas*): an immunohistochemical approach. Comp. Biochem. Physiol. 141C: 151-156, 2005.

Nadeau D, Corneau S, Plante I, Morrow G, Tanguay RM. Evaluation for Hsp70 as a biomarker of effect of pollutants on the earthworm *Lumbricus terrestris*. Cell Stress Chap. 6: 153-163, 2001.

Ohta T. Further examples of evolution by gene duplication revealed through DNA sequence comparisons. Genetics 138: 1331-1337, 1994.

Ohta T, Nei M. Divergent evolution and evolution by the birth-and-death process in the immunoglobulin VH gene family. Mol. Biol. Evol. 11: 469-482, 1994.

Ottaviani E, Franceschi C. The neuroimmunology of stress from invertebrates to man. Prog. Neurobiol. 48: 421-440, 1996.

Ottaviani E, Caselgrandi E, Kletsas D. The CRH-ACTH-biogenic amine axis in invertebrate immunocytes activated by PDGF and TGF-beta. FEBS Lett. 427: 255-258, 1998a.

Ottaviani E, Franchini A, Hanukoglu I. In situ localization of ACTH receptor-like mRNA in molluscan and human immunocytes. Cell. Mol. Life Sci. 54: 139-142, 1998b.

Ottaviani E, Franchini A, Genedani S. ACTH and its role in immune-neuroendocrine functions. A

comparative study. Curr. Pharm. Des. 5: 673-681, 1999.

Ottaviani E, Franchini A, Kletsas D. Platelet-derived growth factor and transforming growth factor-beta in invertebrate immune and neuroendocrine interactions: another sign of conservation in evolution. Comp. Biochem. Physiol. 129C: 295-306, 2001.

Page TJ, Sikder D, Yang L, Pluta L, Wolfinger RD, Kodadek T, *et al*. Genome-wide analysis of human HSF1 signaling reveals a transcriptional program linked to cellular adaptation and survival. Mol. Biosyst. 2: 627-639, 2006.

Park H, Ahn IY, Lee HE. Expression of heat shock protein 70 in the thermally stressed antarctic clam *Laternula elliptica*. Cell Stress Chap. 12: 275-282, 2007.

Park HS, Lee JS, Huh SH, Seo JS, Choi EJ. Hsp72 functions as a natural inhibitory protein of c-Jun N-terminal kinase. EMBO J. 20: 446-456, 2001.

Parsell DA, Lindquist S. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu. Rev. Genet . 27: 437-496, 1993.

Petes LE, Mouchka ME, Milston-Clements RH, Momoda TS, Menge BA. Effects of environmental stress on intertidal mussels and their sea star predators. Oecologia 156: 671-680, 2008.

Piano A, Asirelli C, Caselli F, Fabbri E. Hsp70 expression in thermally stressed *Ostrea edulis*, a commercially important oyster in Europe. Cell Stress Chap. 7: 250-257, 2002.

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

Piano A, Franzellitti S, Tinti F, Fabbri E. Sequencing and expression pattern of inducible heat shock gene products in the European flat oyster, *Ostrea edulis*. Gene 361: 119-126, 2005.

Pirkkala L, Nykanen P, Sistonen L. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J. 15: 1118-1131, 2001.

Polla BS, Stubbe H, Kantengwa S, Maridonneau-Parini I, Jacquier-Sarlin MR. Differential induction of stress proteins and functional effects of heat shock in human phagocytes. Inflammation 19: 363-378, 1995.

Pruski AM, Dixon DR. Heat shock protein expression pattern (HSP70) in the hydrothermal vent mussel *Bathymodiolus azoricus*. Mar. Environ. Res. 64: 209-224, 2007.

Pyza E, Mak P, Kramarz P, Laskowski R. Heat shock proteins (HSP70) as biomarkers in ecotoxicological studies. Ecotoxicol. Environ. Saf. 38: 244-251, 1997.

Radlowska M, Pempkowiak J. Stress-70 as indicator of heavy metals accumulation in blue mussel *Mytilus edulis*. Environ. Int. 27: 605-608, 2002.

Rathinam AV, Chen TT, Grossfeld RM. Cloning and sequence analysis of a cDNA for an inducible 70 kDa heat shock protein (Hsp70) of the American oyster (*Crassostrea virginica*). DNA Seq. 11: 261-264, 2000.

Ravindra K, Bencs L, Van GR. Platinum group elements in the environment and their health risk. Sci. Total. Environ. 318: 1-43, 2004.

Ritossa F. A new puffing pattern induced by temperature shock and DNP in *Drosophila*. Experientia 18: 571-573, 1962.

Roberts DA, Hofmann GE, Somero GN. Heat-shock protein expression in *Mytilus californianus*: acclimatization (seasonal tidal-height comparisons) and acclimation effects. Biol. Bull. 192: 30-32, 1997.

Rouse J, Cohen P, Trigon S, Morange M, Onso-Llamazares A, Zamanillo D, *et al*. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 78: 1027-1037, 1994.

Sanders BM, Pascoe VM, Nakagawa PA, Martin LS. Persistence of the heat shock response over time in the common mussel, *Mytilus edulis*. Mol. Mar. Biol. Biotechnol. 1: 147-154, 1992.

Sanders BM, Martin LS. Stress proteins as biomarkers of contaminant exposure in archived environmental samples. Sci. Total Environ. 139-140: 459-470, 1993.

Sanders BM, Martin LS, Howe SR, Nelson WG, Hegre ES, Phelps DK. Tissue-specific differences in accumulation of stress proteins in *Mytilus edulis* exposed to a range of copper concentrations. Toxicol. Appl. Pharmacol. 125: 206-213, 1994.

Scharf K-D, Hohfeld I, Nover L. Heat stress response and heat stress transcription factors. J. Biosci. 23: 313-329, 1998.

Schroder HC, Hassanein HM, Lauenroth S, Koziol C, Mohamed TA, Lacorn M, *et al*. Induction of DNA strand breaks and expression of HSP70 and GRP78 homolog by cadmium in the marine sponge *Suberites domuncula*. Arch. Environ. Contam. Toxicol. 36: 47-55, 1999.

Singer C, Zimmermann S, Sures B. Induction of heat shock proteins (hsp70) in the zebra mussel (*Dreissena polymorpha*) following exposure to platinum group metals (platinum, palladium and rhodium): comparison with lead and cadmium exposures. Aquat. Toxicol. 75: 65-75, 2005.

Smerdon GR, Chapple JP, Hawkins AJS. The simultaneous immunological detection of four stress-70 protein isoforms in *Mytilus edulis*. Mar. Environ. Res. 40: 399-407, 1995.

Snyder MJ, Girvetz E, Mulder EP. Induction of marine mollusc stress proteins by chemical or physical stress. Arch. Environ. Contam. Toxicol. 41: 22-29, 2001.

Song L, Wu L, Ni D, Chang Y, Xu W, Xing K. The cDNA cloning and mRNA expression of heat shock protein 70 gene in the haemocytes of bay scallop (*Argopecten irradians*, Lamarck 1819) responding to bacteria challenge and naphthalin stress. Fish Shellfish Immunol. 21: 335-345, 2006.

Stefano GB, Cadet P, Zhu W, Rialas CM, Mantione K, Benz D, *et al*. The blueprint for stress can be

found in invertebrates. Neuro. Endocrinol. Lett. 23: 85-93, 2002.

Tirard CT, Grossfeld RM, Levine JF, Kennedy-Stoskopf S. Effect of hyperthermia *in vitro* on stress protein syntesis and accumulation in oyster haemocytes. Fish Shellfish Immunol. 5: 9-25, 1995.

Toyohara H, Hosoi M, Hayashi I, Kubota S, Hashimoto H, Yokoyama Y. Expression of HSP70 in response to heat-shock and its cDNA cloning from Mediterranean blue mussel. Fish. Sci. 71: 327-332, 2005.

Tsukahara F, Yoshioka T, Muraki T. Molecular and functional characterization of HSC54, a novel variant of human heat-shock cognate protein 70. Mol. Pharmacol. 58: 1257-1263, 2000.

Valbonesi P, Ricci L, Franzellitti S, Biondi C, Fabbri E. Effects of Cadmium on MAPK Signalling Pathways and HSP70 Expression in a Human Trophoblast Cell Line. Placenta 29: 725-733, 2008.

van Noort JM. Stress proteins in CNS inflammation. J. Pathol. 214: 267-275, 2008.

Viarengo A, Lowe D, Bolognesi C, Fabbri E, Koehler A. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp. Biochem. Physiol. 146C: 281-300, 2007.

Vijayan MM, Raptis S, Sathiyaa R. Cortisol treatment affects glucocorticoid receptor and glucocorticoid-responsive genes in the liver of rainbow trout. Gen. Comp. Endocrinol. 132: 256-263, 2003.

Vos MJ, Hageman J, Carra S, Kampinga HH. Structural and functional diversities between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families. Biochemistry 47: 7001-7011, 2008.

Wandinger SK, Richter K, Buchner J. The hsp90 chaperone machinery. J. Biol. Chem. 283: 18473-18477, 2008.

Wang TF, Chang JH, Wang C. Identification of the peptide binding domain of hsc70. 18-Kilodalton fragment located immediately after ATPase domain is sufficient for high affinity binding. J. Biol. Chem. 268: 26049-26051, 1993.

Wendelaar Bonga SE. The stress response in fish. Physiol. Rev. 77: 591-625, 1997.

Wheeler JC, Bieschke ET, Tower J. Muscle-specific expression of *Drosophila* hsp70 in response to aging and oxidative stress. Proc. Natl. Acad. Sci. USA 92: 10408-10412, 1995.

Wu C. Heat shock transcription factors: structure and regulation. Annu. Rev. Cell Dev. Biol. 11: 441-469, 1995.

Yamada M, Yamada M, Kiuchi Y, Nara K, Kanda Y, Morinobu S, *et al*. Identification of a novel splice variant of heat shock cognate protein 70 after chronic antidepressant treatment in rat frontal cortex. Biochem. Biophys. Res. Commun. 261: 541-545, 1999.

Yamashita M, Hirayoshi K, Nagata K. Characterization of multiple members of the HSP70 family in platyfish culture cells: molecular evolution of stress protein HSP70 in vertebrates. Gene 336: 207-218, 2004.

Yokoyama Y, Hashimoto H, Kubota S, Kuriyama A, Ogura Y, Mizuta, *et al*. cDNA cloning of Japanese oyster stress protein homologous to the mammalian 78-kDa glucose regulated protein and its induction by heat shock. Fish. Sci. 72: 402-409, 2006.

Yost HJ, Petersen RB, Lindquist S. RNA metabolism: strategies for regulation in the heat shock response. Trends Genet. 6: 223-227, 1990.

Zheng L, He M, Long M, Blomgran R, Stendahl O. Pathogen-induced apoptotic neutrophils express heat shock proteins and elicit activation of human macrophages. J. Immunol. 173: 6319-6326, 2004.

Zhong M, Orosz A, Wu C. Direct sensing of heat and oxidation by Drosophila heat shock transcription factor. Mol. Cell. 2: 101-108, 1998.