ELSEVIER

Identification of cDNAs encoding HSP70 and HSP90 in the abalone *Haliotis tuberculata*: Transcriptional induction in response to thermal stress in hemocyte primary culture

Emilie Farcy [a,b], Antoine Serpentini [a], Bruno Fiévet [b], Jean-Marc Lebel [a,*]

[a] *Laboratoire de Biologie et Biotechnologies Marines-UMR 100 Ifremer, Université de Caen Basse-Normandie.-Esplanade de la Paix-14032 Caen cedex, France*
[b] *Laboratoire de Radioécologie de Cherbourg-Octeville, Institut de Radioprotection et de Sûreté Nucléaire/SECRE, Cherbourg-Octeville, France*

Abstract

Heat-shock proteins are a multigene family of proteins whose expression is induced by a variety of stress factors. This work reports the cloning and sequencing of HSP70 and HSP90 cDNAs in the gastropod *Haliotis tuberculata*. The deduced amino acid sequences of both HSP70 and HSP90 from *H. tuberculata* shared a high degree of homology with their homologues in other species, including typical eukaryotic HSP70 and HSP90 signature sequences. We examined their transcription expression pattern in abalone hemocytes exposed to thermal stress. Real-time PCR analysis indicated that both HSP70 and HSP90 mRNA were expressed in control animals but rapidly increased after heat-shock.
© 2007 Elsevier Inc. All rights reserved.

1. Introduction

Heat-shock proteins (HSP) are highly conserved proteins whose synthesis is enhanced in response to cellular stress, including temperature elevation, tissue trauma, heavy metal toxicity, radiation and infection, or as a result of normal changes associated with cellular development or differentiation (Lindquist and Craig, 1988; Schlesinger, 1990). As molecular chaperones, HSP protect proteins from denaturation and facilitate either the refolding of damaged proteins or, in the case of irreversible damage, their removal through proteolysis (Parsell and Lindquist, 1993, 1994; Sherman and Goldberg, 1996; Hartl, 1996; Bukau and Horwich, 1998; Morimoto, 1998; Frydman, 2001). Heat-shock proteins are generally classified in different families on the basis of their molecular mass. e.g., HSP90, HSP70, HSP20–30, and so on (Feder and Hofmann, 1999). They have been characterized in a range of metazoans, including many aquatic organisms. In molluscs, their involvement in the stress response was reviewed by Sanders (1988, 1993), Gourdon et al. (1998) and Snyder et al. (2001).

The best studied HSPs are the 70-kD protein family (HSP70) because of their role in protein chaperoning (Gething and Sambrook, 1992) and in processes of acquired tolerance (Lindquist and Craig, 1988; Clegg et al., 1998). The HSP70 family includes both heat-inducible (Ingolia and Craig, 1982; Craig et al., 1983) and constitutive proteins. Constitutive and inducible isoforms of the HSP70 family were first reported not only in mammals but also in non-mammalian organisms such as fish (Gornati et al., 2004; Yamashita et al., 2004) and molluscs (Tirard et al., 1995; Laursen et al., 1997; Clegg et al., 1998; Gourdon et al., 2000; Piano et al., 2002, 2004, 2005; Boutet et al., 2003a,b). In molluscs, both isoforms belonging to the HSP70 family are more or less heat-inducible (Piano et al., 2002). The constitutive HSP are expressed under normal conditions and appear to be essential for protein folding or trafficking and regulated proteolysis in unstressed cells (Craig et al., 1983; Lindquist and Craig, 1988; Hightower, 1993). These proteins are faintly or not induced by heat stress. On the other hand, inducible forms are expressed at very low level under normal conditions and over-expressed following stress. For example, heat stress induced the over-expression of a 70 kD isoform that was already present, at very low but detectable level, in the gill and mantle of unstressed *Mytilus edulis* (Chapple et al.,

* Corresponding author. Tel.: +33 2 3156 56 62; fax: +33 2 31 56 53 46.
E-mail address: jean-marc.lebel@unicaen.fr (J.-M. Lebel).

1997). Similarly, temperatures up to 40 °C provoked an over-expression of endogenous HSP70 isoforms in the mussel *Mytilus galloprovincialis* and in the clams *Tapes philippinarum* and *Scapharca inaequivalvis*, without the appearance of any new isoform (Piano et al., 2004).

HSP90 have been much less investigated in molluscs (Tomanek and Somero, 2000, 2002; Ochoa et al., 2002). The members of the 90-kD HSP family are the least understood of the major HSP in terms of their cellular function. HSP90 is a dimer and binds to several cellular proteins, including steroid receptors and protein kinases (Pratt, 1993; Csermely et al., 1998). In addition, HSP90 is also found associated with cytoskeleton proteins such as actin and tubulin (Sanchez et al., 1988; Redmond et al., 1989; Czar et al., 1996; Liang and Macrae, 1997; Garnier et al., 1998). In eukaryotic cells, both 70-kD and 90-kD HSP families exhibited specific homologues in the cytosol and the endoplasmic reticulum (Pelham, 1989; Boorstein et al., 1994; Gupta and Singh, 1994).

The abalone, *Haliotis tuberculata* is a gastropod mollusc with a geographic range in the Eastern Atlantic extending from the English Channel down to the west coast of Africa. Molluscan circulating cells have an important role in defence mechanisms and in tissue repair, and are of major interest in the fields of Ecotoxicology. In this study, we cloned *H. tuberculata* HSP70 and HSP90 cDNA from a hemocyte cDNA library. Then, we used real-time PCR to investigate the expression of HSP70 and HSP90 mRNA in the primary culture of abalone hemocytes exposed *in vitro* to thermal stress.

2. Material and methods

2.1. Animals

Adult wild abalone (*H. tuberculata*), 10–12 cm in shell length, were gathered from the northern Cotentin peninsula (France). They were acclimatised to laboratory conditions for at least 1 month before experimentation, in continuously aerated natural running seawater (15 °C). They were fed weekly with freshly collected local algae (*Palmaria palmata* and *Laminaria digitata*). After an incision in the foot, hemolymph was collected (10–15 mL per animal) using a 20-mL syringe fitted with a 2-gauge hypodermic needle.

2.2. HSP cDNA cloning

2.2.1. Search for a specific fragment using degenerate oligonucleotide

Total RNA was extracted from freshly collected hemocytes using TRI REAGENT (Sigma-Aldrich) and used for purification of polyadenylated-RNA (Dynabeads® mRNA DIRECT Kit from Dynal Biotech.) Poly-A mRNAs (100 ng) were reverse-transcribed using 150 ng oligo(dT) primer, 300 U moloney murine leukemia virus (MMLV) reverse transcriptase (Sigma-Aldrich), RNAse-free dNTP and 40 u RNAsin (Promega).

The cDNAs obtained was used for polymerase chain reaction (PCR) cycling with degenerate primers. These degenerate oligonucleotides were designed on the basis of conserved amino acids of HSP70 and HSP90 from various eukaryotic organisms. The sequences of the forward primers were: *degHSP70_S1*: 5′-CCN GCN TAY TTY AAY GA-3′; *degHSP90_ S1*: 5′-AAR ACN AAR CCN ATH TGG AC-3′ and the sequences of the reverse primers were: *degHSP70_A1*: 5′-TTR TCR AAR TCY TCN CCN CC-3′; *degHSP90_A1*: 5′-GCY TGN GCY TTC ATD AT-3′. The primers were purchased from EUROBIO. PCR amplifications with these degenerate primers were carried out in a Stratagene Thermocycler. Using primers *degHSP70_S1* and *degHSP70_A1*, the PCR for HSP70 was performed with 50 μl of a mixture containing 5 μl of 10× buffer (Tris–HCl 100 mM, KCl 500 mM and 1.0% Triton® X-100, Promega), 4 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of *Taq* DNA polymerase (Promega) and 1 μM of each primer. For HSP70, this mixture was subjected to 40 cycles of amplification (denaturation for 1 min at 95 °C, annealing for 2 min at 44 °C and extension for 1 min at 72 °C). The PCR for HSP90 was performed with the same mixture but with degenerate primers *degHSP90_S1* and *degHSP90_A1* and the annealing temperature was set at 46 °C.

The PCR products were separated on a TAE 1% agarose gel. A single band of approximately 300 bp was obtained for HSP70 and a single band of approximately 1000 bp for HSP90. These PCR products were purified (Wizard SV gel and PCR clean-up system, Promega), and inserted into the PCR®II Topo® vector (TOPO TA cloning kit, Invitrogen) which was used to transform XL1-blue MRF' competent cells. The HSP70 and HSP90 cDNA fragment inserts were sequenced (both strands) by Genome Express.

2.2.2. Construction of cDNA library from abalone hemocytes

Total RNA was isolated from *H. tuberculata* hemocytes by acid guanidinium thiocyanate-phenol-chloroform extraction methods (Chomczynski and Sacchi, 1987). Poly-A RNA was prepared with a mRNA isolation kit (Dynabeads® mRNA DIRECT Kit from Dynal Biotech.) according to the manufacturer's instructions. A cDNA expression library was constructed by starting with 5 μg of poly-A RNA. First-strand synthesis was performed using an oligo(dT) primer with an internal Xho I site and 5-methyl-CTP. cDNA was ligated to EcoRI adaptors and digested with Xho I. cDNA fragments were cloned directionally into the bacteriophage expression vector λ-ZAPII (Stratagene), packaged into phage particles by using a Gigapack III packaging system (Stratagene) and transfected into the host bacterial strain *Escherichia coli* XL-1MRF' according to the manufacturer's instructions (Stratagene). A total of 3.2×10^6 independent clones were recovered.

2.2.3. Amplification of 5′- and 3′-ends by PCR screening of the cDNA library

To amplify the 5′- and 3′-ends of fragments, PCR was performed on phage lysate of the cDNA library with specific primers coupled with primers located on the pBluescript phagemid (Table 1). The PCR was performed with 1 μl of the phage lysate containing 5 μl of 10× High Fidelity Buffer (Tris–HCl 100 mM, KCl 500 mM and 1.0% Triton® X-100, Promega), 2 mM $MgSO_4$, 0.2 mM of each dNTP, 1 U of Platinum® Taq DNA Polymerase High Fidelity (Invitrogen), 0.6 μM of the phagemid-located primer, and 0.2 μM of the gene-specific primer.

Table 1
Primers used for polymerase chain reaction

	Hsp70	Hsp90
Gene-specific	QS1_HSP70	QS1_HSP90
Forward primer	5′ CGGTGAGCGCAATGTTC 3′	5′ CCAGGAAGAATATGCCGAGT 3′
Gene-specific	QA2_HSP70	QA2_HSP90
Reverse primer	5′ CCGCCAAGTGGGTGTCT 3′	5′ CACGGAACTCCAACTGACC 3′
Phagemid-located	Zap 5N	Zap 5N
Forward primer	5′ CCGCTCTAGAACTAGTGGAT 3′	5′ CCGCTCTAGAACTAGTGGAT 3′
Phagemid-located	T7	T7
Reverse primer	5′ GTAATACGACTCACTATAGGGC 3′	5′ GTAATACGACTCACTATAGGGC 3′

The following touchdown amplification cycle was applied using an Eppendorf cycler: a first run of 5 cycles of amplification (denaturation for 30 s at 94 °C, annealing for 1 min at 65 °C and extension for 4 min at 68 °C) was followed by 5 cycles of amplification with annealing temperature set at 60 °C and 25 cycles of amplification with annealing temperature set at 55 °C. The amplification products obtained from this first PCR run were then used for a second nested PCR using the same amplification program and the primers shown in Table 2.

The fragments obtained, respectively 800 bp for the HSP70 5′-end, 1750 bp for the HSP70 3′-end, 1200 bp for the HSP90 5′-end and 1750 bp for the HSP90 3′-end, were purified on a TAE 1% agarose gel and inserted into the plasmid PCR® II Topo® (Invitrogen) as described previously. Plasmid inserts were sequenced (both strands) by Genome Express.

2.3. Primary cell cultures

Hemocytes were cultured as previously described (Lebel et al., 1996; Serpentini et al., 2000). Briefly, after collection, the hemolymph was transferred to a sterile tube and diluted 1:2 in cooled, sterile, modified Alsever's anti-coagulant solution (Bachère et al., 1988) (115 mM glucose; 27 mM sodium citrate; 11.5 mM EDTA; 382 mM NaCl). Hemocytes were rapidly plated at 10.10^6 cells per $80 cm^2$ flask (for mRNA extraction) or at $1.5.10^6$ cells in a 6-well plate (for MTT assay) in which three volumes of sterile artificial seawater were added. The cultures were maintained at 17 °C in an incubator (CO_2 free). After 90 min of incubation, the cells were covered with Hank's 199 sterile medium, modified by the addition of 250 mM NaCl, 10 mM KCl, 25 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM Hepes, 10mM L-glutamine, 100μg/mL streptomycin and 60 μg/mL penicillin G, at a final pH of 7.4. The cells were then kept at

17 °C for 24 h before the experiments. Primary culture of hemocytes can be maintained in such conditions up to ten days.

2.4. Induction test: heat-shock performance

First, primary cultures of hemocytes from the gastropod H. tuberculata were exposed to a 37 °C acute thermal stress in order to study the response kinetics of the HSP70 and HSP90 mRNA levels over a 1-h period at that temperature. In a subsequent experiment, hemocyte primocultures were subjected to a 1-h thermal stress at 25 and 37 °C. The control hemocytes were cultured at 17 °C.

2.5. MTT assay

The cell viability was evaluated by a MTT (3-[4,5-dimethyl-2-thiazol]-2,5-diphenyl-2H-tetrazolium bromide) reduction assay (Mosmann, 1983) adapted to molluscan cell cultures (Domart-Coulon et al., 1994; Lebel et al., 1996; Poncet et al., 2002). The MTT assay is a colorimetric metabolic assay based on mitochondrial dye conversion to assess viability. Briefly, 10% (v/v) of the MTT stock solution (5 mg MTT/mL of PBS) was added to the culture dishes. After 24 h of incubation, an equal volume of isopropanol containing 0.04 N HCl was added to each culture in order to dissolve the converted formazan. Absorbance was then measured at a wavelength of 570 nm with a 630 nm reference.

2.6. mRNA quantification

Total RNA was extracted with TRI reagent according to the manufacturer's instructions (Sigma-Aldrich). The amount and quality of RNA was quantified by measurement of absorbance at 260 and 280 nm in a UV-spectrophotometer. To avoid

Table 2
Primers used for nested PCR

	Hsp70	Hsp90
Gene-specific	QS2_HSP70	HSP90SEQ1S
Forward nested primer	5′ CATCTTTGATCTCGGTGGTG 3′	5′ ACTGTGAGGACCTCATTC 3′
Gene-specific	QA1_HSP70	QA2_HSP90
Reverse nested primer	5′ GCCAAGTGGGTGTCTCCA 3′	5′ CACGGAACTCCAACTGACC 3′
Phagemid-located	OPH	OPH
Forward nested primer	5′ GCTCTAGAACTAGTGGATCCC 3′	5′ GCTCTAGAACTAGTGGATCCC 3′
Phagemid-located	ZAP3	ZAP3
Reverse nested primer	5′ ACTCACTATAGGGCGAATTG 3′	5′ ACTCACTATAGGGCGAATTG 3′

Table 3
Sequences of the primers used for real-time PCR and accession number

Gene	GenBank accession no.	Forward primer	Reverse primer
[a]Actin	AM236595	5′ GGGTGTGATGGTCGGTAT 3′	5′ ACCGTGTTCGATGGGGTA 3′
Ht_HSP70	AM283516	5′ CGGTGAGCGCAATGTTC 3′	5′ CCAAGTGGGTGTCTCCA 3′
Ht_HSP90	AM283515	5′ CCAGGAAGAATATGCCGAGT 3′	5′ CACGGAACTCCAACTGACC 3′

[a] Used to normalize mRNA.

genomic DNA contamination, the total RNA was digested with DNAse I Amplification Grade (Sigma-Aldrich) and an aliquot of DNAse-treated RNA was subjected to PCR with actin primers to check for the absence of genomic DNA.

Reverse transcription was carried out using 500 ng of total RNA treated with DNAse I, 0.5 µg random primers (Promega), 200 U moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega), 12.5 µmol RNAse-free dNTP, and 25 U recombinant RNAsin (Promega). The sequences of the forward and reverse primers for HSP70 and HSP90 mRNA quantification were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Eurogentec (Table 3). Their efficiency was tested using the standard curve method. A dilution range was
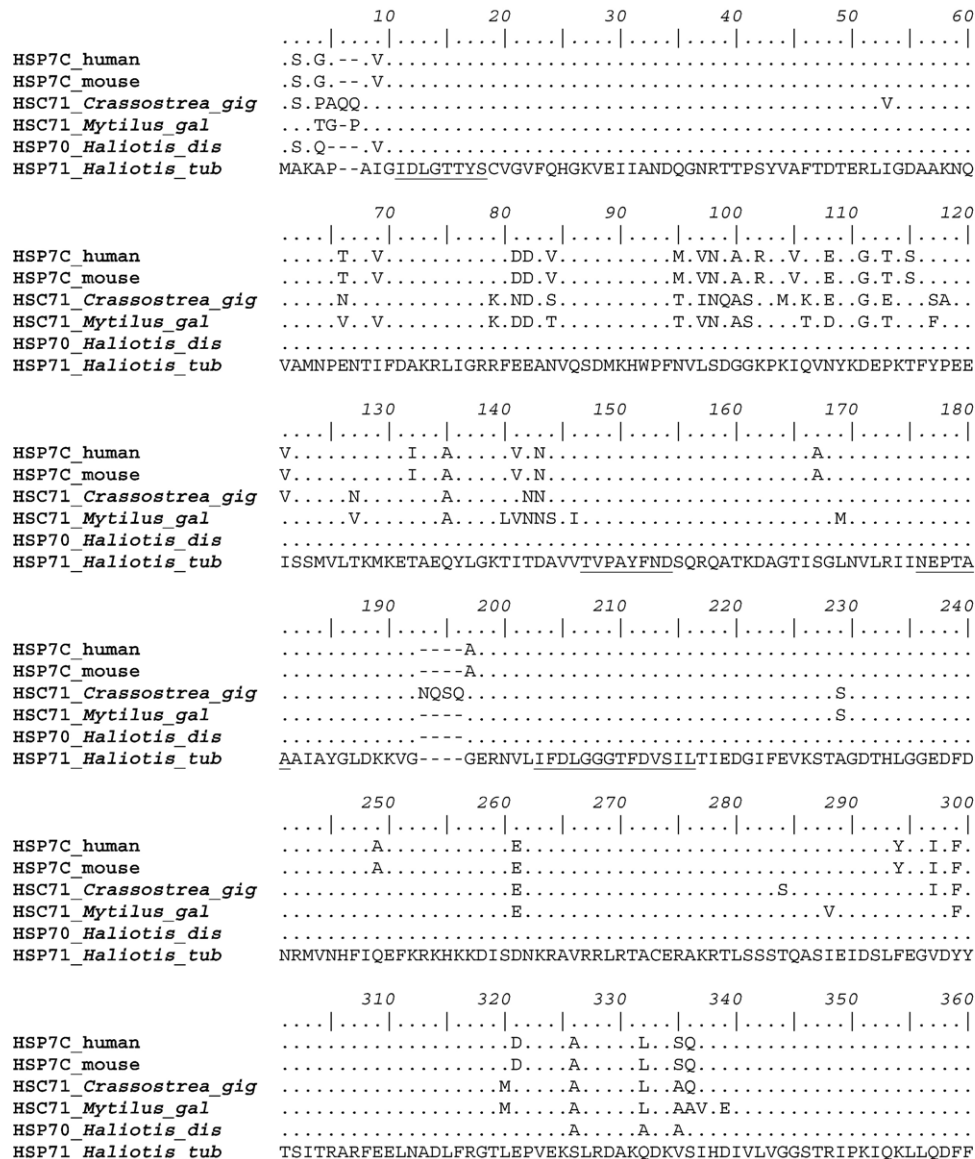
```
                              10        20        30        40        50        60
                       ....|....|....|....|....|....|....|....|....|....|....|....|
        HSP7C_human    .S.G.--.V...................................................
        HSP7C_mouse    .S.G.--.V...................................................
    HSC71_Crassostrea_gig    .S.PAQQ.........................................V......
    HSC71_Mytilus_gal  ...TG-P.....................................................
    HSP70_Haliotis_dis .S.Q---.V...................................................
    HSP71_Haliotis_tub MAKAP--AIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ

                              70        80        90        100       110       120
                       ....|....|....|....|....|....|....|....|....|....|....|....|
        HSP7C_human    .....T..V..........DD.V..........M.VN.A.R..V..E..G.T.S.....
        HSP7C_mouse    .....T..V..........DD.V..........M.VN.A.R..V..E..G.T.S.....
    HSC71_Crassostrea_gig    .....N...........K.ND.S..........T.INQAS..M.K.E..G.E...SA..
    HSC71_Mytilus_gal  .....V..V.........K.DD.T..........T.VN.AS....T.D..G.T...F...
    HSP70_Haliotis_dis .............................................................
    HSP71_Haliotis_tub VAMNPENTIFDAKRLIGRRFEEANVQSDMKHWPFNVLSDGGKPKIQVNYKDEPKTFYPEE

                              130       140       150       160       170       180
                       ....|....|....|....|....|....|....|....|....|....|....|....|
        HSP7C_human    V...........I..A.....V.N......................A..............
        HSP7C_mouse    V...........I..A.....V.N......................A..............
    HSC71_Crassostrea_gig    V.....N.......A.....NN......................................
    HSC71_Mytilus_gal  .......V.......A....LVNNS.I.......................M..........
    HSP70_Haliotis_dis .............................................................
    HSP71_Haliotis_tub ISSMVLTKMKETAEQYLGKTITDAVVTVPAYFNDSQRQATKDAGTISGLNVLRIINEPTA

                              190       200       210       220       230       240
                       ....|....|....|....|....|....|....|....|....|....|....|....|
        HSP7C_human    ..............----A.........................................
        HSP7C_mouse    ..............----A.........................................
    HSC71_Crassostrea_gig    ..........NQSQ.........................S...........
    HSC71_Mytilus_gal  ..........----.........................S...........
    HSP70_Haliotis_dis ..........----..............................................
    HSP71_Haliotis_tub AAIAYGLDKKVG----GERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFD

                              250       260       270       280       290       300
                       ....|....|....|....|....|....|....|....|....|....|....|....|
        HSP7C_human    .......A...........E...........................Y..I.F.
        HSP7C_mouse    .......A...........E...........................Y..I.F.
    HSC71_Crassostrea_gig    ...................E...............S............I.F.
    HSC71_Mytilus_gal  ...................E...................V.........F.
    HSP70_Haliotis_dis .............................................................
    HSP71_Haliotis_tub NRMVNHFIQEFKRKHKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLFEGVDYY

                              310       320       330       340       350       360
                       ....|....|....|....|....|....|....|....|....|....|....|....|
        HSP7C_human    .....................D....A.....L..SQ.................
        HSP7C_mouse    .....................D....A.....L..SQ.................
    HSC71_Crassostrea_gig    ...................M....A.....L..AQ.................
    HSC71_Mytilus_gal  ...................M.....A.....L..AAV.E..............
    HSP70_Haliotis_dis .........................A.....A..A...................
    HSP71_Haliotis_tub TSITRARFEELNADLFRGTLEPVEKSLRDAKQDKVSIHDIVLVGGSTRIPKIQKLLQDFF
```

Fig. 1. Sequence alignments (ClustalW v 1.83). *Haliotis tuberculata* HSP71 protein (*CAK95236*) shares 87% homology with human (*P11142*) and mouse HSC71 protein (*P63017*), 88% with *Crassostrea gigas* HSC71 (*BAD15287*), 88% with *Mytilus galloprovincialis* HSC71 (*CAH0410*) and 96% with *Haliotis discus hannai* HSP70 (*ABC54952*). The characteristic motifs of the HSP70 family are underlined. The characteristic motifs of cytosolic protein are boxed in grey.

```
                            370       380       390       400       410       420
                      ....|....|....|....|....|....|....|....|....|....|....|....|
HSP7C_human           .....N....................S....N............................
HSP7C_mouse           .....N....................S....N............................
HSC71_Crassostrea_gig .....N....................S...........................N..
HSC71_Mytilus_gal     .....N....................S...........................A..
HSP70_Haliotis_dis    ............................................................
HSP71_Haliotis_tub    NGKELCKSINPDEAVAYGAAVQAAILHGDKSEEVQDLLLLDVTPLSLGIETAGGVMTVLI

                            430       440       450       460       470       480
                      ....|....|....|....|....|....|....|....|....|....|....|....|
HSP7C_human           ...................................Y...........L...........
HSP7C_mouse           ...................................Y...........L...........
HSC71_Crassostrea_gig ...................................Y...........L...........
HSC71_Mytilus_gal     ...................................Y...........L...........
HSP70_Haliotis_dis    ...................................Y........................
HSP71_Haliotis_tub    KRNTTIPTKQTQTFTTYSDNQPGVLIQVFEGERAMTKDNNILGKFELTGIPPAPRGVPQI

                            490       500       510       520       530       540
                      ....|....|....|....|....|....|....|....|....|....|....|....|
HSP7C_human           ...............................G..............D.....Q...K........R.
HSP7C_mouse           ...............................G..............D.....Q...K........R.
HSC71_Crassostrea_gig ...............................G...........D..D.......K..Q.....RE
HSC71_Mytilus_gal     ...............................G...............D..K........
HSP70_Haliotis_dis    ............................................................
HSP71_Haliotis_tub    EVTFDIDANGILNVSAVDKSTMKENKITITNDKGRLSKEEIERMVNEAAENYKAEDEKQKD

                            550       560       570       580       590       600
                      ....|....|....|....|....|....|....|....|....|....|....|....|
HSP7C_human           KVSS..S.........A........QG..NDE..QK.L....EI.N...K..T...E....
HSP7C_mouse           KVSS..S.........A........QG..NDE..QK.L....EI.....K..T...E....
HSC71_Crassostrea_gig ..A................D...........G.....L...EEI.K.M.Q....D.E....
HSC71_Mytilus_gal     ..T...S....S....Q.................S...E.M...DEI.K...A.N....E....
HSP70_Haliotis_dis    ............................................................
HSP71_Haliotis_tub    RIQAKNGLESYAFNMKSTVEDEKLKDKISEDDKKTITDKCNDVISWLDSNQLAEKDEFEH

                            610       620       630       640       650       660
                      ....|....|....|....|....|....|....|....|....|....|....|....|
HSP7C_human           Q.....K...........S...MP-....GG-F..---G..P---------.S..A.S...
HSP7C_mouse           Q.....K...........S...MP-....GG-F..---G..P---------.S..A.S...
HSC71_Crassostrea_gig ...................S...PG....--GGMPNFGG...P.G.AP----G...G....
HSC71_Mytilus_gal     ...................S....PG......GGA.GAPG..P.S.----------....
HSP70_Haliotis_dis    ..........................--....GG-M..GMPG....LP.GADGQ.........
HSP71_Haliotis_tub    KQKELEGVCNPIITKLYQAAGGA--GGMPNF-NPG--AAGAGGAGG---APTGGSSGGPT

                            ....|
HSP7C_human           .....
HSP7C_mouse           .....
HSC71_Crassostrea_gig .....
HSC71_Mytilus_gal     .....
HSP70_Haliotis_dis    .....
HSP71_Haliotis_tub    IEEVD
```

Fig. 1 (*continued*).

performed for each pair of primers to obtain a standard curve by plotting the Cycle threshold as a function of the log of the total amount of reverse-transcribed initial RNA. The primers that showed a good level of efficiency ($100 \pm 5\%$) on a range of 4 orders of magnitude of cDNA concentrations were conserved for quantification.

Real-time PCR was performed in a MiQ Cycler (Biorad). All determinations were carried out in duplicate. Controls of no template cDNA were included in the PCR experiments. Amplification was carried out in 96-well plates, in a total volume of 15 μl containing 7.5 μl of 2X iQ SYBR® Green supermix (Biorad), each primer (500 nM final) and cDNA samples obtained from reverse transcription of 5 ng of total RNA. The amplification conditions were 40 cycles of 15 s at 95 °C and 45 s at 60 °C, followed by the protocol for the melting curve: 80 cycles of 10 s with an increase of 0.5 °C between each cycle from 55 °C to 95 °C. The melting curve was used to check if the amplification products had the expected Tm.

The mRNA encoding the cytoskeleton protein actin was used as a reference to normalize HSP mRNA. The expression levels of HSP70 and HSP90 were quantified as the ratios between their mRNA level and actin mRNA. Although actin is often used as a reference, its expression was already shown to be affected under certain experimental conditions such as radiation exposure (Ropenga et al., 2004). The protocol used from RNA quantification to cDNA synthesis was correctly standardized (resulting in a reproducible amount of cDNA). As a result, in the given experimental conditions and tissue, cycle threshold (Ct) for actin, i.e. the number of cycles at which the fluorescence rises appreciably above the background fluorescence, should have been constant if actin was constantly expressed. We checked that the actin variation coefficient Ct was below 10%. This variation

is very low compared to the differential Ct between target mRNA and reference mRNA. Consequently, we can discard the possibility that the observed variation pattern in HSP expression is due to the variation of the reference actin mRNA itself.

2.7. Data analysis

The sequences were analyzed using Infobiogen online tools TRADUC, BLASTP, CLUSTALW, FORPUB (http://www. infobiogen.fr/services/analyse/analyse.php) and the software Bioedit Sequence Alignment Editor v.7.0.4.1 (Hall, 1999).

The statistical analysis was carried out using Statgraphics Centurion XV software (StatPoint, Inc., http://www.statgraphics.com/). Values were expressed as means $\pm$ SD. Since mRNA expression data corresponded to ratios, statistically significant differences between control and heat-shocked hemocytes were determined on log-transformed data using a parametric t-test; $p \leq 0.05$ was accepted as significant and
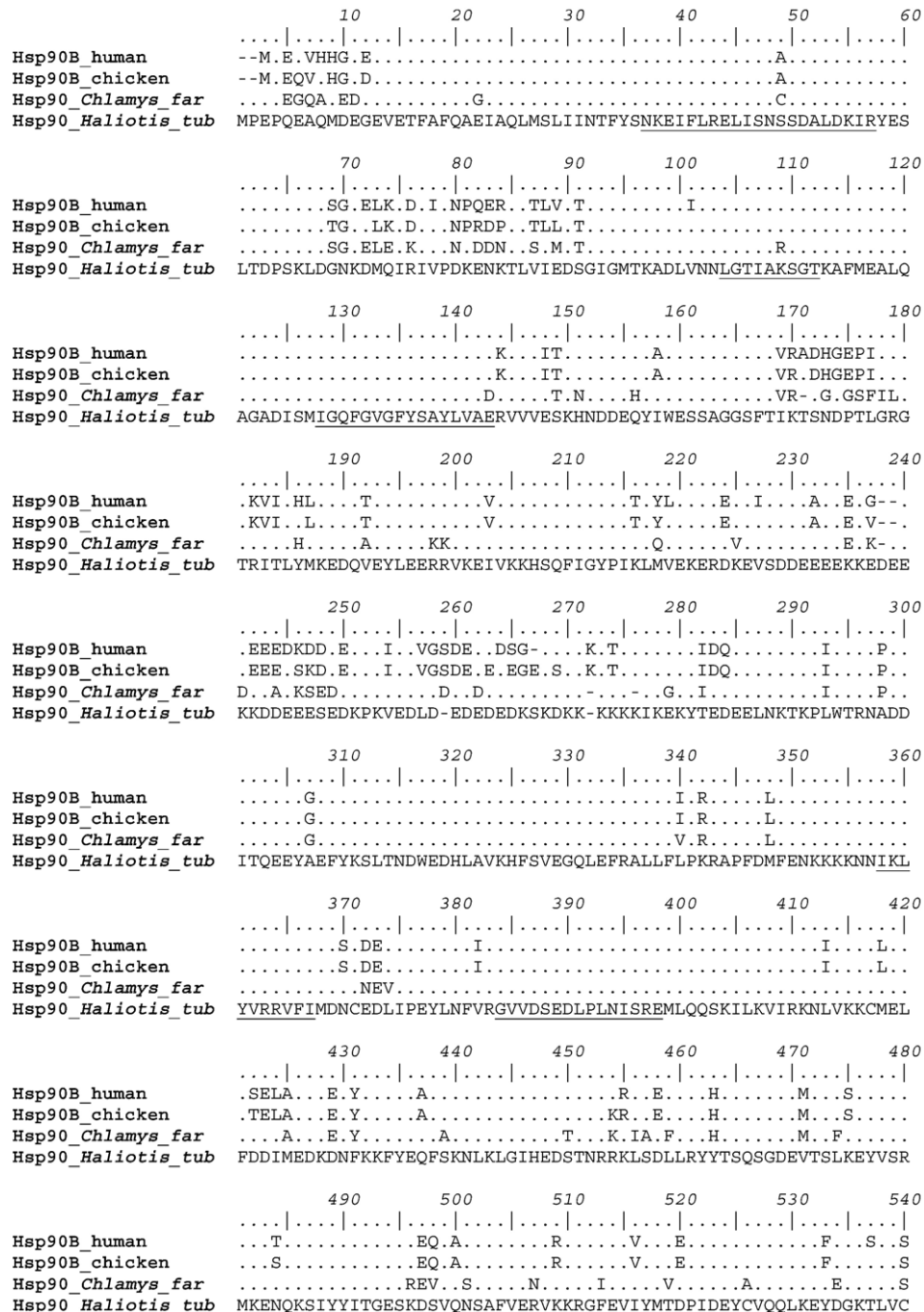
```
                         10        20        30        40        50        60
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human       --M.E.VHHG.E.........................................A...........
Hsp90B_chicken     --M.EQV.HG.D.........................................A...........
Hsp90_Chlamys_far  ....EGQA.ED.........G................................C...........
Hsp90_Haliotis_tub MPEPQEAQMDEGEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNSSDALDKIRYES

                         70        80        90       100       110       120
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human       ........SG.ELK.D.I.NPQER..TLV.T.........I....................
Hsp90B_chicken     ........TG..LK.D...NPRDP..TLL.T.............................
Hsp90_Chlamys_far  ........SG.ELE.K...N.DDN..S.M.T.........R...................
Hsp90_Haliotis_tub LTDPSKLDGNKDMQIRIVPDKENKTLVIEDSGIGMTKADLVNNLGTIAKSGTKAFMEALQ

                        130       140       150       160       170       180
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human       ......................K...IT........A..........VRADHGEPI...
Hsp90B_chicken     ......................K...IT........A..........VR.DHGEPI...
Hsp90_Chlamys_far  .....................D.....T.N....H............VR-.G.GSFIL.
Hsp90_Haliotis_tub AGADISMIGQFGVGFYSAYLVAERVVVESKHNDDEQYIWESSAGGSFTIKTSNDPTLGRG

                        190       200       210       220       230       240
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human       .KVI.HL....T..........V............T.YL....E..I....A..E.G--.
Hsp90B_chicken     .KVI..L....T..........V............T.Y.....E.......A..E.V--.
Hsp90_Chlamys_far  .....H.....A.....KK.................Q........V.........E.K-..
Hsp90_Haliotis_tub TRITLYMKEDQVEYLEERRVKEIVKKHSQFIGYPIKLMVEKERDKEVSDDEEEEKKEDEE

                        250       260       270       280       290       300
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human       .EEEDKDD.E...I..VGSDE..DSG-....K.T.......IDQ........I....P..
Hsp90B_chicken     .EEE.SKD.E...I..VGSDE.E.EGE.S..K.T.......IDQ........I....P..
Hsp90_Chlamys_far  D..A.KSED.........D..D..........-...-..G..I..........I....P..
Hsp90_Haliotis_tub KKDDEEESEDKPKVEDLD-EDEDEDKSKDKK-KKKKIKEKYTEDEELNKTKPLWTRNADD

                        310       320       330       340       350       360
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human       ......G.................................I.R.....L...........
Hsp90B_chicken     ......G.................................I.R.....L...........
Hsp90_Chlamys_far  ......G.................................V.R.....L...........
Hsp90_Haliotis_tub ITQEEYAEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFLPKRAPFDMFENKKKKNNIKL

                        370       380       390       400       410       420
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human       .........S.DE........I...............................I....L..
Hsp90B_chicken     .........S.DE........I...............................I....L..
Hsp90_Chlamys_far  ...........NEV..............................................
Hsp90_Haliotis_tub YVRRVFIMDNCEDLIPEYLNFVRGVVDSEDLPLNISREMLQQSKILKVIRKNLVKKCMEL

                        430       440       450       460       470       480
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human       .SELA...E.Y.....A.................R..E....H.......M...S.....
Hsp90B_chicken     .TELA...E.Y.....A...............KR..E....H.......M...S.....
Hsp90_Chlamys_far  ....A...E.Y.....A...........T..K.IA.F....H.......M..F.....
Hsp90_Haliotis_tub FDDIMEDKDNFKKFYEQFSKNLKLGIHEDSTNRRKLSDLLRYYTSQSGDEVTSLKEYVSR

                        490       500       510       520       530       540
                   ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human       ...T...........EQ.A........R......V...E...........F...S..S
Hsp90B_chicken     ...S...........EQ.A........R......V...E...........F....S
Hsp90_Chlamys_far  ..............REV..S.....N.....I.....V......A.......E....S
Hsp90_Haliotis_tub MKENQKSIYYITGESKDSVQNSAFVERVKKRGFEVIYMTDPIDEYCVQQLKEYDGKTLVC
```

Fig. 2. Sequence alignments (ClustalW v 1.83). *Haliotis tuberculata* HSP90 protein (*CAK95235*) shares 80% homology with human HSP90-beta (*AAH09206*), 78% with chicken HSP90-beta (*Q04619*) and 82% with *Chlamys farreri* HSP90 (*AAR11781*). The characteristic motifs of the HSP90 family are underlined. The characteristic motifs of cytosolic protein are boxed in grey.

```
                            550        560        570        580        590        600
                       ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human           .................M..S..K...N...L............TI.....S........T
Hsp90B_chicken         ................NM..S..K..T...L............TI.....S........T
Hsp90_Chlamys_far      ..............RF...T.EY......V...........T..................
Hsp90_Haliotis_tub     VTKEGLELPEDEEKKKLEEAKAQFEGLCKVMKEILDKKVEKVVVSNRLVTSPCCIVTSQ

                            610        620        630        640        650        660
                       ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human           ...T.................N......M.............E..RQ..E..........V
Hsp90B_chicken         ...T.................N......M.............E..RQ....N.........V
Hsp90_Chlamys_far      ...................S....C.............A.I.S.....GL.....S....V
Hsp90_Haliotis_tub     YGWSANMERIMKAQALRDTSTMGYMAAKKHLEINPDHPIVKTLKEKADADKNDKAVKDLC

                            670        680        690        700        710        720
                       ....|....|....|....|....|....|....|....|....|....|....|....|
Hsp90B_human           V.....A..S........QT.S...Y............EVAA.EPNAAVP..I.......
Hsp90B_chicken         V.....A..S........QT.S...Y............EVIA.ESSIAPP..I.......
Hsp90_Chlamys_far      L......M........E.GT................D..SGAPE.SDENVE.P.......
Hsp90_Haliotis_tub     MLLFETSLLASGFSLEDPTSHANRIHRMIKLGLGIDEDDIPTEATAESATDEMPPLEGDE

                            730
                       ....|....|
Hsp90B_human           .-........
Hsp90B_chicken         .-T.......
Hsp90_Chlamys_far      ..........
Hsp90_Haliotis_tub     DDASRMEEVD
```

Fig. 2 (*continued*).

indicated by the symbol *; ** indicated $p \leq 0.01$; *** indicated $p \leq 0.001$.

3. Results

3.1. Abalone HSP cloning

The complete procedure for cloning and sequencing was repeated three times independently and led to the identification of one sequence each of HSP70 and HSP90 cDNA from the gastropod *H. tuberculata*. The complete Ht_HSP70 and Ht_HSP90 cDNA sequences are available in the EMBL databank (GenBank accession no. AM283516 and AM283515, respectively). For HSP70, a 1953 bp open reading frame which encoded a predicted 651 amino acid polypeptide with a calculated molecular mass of 71 kD cDNA was obtained. For HSP90, the cDNA corresponded to a 2145 bp open reading frame which encoded a predicted 736 amino acid polypeptide with a calculated molecular mass of 84 kD. Nucleic acid sequence analysis revealed the presence of typical ATG start and TAA or TGA stop codons, a putative polyadenylation signal starting at nucleotide 2235 or 2202 for HSP70 or HSP90 cDNA, respectively.

The alignments of the deduced amino acid sequence of *H. tuberculata* HSP70 and HSP90 with metazoan HSP families are shown in Figs. 1 and 2, respectively. A Clustal–Wallis alignment performed on the newly identified HSP proteins indicated that HSP70 and HSP90 proteins from *H. tuberculata* shared high homology with other identified sequences, including higher vertebrate HSP. Indeed, the HSP70 and HSP90 proteins from abalone possessed 87% homology with human HSC71 and 80% homology with human HSP90AB1 protein, respectively.

The deduced amino acid sequences of both Ht_HSP70 and Ht_HSP90 exhibited the characteristic motifs of their respective families. The highly conserved region in both HSP70 and

HSP90 is the amino-terminus (N-term), where an ATP binding domain is located. The carboxy-terminus (C-term) is more divergent as it contains the substrate binding domains that recognize a vast array of proteins. For HSP70, the signature sequences, outlined in Fig. 1, are IDLGTTYS, TVPAYFND, NEPTAA according to Rensing and Maier (1994), and DLGGG according to Gupta and Golding (1993). The consensus pattern GPTIEEVD at the C-terminal end is a specific sequence for cytosolic HSP70 (Boorstein et al., 1994; Demand et al., 1998). The sequence RARFEEL is a signature for eukaryotic non-organelle stress-70 protein according to Rensing and Maier (1994). The Ht_HSP90 protein exhibits the sequences NKEIFL-RELISNSSDALDKIR, LGTIAKSGT, IGQFGVGFYSAYL-VAE, IKLYVRRVFI, GVVDSEDLPLNISRE characteristic of the HSP90 family according to Gupta (1995). It also contains the C-terminal MEEVD sequence, which is a characteristic motif of cytosolic HSP members (Fig. 2).
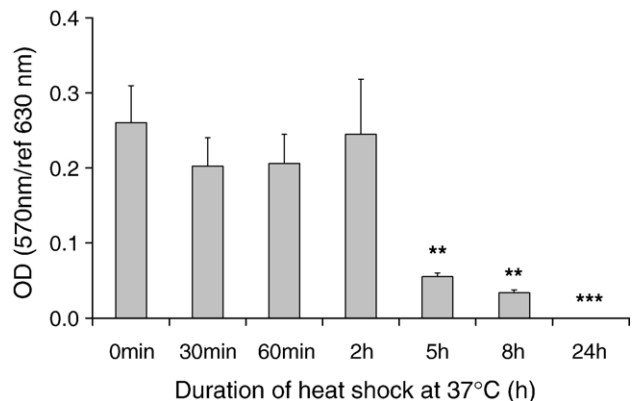

Fig. 3. MTT viability test on primary cultures of hemocytes maintained at 37 °C for a period of 24 h ($n=3$, mean±SD). ** Indicates a significant difference compared to control at time zero ($p<0.01$, *t*-test). *** $p<0.001$.
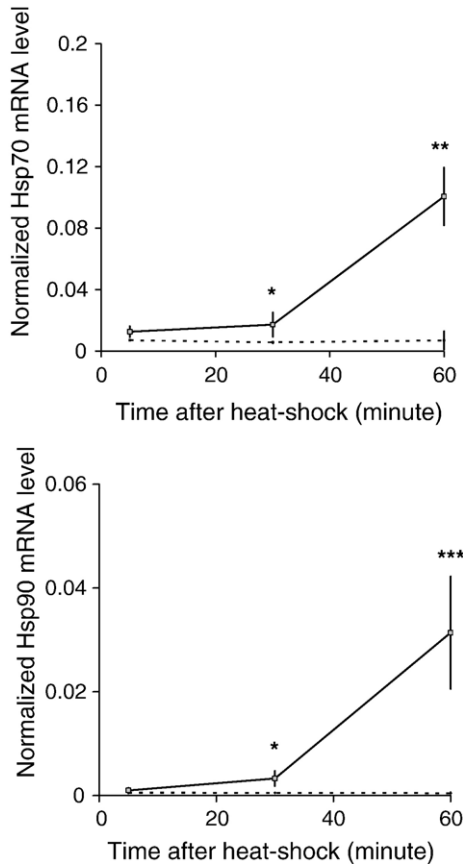
Fig. 4. HSP70 (top) and HSP90 (bottom) mRNA induction kinetics during a 1-h heat-shock at 37 °C ($n=4$; mean±SD). Solid line: heat-shocked hemocytes, dotted line: control hemocytes maintained at 17 °C. * Indicates a significant difference between heat-shocked hemocytes and control hemocytes ($p<0.05$, t-test). ** $p<0.01$. *** $p<0.001$.

3.2. mRNA over-expression following thermal stress in hemocyte primary culture

3.2.1. Determination of cell viability (MTT assay)

The MTT viability test (Fig. 3) indicated that cell viability was maintained for 2 h at 37 °C and then decreased, confirming that 1 h at 37 °C is a sub-lethal heat-shock. From this result, cells were assumed to be viable after 1 h at 17 °C and 25 °C.
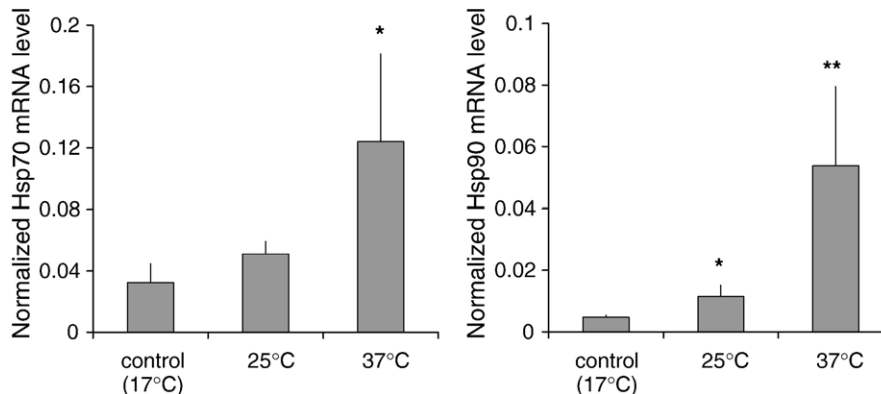
3.2.2. Messenger RNA expression

In the first step, the kinetics of the HSP70 and HSP90 mRNA level changes were investigated. As shown in Fig. 4, HSP mRNA levels were significantly elevated in response to an acute thermal stress. A significant increase of mRNA occurs from 30 min onwards ($n=4$, $p<0.05$, t-test). After 1 h at 37 °C, induction factors between heat-shocked and control cells maintained at 17 °C were about 4-fold for HSP70 and 11-fold for HSP90 ($n=4$, $p<0.01$, t-test).

Repeating the experiment with a heat-shock at 25 °C showed that there is a direct positive correlation between the temperature and the HSP mRNA level (Fig. 5). After 1 h of incubation at 25 °C, the HSP90 mRNA level was significantly increased (2 fold) compared to the control but it increased much more at 37 °C.

4. Discussion

In the present work, the deduced amino acid sequences from the HSP cDNA cloned in abalone hemocytes showed high homology with HSP amino acid sequences from other organisms, including higher vertebrates. Additional lines of evidence supporting their belonging to the HSP family include the identification of several eukaryotic HSP70 and HSP90 signature amino acid sequences (Gupta and Golding, 1993; Gupta and Singh, 1994; Rensing and Maier, 1994; Gupta, 1995). The presence of consensus motifs characteristic of cytoplasmic HSP (Boorstein et al., 1994; Demand et al., 1998) suggests that both identified *H. tuberculata* HSP70 and HSP90 have a cytosolic localization. The cloning and sequencing procedure led to the identification of one sequence each of abalone HSP70 and HSP90 but it must be pointed out that some other isoforms may exist in *H. tuberculata*.

Studies in molluscs emphasized the existence of inducible or constitutive HSP70 isoforms, especially in oysters: *Crassostrea gigas* (Clegg et al., 1998; Gourdon et al., 2000; Boutet et al., 2003b), *Ostrea edulis* (Piano et al., 2002, 2004, 2005; Boutet et al., 2003a) and *Crassostrea virginica* (Tirard et al., 1995). The heat-induced expression of HSP70 isoforms that are absent in control conditions seems to be an original feature of oysters, not reported in other bivalves. Actually, it is more likely that these

Fig. 5. HSP70 (left) and HSP90 (right) mRNA induction after 1 h at 17 °C (control temperature), 25 °C and 37 °C ($n=4$; mean±SD). * Indicates a significant difference between heat-shocked hemocytes and control hemocytes ($p<0.05$, t-test). ** $p<0.01$.

heat-inducible isoforms are present at very low abundance rather than completely absent from control animals. The expression of an apparently inducible HSP70 under control conditions could reflect a difference between abalone and other molluscs, or some unknown stress from which cells had not recovered completely during the 24 h prior to experimentation. In our study, real-time PCR measurements showed that the cloned HSP70 and HSP90 cDNA from *H. tuberculata* are constitutively expressed at baseline temperatures, and are transcriptionally up-regulated when the temperature is increased. This heat-inducible property of these genes confirms that they belong to the HSP family. The question as to whether the sequenced cDNA corresponds to a constitutive or an inducible isoform remains open.

The relation between the HSP70 multigene family and heat stress has been well documented in molluscs. Laursen et al. (1997) subjected a molluscan cell line, originally derived from embryos of the freshwater snail *Biomphalaria glabrata*, to temperature stress and showed the induction of synthesis of several proteins, including one of approximately 70 kD, belonging to the HSP70 gene family. Hemocytes of the oyster *C. virginica* exhibit a strong heat-shock response when subjected to acute temperature elevation, characterized by enhanced synthesis of several proteins, especially one with a molecular mass of 70 kD (Tirard et al., 1995). An increase in HSP70 was observed in heat-shocked oysters *C. gigas*, *C. virginica* and *O. edulis* (Clegg et al., 1998; Gourdon et al., 2000; Rathinam et al., 2000; Hamdoun et al., 2003) and abalones, *Haliotis rufescens* (Snyder et al., 2001) and *Haliotis rubra* (Drew et al., 2001). Our results showed a relationship between the HSP70 mRNA level and the increase of temperature which is in agreement with those studies.

In comparison, the involvement of HSP90 in heat-shock response has not been studied well. Although there exist a few detailed molecular analyses of the genes or mRNA transcripts encoding these proteins, 90-kD stress protein responses in molluscan cells have not been reported. However in octocoral, the expression of the HSP90 gene is up-regulated by thermal stress. Treatment of the animals for 2 h at 4 °C below or above the ambient temperature resulted in a 4.5-fold increase of the mRNA compared to the steady-state level (Wiens et al., 2000). This latter result is in accordance with the transcriptional changes of HSP90 expression level observed in this study.

It is known that natural fluctuation in environmental temperatures and several other physical and chemical parameters can result in the induction of some of the cellular stress responses in mussels and marine snails, in which the HSP family plays an important role (Hofmann and Somero, 1995; Chapple et al., 1998; Tomanek and Somero, 1999; Minier et al., 2000; Hofmann et al., 2002). But, the HSP level is not only an indicator of thermal exposure. Subtidal mussels (*Mytilus* sp.) expressed higher levels of HSP70 in winter than in summer (Roberts et al., 1997). Similarly, Encomio and Chu (2005) observed that total amounts of HSP70 did not correlate positively with seasonal variations in temperature in the oyster *C. virginica*. We monitored the seasonal pattern of HSP family members (HSP70, HSC72, HSP90) at the transcriptional level in *C. gigas*. We found strong seasonal variations mainly characterized by a high HSP mRNA level in winter, between January and March, and a smaller increase in summer between June and August (Farcy et al., in press). Considering those seasonal variations in HSP levels, it may be expected that some differences exist in the response to thermal stress, depending on season. Finally, HSP levels might be differently affected by an acute thermal stress and the gradually increase in the average temperature occurring between winter and summer. Other factors, such as neuroendocrine signaling (Lacoste et al., 2001) or salinity (Werner and Hinton, 2000; Drew et al., 2001), may alter expression of HSP70. Indeed, variable salinity could have a significant effect on the heat-shock response in oysters. In oysters acclimated to lower salinities, temperature effects were more pronounced than in oysters acclimated to higher salinities (Shumway and Koehn, 1982). Kuhl et al. (1992) demonstrated that heat-shock, glucose deprivation, or serotonin exposure in the marine gastropod *Aplysia californica* trigger the synthesis of several nervous system proteins including two HSP70s. An increase in HSP70 concentration was also observed in cadmium-contaminated mussels, *M. edulis*, and limpets, *Collisella pelta* (Sanders, 1988). Conversely, Veldhuizen-Tsoerkan et al. (1991) found no variation in HSP70 levels in sea mussels, *M. edulis*, caged in seawater with various concentrations of cadmium. Snyder et al. (2001) reported a significant increase of HSP90 protein in response to heptachlor exposure in the abalone *H. rufescens*.

In fish, the transcription of HSP90 was found to be partially modulated by plasma cortisol level in primary culture of rainbow trout hepatocytes (Sathiyaa et al., 2001). In early life stages of gilthead sea bream and rainbow trout, food deprivation was also shown to enhance HSP70 and HSP90 protein expression (Cara et al., 2005). As reviewed by Kregel (2002), in mammals, it has been noted that HSPs can also play a role in apoptosis. HSP27, HSP70 and HSP90 proteins are predominantly anti-apoptotic, whereas HSP60 is proapoptotic. Moreover, it appears that these HSPs function at multiple points in the apoptotic signaling pathway to elicit this response (Garrido et al., 2001).

Although numerous studies have used HSP protein levels as a biomarker, to our knowledge, there are few reports showing relative levels of the biomarker transcript (Laursen et al., 1997; Piano et al., 2004, 2005). As underlined by Kregel (2002) in higher vertebrate models, an important consideration regarding HSP70 regulation involves the apparent discordance between transcription of the message and HSP70 translation. There is evidence suggesting that transcriptional activation of the HSP70 gene may be not be paralleled by protein synthesis. For instance, in cell culture experiments, HSP70 mRNA can increase in response to a stress challenge, although there is little HSP70 protein produced (Bruce et al., 1993; Hensold et al., 1990). A follow up of this work on the characterization of abalone HSP family members will be to investigate the response of hemocytes to thermal stress at the protein level.

Acknowledgement

References

Bachère, E., Chagot, D., Grizel, H., 1988. Separation of *Crassostrea gigas* hemocytes by density gradient centrifugation and counterflow centrifugal elutriation. Dev. Comp. Immunol. 12, 549–559.

Boorstein, W.R., Ziegelhoffer, T., Craig, E.A., 1994. Molecular evolution of the HSP70 multigene family. J. Mol. Evol. 38, 1–17.

Boutet, I., Tanguy, A., Moraga, D., 2003a. 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.

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

Bruce, J.L., Price, B.D., Coleman, N., Calderwood, S.K., 1993. Oxidant injury rapidly activates the heat shock transcription factor but fails to increase levels of heat shock proteins. Cancer Res. 53, 12–15.

Bukau, B., Horwich, A.L., 1998. The Hsp70 and Hsp60 chaperone machines. Cell 92, 351–366.

Cara, J.B., Aluru, N., Moyano, F.J., Vijayan, M.M., 2005. Food-deprivation induces HSP70 and HSP90 protein expression in larval gilthead sea bream and rainbow trout. Comp. Biochem. Physiol. B 142, 426–431.

Chapple, J.P., Smerdon, G.R., Hawkins, A.J.S., 1997. Stress-70 protein induction in *Mytilus edulis*: tissue-specific responses to elevated temperature reflect relative vulnerability and physiological function. J. Exp. Mar. Biol. Ecol. 217, 225–235.

Chapple, J.P., Smerdon, G.R., Berry, R.J., Hawkins, A.J.S., 1998. Seasonal changes in stress-70 protein levels reflect thermal tolerance in the marine bivalve *Mytilus edulis* L. J. Exp. Mar. Biol. Ecol. 229, 53–68.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Clegg, J.S., Uhlingher, K.R., Jackson, S.A., Cherr, G.N., Rifkin, E., Friedman, C.S., 1998. Induced thermotolerance and heat shock protein-70 family in Pacific oyster *Crassostrea gigas*. Mol. Mar. Biol. Biotechnol. 7, 21–30.

Craig, E.A., Ingolia, T.D., Manseau, L.J., 1983. Expression of *Drosophila* heat-shock cognate genes during heat-shock and development. Dev. Biol. 99, 418–426.

Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z., Nardai, G., 1998. The 90-kDa molecular chaperone family: structure, function and clinical applications. A comprehensive review. Pharmacol. Ther. 79, 129–168.

Czar, M.J., Welsh, M.J., Pratt, W.B., 1996. Immunofluorescence localization of the 90-kDa heat-shock protein to cytoskeleton. Eur. J. Cell Biol. 70, 322–330.

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

Domart-Coulon, I., Doumenc, D., Auzoux-Bordenave, S., Le Fichant, Y., 1994. Identification of media supplements that improve the viability of primarily cell cultures of *Crassostrea gigas* oysters. Cytotechnology 16, 109–120.

Drew, B., Miller, D., Toop, T., Hanna, P., 2001. Identification of expressed Hsp's in blacklip abalone (*Haliotis rubra*, Leach) during heat and salinity stresses. J. Shellfish Res. 20, 695–703.

Encomio, V.G., Chu, F.L.E., 2005. Seasonal variation of heat shock protein 70 in eastern oysters (*Crassostrea virginica*) infected with *Perkinsus marinus* (Dermo). J. Shellfish Res. 24, 167–175.

Farcy, E., Voiseux, C., Lebel, J.M., Fiévet B., in press. 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.

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

Frydman, J., 2001. Folding of newly translated proteins in vivo: the role of molecular chaperones. Annu. Rev. Biochem. 70, 603–649.

Garnier, C., Barbier, P., Gilli, R., Lopez, C., Peyrot, V., Briand, C., 1998. Heat-shock protein 90 (hsp90) binds in vitro to tubulin dimer and inhibits microtubule formation. Biochem. Biophys. Res. Commun. 250, 414–419.

Garrido, C., Gurbuxani, S., Ravagnan, L., Kroemer, G., 2001. Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem. Biophys. Res. Commun. 286, 433–442.

Gething, M.J., Sambrook, J., 1992. Protein folding in the cell. Nature 355, 33–45.

Gornati, R., Papis, E., Rimordi, S., Trova, G., Sbroglia, M., Bernardini, G., 2004. Rearing density influences the expression of the stress-related genes in sea bass (*Dicentrarchus labrax*, L.). Gene 341, 111–118.

Gourdon, I., Guérin, M.C., Torreilles, J., 1998. Cellular and molecular mechanisms of the stress response from marine bivalvia. C.R. Soc. Biol. 192, 749–774.

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

Gupta, R.S., 1995. 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.

Gupta, R.S., Golding, G.C., 1993. Evolution of Hsp70 gene and its implications regarding relationships between archaebacteria, eubacteria, and eukaryotes. J. Mol. Evol. 37, 573–582.

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

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95–98.

Hartl, F.U., 1996. Molecular chaperones in cellular protein folding. Nature 381, 571–580.

Hamdoun, A., Cheney, D., Cherr, G., 2003. 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.

Hensold, J.O., Hunt, C.R., Calderwood, S.K., Housman, D.E., Kingston, R.E., 1990. DNA binding of heat shock factor to the heat shock element is insufficient for transcriptional activation in murine erythroleukemia cells. Mol. Cell. Biol. 41, 1600–1608.

Hightower, L.E., 1993. A brief perspective on the heat-shock response and stress proteins. Mar. Environ. Res. 35, 79–83.

Hofmann, G., Somero, G., 1995. 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.

Hofmann, G.E., Buckley, B.A., Place, S.P., Zippay, M.L., 2002. Molecular chaperones in ectothermic marine animals: biochemical function and gene expression. Integr. Comp. Biol. 42, 808–814.

Ingolia, T.D., Craig, E.A., 1982. *Drosophila* gene related to the major heat shock-induced gene is transcribed at normal temperatures and not induced by heat shock. Proc. Natl. Acad. Sci. U S A 79, 525–529.

Kregel, K.C., 2002. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J. Appl. Physiol. 92, 2177–2186.

Kuhl, D., Kennedy, T.E., Barzilai, A., Kandel, E.R., 1992. Long-term sensitization training in *Aplysia* leads to an increase in the expression of BiP, the major protein chaperon of the ER. J. Cell Biol. 119, 1069–1076.

Lacoste, A., De Cian, M.C., Cueff, A., Poulet, S.A., 2001. Noradrenaline and alpha-adrenergic signalling induce the Hsp70 gene promoter in mollusc immune cells. J. Cell Sci. 114, 3557–3564.

Laursen, J.R., Liu, H., Wu, X.J., Yoshino, T.P., 1997. 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.

Lebel, J.M., Giard, W., Favrel, P., Boucaud-Camou, E., 1996. Effects of different vertebrate growth factors on primary cultures of hemocytes from the gastropod mollusc, *Haliotis tuberculata*. Biol. Cell 86, 67–72.

Liang, P., Macrae, T.H., 1997. Molecular chaperones and the cytoskeleton. J. Cell Sci. 110, 1431–1440.

Lindquist, S., Craig, E.A., 1988. The heat-shock proteins. Annu. Rev. Genet. 22, 631–677.

Minier, C., Borghi, V., Moore, M.N., Porte, C., 2000. Seasonal variation of MXR and stress proteins in the common mussel, *Mytilus galloprovincialis*. Aquat. Toxicol. 50, 167–176.

Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones and negative regulators. Genes Dev. 12, 3788–3796.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Ochoa, G.H., Clark, Y.M., Matsumoto, B., Torres-Ruiz, J.A., Robles, L.J., 2002. Heat shock protein 70 and heat shock protein 90 expression in light- and dark-adapted adult octopus retinas. J. Neurocytol. 31, 161–174.

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

Parsell, D.A., Lindquist, S., 1994. Heat Shock Proteins and Stress Tolerance. In: Morimoto, R., Tissieres, I., Georgopoulos, A. (Eds.), The Biology of Heat Shock Proteins and Molecular Chaperones, vol. 26. Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y, pp. 457–494.

Pelham, H.R.B., 1989. Control of protein exit from endoplasmic reticulum. Annu. Rev. Cell Biol. 5, 1–23.

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

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

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

Poncet, J.M., Serpentini, A., Boucaud-Camou, E., Lebel, J.M., 2002. Cryopreservation of mantle dissociated cells from *Haliotis tuberculata* (Gastropoda) and post-thawed primary cells cultures. Cryobiology 44, 38–45.

Pratt, W.B., 1993. The role of heat shock proteins in regulating the function, folding and trafficking of the glucocorticoid receptor. J. Biol. Chem. 268, 21455–21458.

Rathinam, A.V., Chen, T.T., Grossfeld, R.M., 2000. 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.

Redmond, T., Sanchez, E.R., Bresnick, E.H., Schlesinger, M.J., Toft, D.O., Pratt, W.B., Welsh, M.J., 1989. Immunofluorescence colocalization of the 90-kDa heat-shock protein and microtubules in interphase and mitotic mammalian cells. Eur. J. Cell Biol. 50, 66–75.

Rensing, S.A., Maier, U.G., 1994. Phylogenetic analysis of the stress-70 protein family. J. Mol. Evol. 39, 80–86.

Roberts, D.A., Hofmann, G.E., Somero, G.N., 1997. Heat-shock protein expression in *Mytilus californianus*: acclimatization (seasonal and tidal-height comparisons) and acclimation effects. Biol. Bull. 192, 309–320.

Ropenga, A., Chapel, A., Vandamme, M., Griffiths, N.M., 2004. Use of reference gene expression in rat distal colon after radiation exposure: a caveat. Radiat. Res. 161, 597–602.

Sanchez, E.R., Redmond, T., Scherrer, L.C., Bresnick, E.H., Welsh, M.J., Pratt, W.B., 1988. Evidence that the 90-kilodalton heat shock protein is associated with tubulin-containing complexes in L cell cytosol and in intact PtK cells. Mol. Endocrinol. 2, 756–760.

Sanders, B.M., 1988. The role of the stress proteins response in physiological adaptation of marine molluscs. Mar. Environ. Res. 24, 207–210.

Sanders, B.M., 1993. Stress proteins in aquatic organisms: an environmental perspective. Crit. Rev. Toxicol. 23, 49–75.

Sathiyaa, R., Campbell, T., Vijayan, M.M., 2001. Cortisol modulates HSP90 mRNA expression in primary cultures of trout hepatocytes. Comp. Biochem. Physiol. B 129, 679–685.

Schlesinger, M.J., 1990. Heat shock proteins. J. Biol. Chem. 265, 12111–12114.

Serpentini, A., Ghayor, C., Hebert, V., Galéra, P., Pujol, J.P., Boucaud-Camou, E., Lebel, J.M., 2000. *De novo* synthesis and identification of collagen transcripts in hemocytes from the gastropod mollusc, *Haliotis tuberculata*. J. Exp. Zool. 287, 275–284.

Sherman, M.Y.S., Goldberg, A.L., 1996. Involvement of Molecular Chaperones in Intracellular Protein Breakdown. In: Feige, U., Morimoto, R., Yahara, I., Polla, B. (Eds.), Stress-Inducible Cellular Responses. Birkhäuser Verlag, Basel, pp. 57–78.

Shumway, S.E., Koehn, R.K., 1982. Oxygen consumption in the American oyster *Crassostrea virginica*. Mar. Ecol., Prog. Ser. 9, 59–68.

Snyder, M.J., Girvetz, E., Mulder, E.P., 2001. Induction of marine mollusc stress proteins by chemical or physical stress. Arch. Environ. Contam. Toxicol. 41, 22–29.

Tirard, C.T., Grossfeld, R.M., Levine, J., Kennedy-Stoskopf, S., 1995. Effect of hyperthermia in vitro on stress protein synthesis and accumulation in oyster hemocytes. Fish Shellfish Immunol. 5, 9–25.

Tomanek, L., Somero, G.N., 1999. Evolutionary and acclimation induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. J. Exp. Biol. 202, 2925–2936.

Tomanek, L., Somero, G.N., 2000. Time course and magnitude of synthesis of heat-shock proteins in congeneric marine snails (genus *Tegula*) from different tidal heights. Physiol. Biochem. Zool. 73, 249–256.

Tomanek, L., Somero, G.N., 2002. Interspecific- and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*): implications for regulation of Hsp gene expression. J. Exp. Biol. 205, 677–685.

Veldhuizen-Tsoerkan, M.B., Holwerda, D.A., De Bont, A.M.T., Smaal, A.C., Zandee, D.I., 1991. A field study on stress indices in the sea mussel, *Mytilus edulis*: application of the stress approach in biomonitoring. Arch. Environ. Contam. Toxicol. 21, 497–504.

Werner, I., Hinton, D.E., 2000. Spatial profiles of Hsp70 proteins in Asian clam (*Potamocorbula amurensis*) in northern San Francisco Bay may be linked to natural rather than anthropogenic stressors. Mar. Environ. Res. 50, 3379–3384.

Wiens, M., Ammar, M., Nawar, A., Koziol, C., Hassanein, H., Eisinger, M., Müller, I., Müller, W., 2000. Induction of heat-shock (stress) protein gene expression by selected natural and anthropogenic disturbances in the octocoral *Dendronephthya klunzingeri*. J. Exp. Mar. Biol. Ecol. 245, 265–276.

Yamashita, M., Hirayoshi, K., Nagata, K., 2004. Characterization of multiple members of the Hsp70 family in platyfish culture cells: molecular evolution of stress protein Hsp70 in vertebrates. Gene 336, 207–218.