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Comparison of heat-shock responses between the hydrothermal vent shrimp *Rimicaris exoculata* and the related coastal shrimp *Palaemonetes varians*

Delphine Cottin [a], Bruce Shillito [a], Thomas Chertemps [b], Sven Thatje [c], Nelly Léger [a], Juliette Ravaux [a,*]

[a] Université Pierre et Marie Curie-Paris 06 (UPMC), UMR 7138, Equipe Adaptations aux Milieux Extrêmes, 7 Quai Saint-Bernard, 75005 Paris, France
[b] UPMC UMR-A 1272, Physiologie de l'Insecte: Signalisation et Communication, 7 Quai Saint-Bernard, 75005 Paris, France
[c] National Oceanography Centre, Southampton, School of Ocean and Earth Science, University of Southampton, European Way, Southampton SO14 3ZH, UK

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ABSTRACT

The deep-sea vent shrimp *Rimicaris exoculata* is believed to occur at the hot end of the hydrothermal biotope in order to provide essential elements to its epibiosis. Because it is found close to hot venting water, *R. exoculata* lives in a highly fluctuating environment where temperature (2–40 °C in the swarms) can exceed its critical maximal temperature (33–38.5 ± 2 °C). In order to understand how this vent shrimp copes with hyperthermia, we compared its molecular heat stress response following an acute but non-lethal heat-shock (1 h at 30 °C) with that of its monophyletic shallow-water relative, the shrimp *Palaemonetes varians*, known to frequently undergo prolonged exposure at temperatures up to 30 °C in its natural environment during summer. We isolated four isoforms of heat-shock proteins 70 (HSP70) in *R. exoculata* (2 constitutive and 2 inducible isoforms) and two isoforms in *P. varians* (1 constitutive and 1 inducible isoform) and quantitatively compared their magnitude of induction at mRNA level, using real-time PCR, in the case of experimentally heat-stressed shrimps, with respect to control (unstressed) animals. Here, we report the first quantification of the expression of multiple *hsp70* genes following heat stress in a deep-sea vent species living at 2300 m depth. Our results show a strong increase of *hsp70* inducible genes in the vent shrimp (~400-fold) compared to the coastal shrimp (~15-fold). We therefore propose that, the highly inducible molecular response observed in *R. exoculata* may contribute to the ability of this species to tolerate thermal extremes.

1. Introduction

Hydrothermal vents are highly dynamic deep-sea environments characterized by great spatial and temporal heterogeneity. In fact, as a result of turbulent mixing between the hot hydrothermal fluid (350 °C) and the cold ambient sea water (2 °C), species colonizing black smoker chimney walls may be subjected to rapid and severe changes of temperature in their environment (Tunnicliffe, 1991). To survive in this highly fluctuating thermal habitat, these vent species may have developed specific adaptations at behavioural and/or physiological levels (Childress and Fisher, 1992; Somero, 1992; Fisher, 1998).

The alvinocarid shrimp, *Rimicaris exoculata* (Williams and Rona, 1986) is one of the most abundant invertebrates (1000–3000 ind. m^{-2}) at the Mid-Atlantic Ridge vent sites, forming dense swarms on the walls of active chimneys (Van Dover et al., 1988; Gebruk et al., 2000; Desbruyères et al., 2001). This species is believed to fulfil most of its nutritional needs by feeding from the abundant bacterial epibiosis hosted within its gill chambers, and may therefore be constrained to maintain close proximity to the hydrothermal fluid (Segonzac et al., 1993; Rieley et al., 1999; Zbinden et al., 2004). Indeed, the epibionts are believed to be chemoautotrophic sulphur-oxidizers, and mesophilic temperatures (>20 °C) have been suggested to be important for carbon fixation (Wirsen et al., 1993; Gebruk et al., 2000). Adaptation to elevated temperature may thus constitute a selective advantage for the exploitation of H_2S that is needed to support the chemolithoautotrophic metabolism of its symbiont (Somero, 1992). Discrete *in situ* temperature measurements ranging from 2 to 40 °C have been reported within the swarms of *R. exoculata* and up to 70 °C only several centimetres away from the swarms (Van Dover et al., 1988; Gebruk et al., 1993; Segonzac et al., 1993; Desbruyères et al., 2000, 2001; Schmidt et al., 2008). Gebruk et al. (2000) also reported that up to 30% of collected specimens were damaged (scalded cuticle) by heat exposure. However, recent *in vivo* experiments revealed that *R. exoculata* does not tolerate sustained exposures to temperatures in the 33–38.5 ± 2 °C range (Ravaux et al., 2003; Shillito et al., 2006). The adaptation strategy with respect to hyperthermia remains therefore to be explored in this vent shrimp.

To understand the underlying mechanisms through which high thermal tolerance is conferred to deep-sea vent fauna, it is important to know how their biological processes differ from their shallow-water relatives (Somero, 1992; Childress and Fisher, 1992; Mestre et al., 2009). In this study, we propose to compare the molecular heat stress response

* Corresponding author. Tel.: +33 1 44 27 35 02; fax: +33 1 44 27 52 50.
E-mail address: juliette.ravaux@snv.jussieu.fr (J. Ravaux).

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of the vent shrimp *R. exoculata* to a sustained exposure at a temperature close to its upper thermal limit (30 °C), with that of the coastal salt marsh shrimp *Palaemonetes varians* (Leach, 1814), which is known to frequently undergo such severe heat stress in its natural environment. This comparison is particularly relevant since *P. varians* also lives in a fluctuating thermal habitat (Healy, 1997) where the reported temperature minima (0–2 °C) and maxima (30–33 °C) are comparable with those suggested to be encountered by the vent shrimp in its habitat (Lofts, 1956; Jefferies, 1964). Moreover, a study has recently shown, within the Caridea infraorder, a monophyletic relationship between palaemonid and alvinocarid shrimps (Tokuda et al., 2006).

We focused our investigations on a well-known family of stress biomarkers, the HSP70, since an immunodetection has already revealed an induction of these proteins in *R. exoculata* following a brief exposure at 25 °C (Ravaux et al., 2003). HSP70 proteins are known to function as chaperones, assisting in protein folding in response to a wide variety of stressors (Feder and Hofmann, 1999; Sørensen et al., 2003; Mayer and Bukau, 2005). They are among the most prominent proteins induced by heat and their role in conferring increased heat tolerance has been well established (Sanders, 1993; Parsell and Lindquist, 1993; Feder and Hofmann, 1999). In the HSP70 multigenic family, some members are expressed at extremely low level under normal conditions and increase significantly in response to stressors (stress-inducible proteins, HSP70) whereas others (cognate stress proteins, HSC70) are constitutively expressed under normal conditions but may be up-regulated under stress conditions (Lindquist and Craig, 1988; Feder and Hofmann, 1999). Presently, few data are available on HSP70 for vent species (Ravaux et al., 2003, 2007; Pruski and Dixon, 2007; Leignel et al., 2007; Cottin et al., 2008) and to our knowledge, only two studies deal with HSP70 gene regulation (Boutet et al., 2009; Ravaux et al., 2009).

Our investigations were initiated by Ravaux et al. (2007) who provided the first HSP70 sequences of hydrothermal vent species, one of which (*hsp70* form1) belonging to *R. exoculata*. We here characterized 3 additional cDNA belonging to the HSP70 family (two *hsc70* and one *hsp70*) in *R. exoculata* and the first HSP70 sequences (one *hsc70* and one *hsp70*) in *P. varians*, and we propose to quantitatively compare their magnitude of induction at mRNA level, using real-time PCR, in the case of experimentally heat-stressed shrimps, with respect to control (unstressed) animals.

2. Materials and methods

2.1. Animal collection

Rimicaris exoculata specimens were collected during the cruise «Momardream» (R/V *Pourquoi pas*?, Nautile submersible, July 2007) along the Mid-Atlantic ridge at the Rainbow vent site (36°14′N, 33°54′ W, 2300 m depth). Animals were sampled with a suction device operated by the submersible's hydraulic arm, and stored inside insulated Perspex cylinders until further transfer to the ship. Most of the shrimps survived the collection trauma, and live adult specimens (4 to 6 cm in length) were immediately re-pressurized inside the pressure vessel IPOCAMP for *in vivo* experiments (For details see Ravaux et al., 2003 and Cottin et al., 2008). Less than 2 h passed between the time the samples began decompression (submersible ascent) and the moment they were re-pressurized.

Adult specimens of *P. varians* (4 to 5 cm in length) were randomly caught with a fishnet from salt marsh ponds of the British coast near Lymington (Hampshire, England, 50°45′N, 1°32′W) in November 2007. Freshly collected shrimps were then kept for 7 days at 10 °C, in 10 L PVC-tanks with filtered sea water (35 ppt salinity) under continuous aeration, and an artificial day–night cycle. During this acclimatization period, animals were fed with fish pellet, and half of the water was replaced every 2 days by fresh 10 °C filtered sea water.

2.2. Heat-shock experiments

2.2.1. Time course study of hsp70 in P. varians following a sudden heat-shock

A time course study of *hsp70* expression levels was carried out on *P. varians* specimens following a sudden heat stress (1 h at 28 °C; Fig. 1A) in order to detect the time of the peak response. This experiment was performed using temperature-controlled seawater tanks (one tank set at 10 °C and one tank set at 28 °C). Briefly, 10 °C-acclimatized shrimps (a total of 40 individuals) were suddenly transferred into a 28 °C-seawater tank with continuous aeration for 1 h, and then put back at reference temperature (10 °C tank). Ten individuals were then sampled and frozen 30 min, 2 h, 4 h, and 6 h after the heat-shock, in order to measure their *hsp70* levels. Reference specimens (a total of 40 individuals) which had been maintained at 10 °C constant temperature were also sampled and frozen at each duration point (10 individuals per point).

2.2.2. Ramp-shock experiments in P. varians and R. exoculata

A ramp-shock experiment was carried out on *P. varians* and *R. exoculata* specimens. These experiments were performed inside the incubator IPOCAMP at *in situ* pressure for both species in order to obtain comparable heating rate (Fig. 1B). The experiment was carried out at atmospheric pressure on a total of 30 specimens for the coastal shrimp and at 23 MPa on a total of 42 specimens for the hydrothermal vent shrimp, in flow-through mode (20 l h^{-1} flow rates). The temperature of the flowing seawater (filtered at 0.4 μm) is measured constantly in the inlet and outlet lines (±1 °C). Animals were maintained 5 h at 10 ± 1 °C, then the water temperature was ramped to 28 ± 2 °C (at a rate of 0.2 °C/min) for *P. varians* and to 30 ± 2 °C for *R. exoculata* (at a rate of 0.3 °C/min). Shrimps were kept at this elevated water temperature for 1 h, after which the temperature was

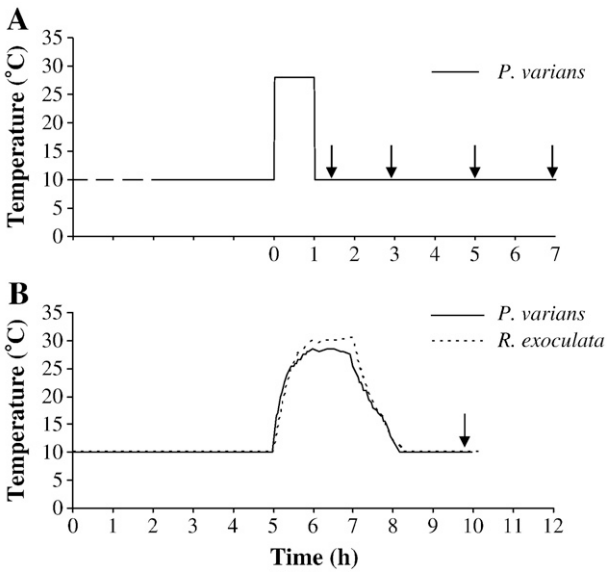

Fig. 1. (A) Temperature profile of the heat-shock experiment performed on *P. varians* shrimps using temperature-controlled water baths. 10 °C-acclimatized shrimps were suddenly immersed in a 28 °C-water bath for 1 h and then put back at 10 °C. Animals were then sampled 30 min, 2 h, 4 h and 6 h after the heat-shock (see black arrows) and rapidly frozen in liquid nitrogen. (B) Temperature profiles of heat-shock experiments performed on *R. exoculata* (broken line) and *P. varians* (solid line) specimens inside the pressurized IPOCAMP vessel. The animals were maintained for 5 h at 10 °C (±1 °C) and subsequently ramped to 28 °C for *P. varians* and to 30 °C for *R. exoculata*. After 1 h at this elevated temperature, the shrimps were given two hours recovery at 10 °C (black arrow) and subsequently sampled and immediately frozen in liquid nitrogen for further molecular analysis. Temperature values (±2 °C during heating events) correspond to the mean temperature obtained from T1 (upstream temperature of the water flow) and T2 (downstream temperature of the water flow) probes (see Ravaux et al., 2003 for more details).

reduced to 10 °C, and maintained as such for 2 more hours, until the end of the experiment. It had previously been shown that a temperature of 30 °C is non-lethal for *R. exoculata* specimens (Ravaux et al., 2003). In the case of *P. varians*, a preliminary heating experiment revealed a 40% mortality rate (12 dead animals on a total of 30 experimented shrimps) following a 1 h exposure at 35 °C (data not shown). In view of this result, heating experiments on the coastal shrimp were finally carried out at lower temperature (28 ± 2 °C). At the end of the experiments, shrimps were rapidly dissected and frozen in liquid nitrogen for further analyses. Less than 10 min passed between the end of the experiments and the moment the last shrimp was frozen. Reference animals (48 specimens of *R. exoculata* and 31 specimens of *P. varians*) were maintained for the same duration as for the heating experiments (about 10 h) at a constant temperature of 10 °C.

2.3. RNA extraction and reverse transcription

Tissues from shrimp abdomens, with their cuticle, were ground in liquid nitrogen. The powder was homogenized in Trizol reagent (Invitrogen), and total RNA was isolated according to the manufacturer's instructions and quantified by spectrophotometry. RNA integrity was then evaluated by electrophoresis in a 1.2% agarose gel under denaturing conditions. Subsequently, single-stranded cDNA were synthesized from 3 µg of shrimp abdomen total RNA using oligo $(dT)_{18}$-anchor primer and MMLV reverse transcriptase (200 units, Promega, Lyon, France). The reaction mixture contained dNTP mix (Promega), RNasin (Promega), oligo$(dT)_{18}$-anchor primer (3′RACE) or a specific HSP70 primer (5′RACE), sterile water and RNA template dissolved in a final volume of 25 µl. This solution was heated at 65 °C for 5 min before addition of the enzyme and incubation for 1 h at 37 °C and 15 min at 75 °C.

2.4. cDNA amplification and rapid amplification of the 3′ and 5′ cDNA ends (RACE) for hsp70 in R. exoculata and P. varians

The cDNA encoding putative *hsp70* genes in *R. exoculata* (*hsp70* form2 and *hsc70* form3) and *P. varians* (*hsp70* form1 and *hsc70* form2) were amplified by PCR amplification, using the degenerated primers HSP1, HSP2, HSP3 and HSP4 (Table 1). PCR amplifications were performed following a previously published protocol (Ravaux et al., 2007). The 5′ and 3′ ends of *R. exoculata hsp70* cDNA were obtained using a SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) with univP and nested primers and the specific primers Rim8 and Re4 for *hsp70* form2, and Rex2 and Rex3 for *hsc70* form3 (Table 1). The almost complete *P. varians hsp70* cDNAs were obtained by performing nested PCR using nested specific primers (Var3 for *hsp70* form1 and Vari1, Vari3 for *hsc70* form2) and the 5′ Primer or 3′ Primer designed from multiple alignments of homologous sequences (Cottin et al., 2008). The PCR products were purified, subcloned, sequenced and identified as previously described (Ravaux et al., 2007). The resulting nucleotide sequences were then deposited in the GenBank database under the accession numbers FJ875280 (*hsp70* form2), FJ268954 (*hsc70* form3) for *R. exoculata* and FJ356149 (*hsp70* form1) and FJ875279 (*hsc70* form2) for *P. varians*. A cDNA coding for a fourth isoform of HSP70 proteins was obtained for *R. exoculata* following a suppression subtractive hybridization procedure (using different stressors) performed in our lab but not described in this paper. This sequence, named *hsc70* form4, was also deposited in GenBank under the accession number FJ654506.

Table 1
Nucleotide sequences of primers used in polymerase chain reaction for the amplification of reference genes and *hsp70* sequences in *Palaemonetes varians* and *Rimicaris exoculata*.

Primer	Direction	Sequences (5′–3′)
HSP1	F	AAGGTGGARATCATCATCGCCAAYGAYCARGG
HSP2	F	TAYGTNGCVTTCACNGACAC
HSP3	R	AGGTTGTTGTCCTTGGTCATYGC
HSP4	R	TAGAARTCRATRCCYTCGAASAGACAGTC
Rim8	F	GCAACACCACCATCCCAACCAA
Re4	R	ATTGGCATCTATGTCAAAGGTC
Rex2	F	AAAGACCCATCCGAGAACAAGCGT
Rex3	R	AAGGTTTGGGTTTGCTTCGTGGGA
Var3	F	CTTGACGTGGCTCCTCTTTC
Vari1	F	CCTGGCGTGCTAATTCAAGT
Vari3	F	TGACGAGAAGCAGCGAGAGCGTA
5′ primer	F	ATGGCVAAGGCAMGYGCTGTSGGTAT
3′ primer	R	TTASTCRACTTCTCRATGGTGGG
UnivP	F/R	CTAATACGACTCACTATAGGGCAAGCAGTGGTATC AACGCAGAGT
Nested	F/R	AAGCAGTGGTATCAACGCAGAGT

Nucleotide codes: R = A/G, Y = C/T, N = A/T/G/C, S = G/C. F, forward; R, reverse

2.5. Sequence analysis

A dataset of the available crustacean HSP70 nucleotide sequences was built from GenBank database, and was subsequently aligned with ClustalW. The ambiguously aligned regions were removed, leading to 1692 nucleotide positions. The sequences that proved too short to provide relevant information for tree-building purposes were removed (including *R. exoculata hsp70* form 4), leading to a final dataset of 31 sequences that were rooted with the 3 branchiopods sequences from *Artemia franciscana* (GenBank accession no. AF427596), *Daphnia magma* (GenBank accession no. EU514494) and *Moina macrocopa* (GenBank accession no. EU514495). Phylogenetic relationships were then reconstructed by probabilistic methods, namely Maximum Likelihood (PHYML, Guindon and Gascuel, 2003) using the SYMIG model. Robustness of the topology was assessed by 100 bootstrap replicates.

2.6. Real-time PCR analysis of hsp70 in R. exoculata and P. varians shrimps

2.6.1. DNAse treatment and reverse transcription

Real-time PCR was used to accurately evaluate in both control and heat-shocked individuals the expression levels of the *hsp70* genes identified in *R. exoculata* and *P. varians*. For each individual, 4 µg of total RNA was treated to remove DNA contamination by using the Turbo-DNAse kit (Ambion, Foster city, CA, USA) and then reversely transcribed to cDNA using the oligo$(dT)_{18}$ primer and the Superscript II reverse trancriptase kit (200 units, Invitrogen) according to the manufacturer's instructions. Amplification of *hsp70* transcripts characterized in *R. exoculata* and *P. varians* was performed on cDNAs of single individual for control and heat-exposed shrimps (n = at least 3 individuals for each treatment). The specific primer pairs designed for the amplification of *hsp70* transcripts in both species are shown in Table 2.

2.6.2. Reference gene selection and primer design

The expression of 4 (ß-actin, GAPDH, *Rpl8* and *18S* gene) and 3 reference genes (ß-actin, GAPDH, *Rpl8*) were analysed for *R. exoculata* and *P. varians*, respectively. Based on crustacean genes in databases, degenerate primers were designed for *GAPDH* and *Rpl8* genes using the Eprimer3 software (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=eprimer3) considering conserved protein domains (Table 1). The resulted amplicons were then cloned using the TOPO TA cloning kit (Invitrogen) and sequenced (GATC Biotech, France). The corresponding sequences were deposited in GenBank (*Rimicaris* GAPDH FJ875278, *Rimicaris* Rpl8 FJ875277, *Palaemonetes* GADPH GQ120565, and *Palaemonetes* Rpl8 GQ120564). Subsequently, specific primers were designed and primer optimal annealing temperature was optimized using standard RT-PCR tests. For the *18S* and the ß-actin reference gene,

Table 2
Primer sequences (F: forward; R: reverse) used for the real-time PCR analysis of *Rimicaris exoculata* and *Palaemonetes varians* genes.

Primer	Direction	Sequences (5′–3′)
Rimicaris hsp70 form1	F	TAAGGACAATATGAAGCAGCAGC
	R	AAATACAGAATGACAAAACACAG
Rimicaris hsp70 form2	F	ACTGAAGAGGCTGGAAGGAAGC
	R	GACGTAAGTGAATGAAGATGAAT
Rimicaris hsp70 form3	F	AAGGTTTGGGTTTGCTTCGTGGGA
	R	TAGCGGGTGGCTCCACAAGGATT
Rimicaris hsp70 form4	F	CAGAACAGGCTCACCCCAGAAG
	R	GTTTGGCCCCCAACTTCTCCT
Palaemonetes hsp70 form1	F	CCAGCCGTCACCATCCAGGTGT
	R	GCGGTCGATGTCCTCCTTGCTG
Palaemonetes hsp70 form2	F	TTCCTGAGGAGGATCGCAAA
	R	CATTCCACCAGGAGGAGCAC
Rimicaris GAPDH	F	CCCCTTCATTGCTCTGGACTAC
	R	ACCACCTTGGAAGTGAGCAGA
Rimicaris 18S	F	GCTGTGGATTGTAGGCCATGCGCCTAC
	R	GGCTGCTGGCACCAGACTTGCCCTCCAA
Rimicaris ß-actin	F	ACCAGTGCTGAACGTGAGATCG
	R	TCTCGTGGACACCAGCAGATTC
Rimicaris Rpl8	F	GAAGCTCCCATCAGGTGCCAAGAA
	R	TTGTTACCACCACCGTGAGGATGC
Palaemonetes GAPDH	F	AAGGGCGCCGAGGTTGTTGCTGTAA
	R	GCACCAGCCTTGCTCCATGGAATGT
Palaemonetes ß-actin	F	ACCAGTGCTGAACGTGAGATCG
	R	TCTCGTGGACACCAGCAGATTC
Palaemonetes Rpl8	F	TCCCGGTCGTGGTGCACCTATT
	R	GACGGCCTCGGTCACCAGTCTTT

specific primers were designed using sequences in GenBank (*Rimicaris* ß-actin FJ410324, *Palaemonetes* ß-actin FJ654525, *Rimicaris* 18S AM087652) (Table 2).

2.6.3. Real-time quantitative RT-PCR

All real-time quantitative RT-PCR reactions were performed on the LightCycler® 480 Real-Time PCR Detection System (Roche, France). Each 12.5 µl reaction consisted of 6.25 µl LightCycler® 480 Sybr Green I Master (Roche, France), 2.5 µl diluted cDNA, 1.25 µl of forward and reverse primer and 1.25 µl of sterile water. PCR conditions were 1 cycle of 94 °C for 13.5 min followed by 45 cycles of (94 °C 30 s, 56 °C 30 s, and 72 °C 30 s) for *R. exoculata* cDNAs and 35 cycles of (94 °C 30 s, 62 °C 30 s, and 72 °C 30 s) for *P. varians* cDNAs. After PCR, a melting curve analysis was performed in order to demonstrate the specificity of the PCR products. All primer pairs tested generated a single and discrete peak in the dissociation curve (data not shown). A negative control and a 5-fold dilution series protocol of pooled cDNAs (from both control and hyperthermic treatments) were included in each run. The 5-fold dilution series were used to construct a relative standard curve to determine the PCR efficiencies and for further quantification analysis. In all experiments, all primer pairs gave amplification efficiencies of 90–100%. Each reaction was run in triplicate (technical replicate).

2.6.4. Determination of the best reference gene and data analysis

Data were analysed with the LightCycler® 480 software (Roche, France) and the crossing point values (Cp-values) were first determined for the reference genes. The average Cp value of each triplicate reaction was then used for subsequent analysis with the geNorm, Normfinder, and BestKeeper programs in order to determine the best reference gene. This approach relies on the principle that the expression of a perfect reference gene should be identical in all samples, independently of experimental conditions. For the comparison of control and heat-exposed individuals, the *RPL8* gene was considered as displaying consistent expression in both *R. exoculata* and *P. varians* shrimps and therefore was determined to be the best reference gene for both species. Subsequently, the expression of *hsp70* genes was normalized to geometric means of this reference and the mean normalized gene expression of each triplicate reaction was then calculated with the Q-Gene software.

2.7. Statistical analysis

A Mann–Whitney Test was used to compare differences between heat-shocked and control samples for *hsp70* genes in shrimps. All analyses were performed using STATISTICA version 7.0 (Statsoft) and significance levels were $p<0.05$ (*).

3. Results

3.1. Shrimp survival

Most of the *R. exoculata* specimens originating from Rainbow (2300 m depth) appeared to withstand the decompression events during the collection process, except for some individuals which may have been damaged by the suction sampler. At 23 MPa, almost all animals were alive either after the reference experiment (92% of survival after 10 h at 10 °C) or after the heat exposure (88% of survival after a 1 h exposure at 30 °C, 10 h total duration).

No mortality was observed for *P. varians* specimens maintained at 10 °C either during the acclimatization period (7 days) in PVC-tanks or after the experiment performed inside IPOCAMP (10 h). Moreover, all the specimens were still alive and relatively active at the end of the heating experiments (1 h at 28 °C) even after 6 h of recovery.

3.2. Description of shrimp HSP70 sequences

One complete cDNA (*hsc70* form3) and two partial cDNAs (*hsp70* form2 and *hsc70* form4) were obtained for *R. exoculata* (see Fig. S1 in the Supplementary data files). The cDNA of *R. exoculata hsp70* form2 is 1978 nucleotides in length (601 amino acid), including a 1806 nt coding region and a 3′ UTR of 172 nt with a polyadenylation signal sequence (AATAAA at position 1935). The full-length cDNA of *R. exoculata hsc70* form3 is 2262 bp in length with a single reading frame of 1965 nt (654 amino acid) and a 273 nt-long 3′ UTR, which contains the consensus polyadenylation signal at position 2222. Finally, the partial cDNA *hsc70* form4 is 428 nt in length (142 amino acid).

Two partial cDNA were obtained for *P. varians*. The cDNA of *P. varians hsp70* form1 is a single reading frame of 1687 nt (567 amino acid) and the cDNA of *P. varians hsc70* form2 is a 1840 nt ORF (613 amino acid).

3.3. Relationships among crustacean HSP70

Two groups of sequences, which are supported by high bootstrap percentages values, are evidenced in the tree (Fig. 2). The first group, including *R. exoculata* form 3 and *P. varians* form 2 sequences (identified below as HSC70), would correspond to constitutively expressed 70 kDa HSP, since nine of these sequences were clearly identified as HSC70. The second group, including *R. exoculata* form 1, *R. exoculata* form 2 and *P. varians* form 1 sequences, would therefore correspond to heat-inducible forms, since four of these sequences are identified as HSP70 (see below).

3.4. Time course of hsp70 mRNA expression in P. varians

A time course study of *hsp70* expression levels was performed in *P. varians* following a rapid transfer of the shrimps from 10 °C to 28 °C. Fig. 3 represents the expression level of *hsp70* form1 and *hsc70* form2 for various time points of recovery: 30 min, 2 h, 4 h and 6 h after the 1 h-heat-shock. The expression level of *hsp70* form1 was higher in heat-shocked samples than in control samples at each time of recovery. This differential expression increased from 15-fold at 30 min to 26-fold at 2 h and to 54-fold, 4 h after the heat-shock.

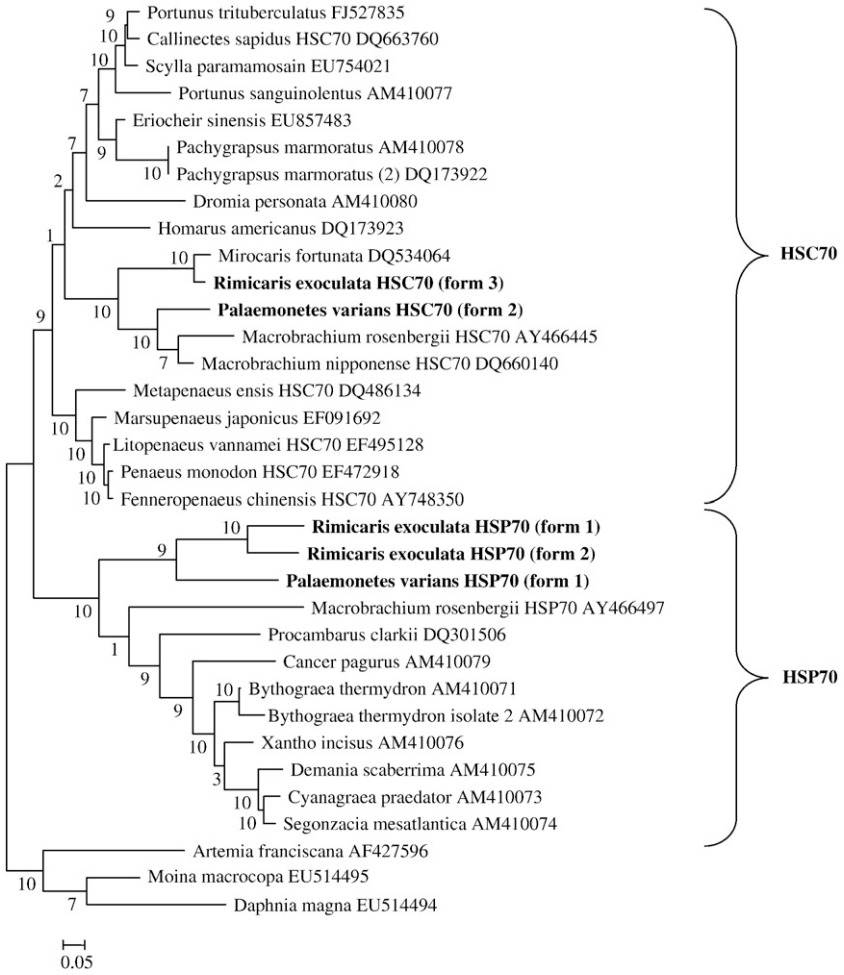

Fig. 2. Phylogenetic tree showing the relationships between the *hsp70* nucleotide sequences of decapod crustaceans. The *hsp70* sequences of 34 crustaceans were analysed through Maximum Likelihood (PHYML, Guindon and Gascuel, 2003) under a SYMIG model. The sequences that were clearly assigned to inducible forms (HSP70) or constitutive forms (HSC70) through expression analyses are annotated on the tree. The accession number of the nucleotide sequence is provided after each species name, and the sequences analysed in this paper are identified in bold. The values indicated on the branches correspond to bootstrap percentages (BP). According to this tree, there is a functional grouping of the sequences identified as HSP70 and HSC70.

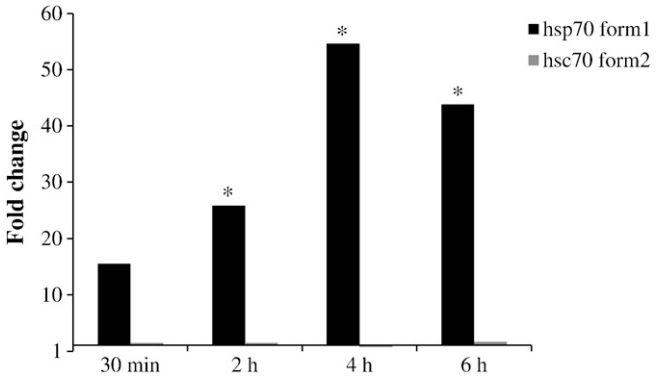

Fig. 3. Time course study of *hsp70* expression levels in *Palaemonetes varians* shrimps. The levels of *hsp70* form1 (black columns) and *hsc70* form2 (grey columns) were detected at different times of recovery following a sudden heat exposure at 28 °C for 1 h (see Materials and methods). Expression level of *hsp70* were normalized to the corresponding *RPL8* abundance and expressed as the ratio to control (constant temperature of 10 °C), considered as equal to 1. All amplifications were reproduced in triplicate and values correspond to the mean normalized expression (± SEM) of at least 3 independent samples ($n = 3$ or 4 individuals). To compare data, Mann–Whitney Test were used and the asterisk above the bars indicates a significant difference between the mean normal expression of control and heat-shocked samples ($^*p < 0.05$) of the same time point.

Finally, the expression of *hsp70* form1 decreased to 43-fold, 6 h after stress. On the contrary, whatever the time point considered, no significant changes in *hsc70* form2 levels (1.4-fold at 30 min, 1.4-fold at 2 h, 0.7-fold at 4 h and 1.6-fold at 6 h) was observed between heat-shocked and control samples.

3.5. Expression analysis of hsp70 genes in R. exoculata and P. varians

Using real-time PCR, changes in *hsp70* expression levels were assessed in *R. exoculata* and *P. varians* specimens following the heat-shock experiments performed inside IPOCAMP. Fig. 4A shows the expression levels obtained for *hsp70* form1, *hsp70* form2, *hsc70* form3 and *hsc70* form4 in control (10 °C) and heat-shocked *R. exoculata* (30 °C). A significant difference between control and heat-shocked *R. exoculata* specimens was observed for *hsp70* form1 and *hsp70* form2 genes (Mann–Whitney Test, $p < 0.05$), with a strong increase of expression levels after heat stress, reaching 373-fold for *hsp70* form1 and 132-fold for *hsp70* form2. On the contrary, the expression levels of *hsc70* form3 (1.9-fold) and *hsc70* form4 (0.7-fold) observed in heat-shocked samples were not statistically different from those detected under control conditions, suggesting that these genes were not significantly affected by thermal stress.

The expression levels of the two *hsp70* genes (*hsp70* form1 and *hsc70* form2) identified in the coastal shrimp *P. varians*, for control (10 °C) and heat-exposed shrimps (28 °C), are shown in Fig. 4B. The

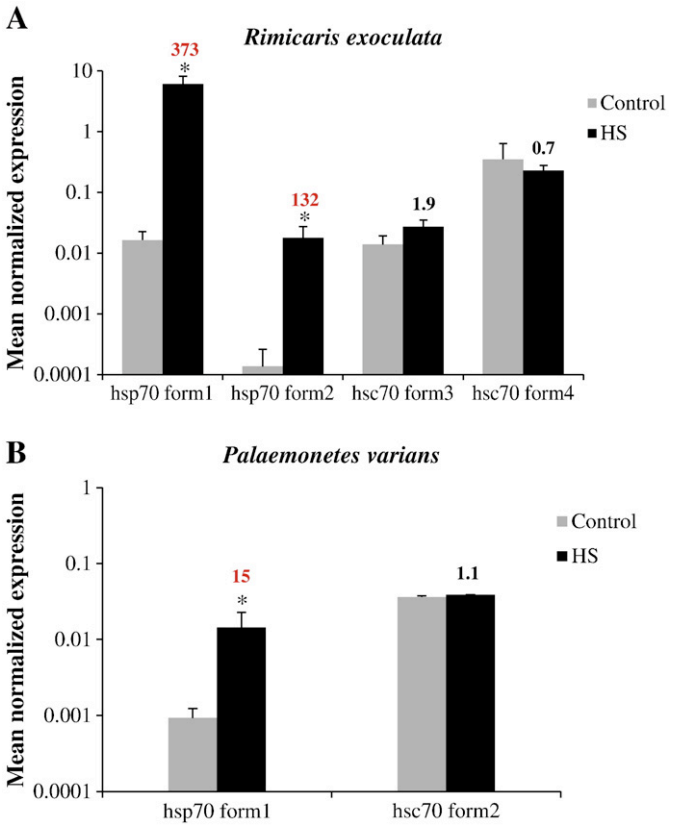

Fig. 4. Expression levels of *hsp70* genes obtained by qPCR in *R. exoculata* (A) and *P. varians* (B) for control (grey columns; 10 °C for both species) and heat-shocked (HS; black columns; 30 ± 2 °C for *R. exoculata* and 28 ± 2 °C for *P. varians*) specimens. For both species, the expression level of genes was measured 2 h after the heat-shock and normalized to the corresponding *RPL8* abundance. All amplifications were reproduced in triplicate and values correspond to the mean normalized expression (± SEM) of at least 3 independent samples ($n = 3$ or 4 individuals). To compare data, Mann–Whitney Test were used and the asterisk indicates a significant difference between the mean normal expression of control and heat-shocked samples (*$p < 0.05$). For each gene, the number above the black columns indicated the fold of induction obtained after heat stress.

expression of *hsp70* form1 was found to significantly increase following the heat-shock (15-fold; Mann–Whitney Test, $p < 0.05$), whereas no drastic change was observed for *hsc70* form2 (1.1-fold; Mann–Whitney Test, $p > 0.05$) compared to control samples.

4. Discussion

4.1. Characterization of R. exoculata and P. varians HSP70 sequences

As expected, several general eukaryotic HSP70 family motifs were identified in the amino acid sequences of *R. exoculata* and *P. varians* confirming their belonging to the HSP70 family (see Fig. S1). Several structural characteristics were proposed to differentiate constitutive and inducible isoforms. Some authors have suggested that cytosolic eukaryotic HSC70 has GGMP repeats in the C-terminal region (Prapapanish et al., 1996; Liu et al., 2004; Wu et al., 2008). Both *R. exoculata hsc70* form3 and *P. varians hsc70* form2, which, according to our expression analysis encode HSC70 (see below), contain this motif at position 624 (see Fig. S1). However, in *R. exoculata hsc70* form3, the tetrapeptide occurs only once (amino acid 624–628), whereas it is repeated twice in *P. varians hsc70* form2 (amino acid 624–632). Moreover, such motifs were found in the HSP70 cDNA sequence of the copepod *Trigriopus japonicus* (four GGMP repeats in the C-terminal region) whereas expression analysis by real-time PCR clearly indicates that this sequence corresponds to a heat-inducible HSP70 (Rhee et al., 2009). Despite the difficulty of identifying a specific motif for HSP70 vs. HSC70 sequences, the nucleotide sequences of the inducible and constitutive forms appeared in separate groups in the phylogenetic tree (Fig. 2). This tree can thus prove useful as a predictive tool for an *a priori* annotation of *hsp70* sequences in decapod crustaceans, especially since the phylogeny obtained is congruent with other phylogeny of Decapoda constructed using nuclear or mitochondrial DNA (Tsang et al., 2008). Nevertheless, gene expression studies after stress exposures still seem to be the only way to unambiguously distinguish *hsp70* and *hsc70*.

4.2. Thermal characteristics of P. varians

The palaemonid shrimp *P. varians* is found in very shallow waters and is particularly abundant in areas of salt marsh, where large and acute daily fluctuations of water temperature have been reported (about 10 °C per 24 h) (Nugegoda and Rainbow, 1989; Nielsen and Hagerman, 1998). In addition, this species is known to undergo a wide seasonal gradient of temperature in its environment (from 0 °C in December to up to 33 °C in July) (Lofts, 1956; Jefferies, 1964; Healy, 1997). For the specimens used in this study, which were collected during winter, a reference temperature of 10 °C is assumed to be tolerated by the animals, since first it is close to the water temperature measured at the time of sampling (8 °C) and second it is consistent with the mean temperature suggested to be encountered in the natural habitat of *P. varians*, i.e. 10–15 °C (Lofts, 1956; Jefferies, 1964). On the contrary, our heat-shock temperature of 28 °C is close to the environmental maxima reported for *P. varians* (Lofts, 1956; Healy, 1997) and, in view of the 40% mortality rate observed at the end of our preliminary heating experiment (35 °C for 1 h), a 1 h exposure at 28 °C may be considered as a severe thermal stress for the animals.

One physiological strategy to cope with thermal stress is the synthesis of heat-shock proteins (HSPs), which prevent protein aggregation and facilitate removal of damaged proteins (Parsell and Lindquist, 1993; Feder and Hofmann, 1999). The expression of HSP70 proteins is thus frequently used as a bioindicator of stress. In this study, we first followed through time the *hsp70* expression levels of *P. varians* after a sudden immersion in a 28 °C-water (Fig. 1A) in order to detect the time of the peak response. Real-time PCR analyses showed a differential regulation of the two *hsp70* genes since a rapid increase of inducible *hsp70* form1 (15-fold at 30 min, 25-fold at 2 h) with a maximum level at 4 h (~54-fold) was observed, while no significant change was detected for *hsc70* form2 (see Fig. 3). We thus identified the *hsp70* form1 of *P. varians* as a gene encoding a heat-inducible HSP70 protein and the *hsc70* form2 as a gene encoding a HSC70 protein. We can also assume that the exponential phase of the stress response is situated between 30 min and 4 h after heat-shock since the induction levels of *hsp70* form1 tends to decrease at 6 h (43-fold). Secondly, we explored the capacity of *P. varians* to respond to a 28 °C ramp-shock (Fig. 1B) by measuring the levels of *hsp70* isoforms through real-time PCR. The results confirmed the differential regulation of the two *hsp70* genes since an up-regulation of *hsp70* form1 (~15-fold) was demonstrated, while no significant change was observed for *hsc70* form2 (1.1-fold) (Fig. 3B). Surprisingly, after 2 h of recovery, we observed an expression level of *hsp70* form1 about 2-fold less in the ramp-shock experiment than in the sudden immersion experiment. It thus appears that the heating rate may influence the expression level of inducible *hsp70* genes in *P. varians*. Since the magnitude of HSP synthesis may be proportional to the severity of the heat stress (DiDomenico et al., 1982; Tomanek and Somero, 2000), it seems that, for a 1 h-heat-shock at 28 °C, a rapid heating rate (sudden immersion) induce a higher level of stress than a slow heating rate (0.3 °C/s) in *P. varians* specimens. This observation appears consistent with the conditions of exposure to heat stress found in the habitat of *P. varians* in which the temporal gradient is principally driven by the tidal rhythm. For an accurate comparison of stress level between

P. varians and *R. exoculata*, the heat-shock experiment in *R. exoculata* was therefore performed with comparable heating rates and the *hsp70* expression was detected after 2 h of recovery (exponential phase of the stress response).

4.3. Comparison of hsp70 expression between the vent shrimp R. exoculata and the coastal shrimp P. varians

The reference experiments for *R. exoculata* were performed at *in situ* pressure (23 MPa), and at a reference temperature of 10 °C, according to our previous work (Ravaux et al., 2003) and to the mean temperature values recorded in the shrimp microenvironment, 10–15 °C (Segonzac et al., 1993; Desbruyères et al., 2001; Schmidt et al., 2008). We can therefore assume that, with a 92% survival rate with no drastic change in general behaviour, our reference conditions are tolerated by the animals. On the contrary, and in view of the thermal resistance of *R. exoculata* (33–38.5 ± 2 °C) (Ravaux et al., 2003; Shillito et al., 2006), our heat-shock experiment (30 °C-exposure for 1 h) may be considered as sufficient to induce stress in the shrimps.

Both hydrothermal and coastal species live in highly fluctuating environments where temperature can regularly approach their lethal limits (Gebruk et al., 1993; Shillito et al., 2006; Helmuth and Hofmann, 2001; and the present work). One of the objectives of this study was to compare the stress response of the hydrothermal vent shrimp with a related coastal species following a similar severe thermal stress in order to characterize the adaptation mechanisms of *R. exoculata* regarding hyperthermia. For a relative comparison between species, quantification of *hsp70* genes expression was performed using real-time PCR, which is currently the most sensitive method to quantify gene expression (Bustin, 2000). Furthermore, total RNAs were extracted from the same tissues (abdomen muscles) in both shrimps, since the expression of HSP70 is known to be tissue-specific (Wang et al., 2007; Wu et al., 2008). The results for *R. exoculata* show a dramatic increase of *hsp70* form1 (373-fold) and *hsp70* form2 (132-fold) expression levels following a 30 °C-exposure whereas the expression of *hsc70* form3 (1.9-fold) and *hsc70* form4 (0.7-fold) were not significantly affected (Fig. 4A). This shows that *hsp70* form1 and *hsp70* form2 encode heat-inducible forms while *hsc70* form3 and *hsc70* form4 are coding for constitutive forms. Thus, for comparable heat stress conditions, the shrimp *R. exoculata* displays, for at least two genes, considerably higher levels of inducible HSP70 mRNA than the coastal shrimp. In fact, when compared to the expression of *P. varians hsp70* form1, the expression of *R. exoculata hsp70* form1 is 25-fold higher and the expression of *R. exoculata hsp70* form2 is 9-fold higher. Since induction of HSP70 proteins is essential to reduce the damaging effects of heat stress (Feder and Hofmann, 1999; Parsell and Lindquist, 1993; Sørensen et al., 2003), the highly inducible molecular response observed in *R. exoculata* may contribute to the ability of this species to tolerate extreme temperature, while the lower inducibility in *P. varians* suggests that either it is more susceptible to cellular damages, or bears other defence mechanisms with respect to hyperthermia. The combined protection of the two inducible *hsp70* genes in *R. exoculata* may also help this shrimp cope with the stressful thermal conditions of hydrothermal vent habitats.

To our knowledge, studies that have measured *hsp70* mRNA after thermal stress in marine species by real-time PCR report levels of induction ranging from less than 10-fold (Voznesensky et al., 2004; Cellura et al., 2006; Park et al., 2007; Rhee et al., 2009) to thousand-folds (Ojima et al., 2005a; Osovitz and Hofmann, 2005; Clark et al., 2008). The levels of *hsp70* mRNA detected for *R. exoculata* are comparable with those of a cold-water fish, the rainbow trout *Oncorhynchus mykiss* (480-fold after a 3 h-heat-shock at 28 °C; Ojima et al., 2005a), for which a 50% mortality rate was shown after 1 week at 26 °C (Ojima et al., 2005b). Moreover, it is also in the same range of another cold-water species, the purple sea urchin *Strongylocentrotus purpuratus* (250-fold after a 30 min-heat-shock at 26 °C), known to routinely experience temperature near 4 °C in its natural habitat (Osovitz and Hofmann, 2005). Finally, *hsp70* genes expression levels higher than *R. exoculata* were reported in the Antarctic marine mollusc, *Nacella concinna* (almost 2000-fold after a 2 h-heat-shock at 20 °C), which used to live at low (−1.86 to +1 °C) and stable seawater temperatures (Clark et al., 2008). On the contrary, the level measured for *P. varians hsp70* form1 is similar to that of the mussel *Mytilus galloprovincialis* (6-fold induction after a 90 min-heat-shock at 30 °C; Cellura et al., 2006) or the abalone *Haliotis tuberculata* (4-fold after a 1 h-heat-shock at 37 °C; Farcy et al., 2007), two other thermally-challenged species. It thus appears that, as opposed to *P. varians*, the level expression obtained in *R. exoculata* is comparable to species that rarely undergo heat stress in their habitat. Moreover, low levels of *hsp70* gene have been recently reported in natural populations of *R. exoculata* (Ravaux et al., 2009). From these results, we can suggest that *R. exoculata* rarely encounters sustained temperature close to its thermal limits *in situ*. This mobile species may therefore avoid prolonged contacts with high temperature zones through a behavioural response, while a strong molecular response may be triggered in case of occasional and unpredictable thermal extremes. Further studies are needed to support this hypothesis. For example, additional experiments will be necessary to determine the existence of an exposure duration threshold (a few minutes) at elevated temperature (30 °C and more), which would result in lower expression of inducible *hsp70* genes in *R. exoculata*. Moreover, the optimal temperature necessary for an efficient carbon fixation by the epibionts has to be specified in order to understand better the thermoregulatory behaviour of this shrimp.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jembe.2010.06.008.

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