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

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**A B S T R A C T**

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.

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**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 (Wirsing et al., 1993; Gebruk et al., 2000). Adaptation to elevated temperature may thus constitute a selective advantage for the exploitation of H₂S 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...
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 (Lofte, 1956; Jeffries, 1964). Moreover, a study has recently shown, within the Caridina infraorder, a monophyletic relationship between palamoid 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 levels 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 (Bouet 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* form 1) 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 «Monardream» (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 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 (201 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](https://example.com/fig1.png)

**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) 10-12-anchor primer and MMLV reverse transcriptase (200 units, Promega, Lyon, France). The reaction mixture contained dNTP mix (Promega), RNasin (Promega), oligo(dt) 10-12-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 R4 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 Var1, Var3 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 FJ752800 (hsp70 form2), FJ268954 (hsc70 form3) for *R. exoculata* and FJ356149 (hsp70 form1) and FJ752779 (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.

### 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. AF275961), Daphnia magna (GenBank accession no. EU149494) and Moina macrocopa (GenBank accession no. EU149495). 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 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) 12-mer primer and the Superscript II reverse transcriptase 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.

**Table 1**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Sequences (5’-3’)</th>
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<td>F</td>
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</tr>
<tr>
<td>HSP2</td>
<td>F</td>
<td>TAYTGNGCTTCAGGCAAGAC</td>
</tr>
<tr>
<td>HSP3</td>
<td>R</td>
<td>AGGGTTGTGCTGCTGATYG</td>
</tr>
<tr>
<td>HSP4</td>
<td>R</td>
<td>TAGAACRTRRACCTGGTTAGACAGTC</td>
</tr>
<tr>
<td>Rim8</td>
<td>F</td>
<td>GCAACGACCATGCTCAACAA</td>
</tr>
<tr>
<td>Rex4</td>
<td>R</td>
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<tr>
<td>Rex2</td>
<td>F</td>
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</tr>
<tr>
<td>Rex3</td>
<td>F</td>
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</tr>
<tr>
<td>Var3</td>
<td>F</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Var3</td>
<td>F</td>
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<tr>
<td>5’ primer</td>
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</tr>
<tr>
<td>3’ primer</td>
<td>F</td>
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</tr>
<tr>
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<td>F/R</td>
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</tr>
<tr>
<td>Nested</td>
<td>F/R</td>
<td>AGACTGCTGATCAACAGCAGAG</td>
</tr>
</tbody>
</table>

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Nucleotide codes: R=A/G, Y=C/T, N=A/T/G/C, S=G/C. F, forward; R, reverse
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specific primers were designed using sequences in GenBank (Rimicaris 8-actin FJ410324; Palaemonetes 8-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, 72 °C 30 s) for each run. The 5-fold dilution series were used to construct a relative standard curve to determine the PCR 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 calculated with the Q-Gene software.

2.7. Statistical analysis

A Mann–Whitney Test was used to test 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 30 °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.
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
expression of hsp70 form1 was found to significantly increase following the heat-shock (15-fold; Mann–Whitney Test, \( p \approx 0.05 \)), 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 GMP 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 Triprops jucnus (four GMP 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 palamond 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 Hageman, 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) (Lofls, 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).

Surprisingly, 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 shrimp.

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; Helmhut 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. Thus, total RNAs were extracted from the same tissues (abdomen muscles) in both shrimps, since the expression of HSP70 is known to be tissue-specific. Thus, total RNAs were extracted from the same tissues (abdomen muscles) in both shrimps, since the expression of HSP70 is known to be tissue-specific.

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 Halitiotis 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.

Acknowledgements

We wish to thank the captain and crew of the R/V Pourquoi pas?, the crew of the Nautilis subsensible, and the chief scientists of the Momardreck cruise, Françoise Gail and Jérôme Dyment. We are grateful to Magali Zbinden and Jean-Yves Toullèc for their help in experimental work on board. We also wish to thank Philippe Lopez for his help in sequence analysis. We acknowledge funding support from the GDR Ecchis and the European Community program EXOCET/D. This work was supported by the Marine Biodiversity and Ecosystem Functioning Network of Excellence MarBEF (contract no. GOCE-CT-2003-505446) of the FP6, and a research grant from the Total Foundation (“Abyss2100”). [SS]

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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