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Heat-shock response and temperature resistance in the deep-sea vent shrimp *Rimicaris exoculata*

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Summary

The shrimp *Rimicaris exoculata* swarms around hydrothermal black smoker chimneys at most vent sites along the Mid-Atlantic Ridge. This species maintains close proximity to the hydrothermal fluid, where temperatures can reach 350°C and steep thermal and chemical gradients are expected. We performed *in vivo* experiments in pressurized aquaria to determine the upper thermal limit [critical thermal maximum (CT_{max})] of *R. exoculata* and to investigate some characteristics of the shrimp stress response to heat exposure. These experiments showed that the shrimp does not tolerate sustained exposure to temperatures in the 33–37°C range (CT_{max}). A heat-inducible stress protein belonging to the hsp70 family was identified in *R. exoculata*, and its synthesis threshold induction temperature is below 25°C. The *R. exoculata* optimal thermal habitat may thus be restricted to values lower than previously expected (<25°C).

Key words: hydrothermal vent, thermal stress, Crustacea, Caridae, IPOCAMP, chaperone, *Rimicaris exoculata*, shrimp, heat shock.

Introduction

Caridean shrimps, belonging to the family Alvinocarididae, dominate the vagile megafauna at most Mid-Atlantic Ridge hydrothermal vent sites (Desbruyères et al., 2001). One species, *Rimicaris exoculata* (Williams and Rona, 1986), is particularly abundant, forming dense swarms (>3000 individuals m^{-2}) around the chimneys expelling superheated sulfide-loaded fluid (Segonzac et al., 1993; Polz et al., 1998; Gebruk et al., 2000). This species supports a highly specialized bacterial epibiosis within its gill chamber, which constitutes the major, if not unique, diet source of *R. exoculata* adults (Casanova et al., 1993; Gebruk et al., 2000). The epibionts are believed to be chemoautotrophic sulfur-oxidizers (Wirsen et al., 1993), and mesophilic temperatures (>20°C) have been suggested to be important for carbon fixation (Wirsen et al., 1993; Gebruk et al., 2000). Whatever the reason may be for maintaining close proximity to hydrothermal fluid (temperature or sulfide concentration), *R. exoculata* must avoid exposure to lethal temperature areas. Temperatures nearing 40°C have been reported within shrimp swarms and up to 70°C several centimeters away from the swarms on the chimney wall (Gebruck et al., 1993; Segonzac et al., 1993). However, nothing is known regarding the effects of sustained exposure to such maxima for the shrimps. In the case of other epibiont-bearing species at East Pacific Rise vents, tolerance to much higher sustained temperatures (60–80°C) has been suggested for the annelid worm *Alvinella pompejana* (Cary et al., 1998), whereas it was shown not to exceed 40°C in the case of *Hesiolyra bergi*, another epibiosis-supporting invertebrate that shares a similar thermal micro-environment (Shillito et al., 2001).

The exposure of organisms to elevated temperature induces the expression of heat-shock proteins (hsp; Lindquist, 1986). In most organisms studied so far, hsp70 proteins are among the most prominent proteins induced by heat, and these proteins do play a central role in tolerance to high temperatures, as they allow cell survival during and after thermal stress (reviewed in Parsell and Lindquist, 1993). Most research on these proteins has focused on cellular and molecular aspects, but there is a growing interest in approaching the organismal, ecological and evolutionary aspects of the stress response (reviewed by Feder and Hofmann, 1999; Queitsch et al., 2002). Because *R. exoculata* lives in a complex and highly fluctuating thermal environment and is thought to undergo frequent harsh temperature conditions on the chimney wall, it provides a useful biological system for stress-response studies.

The central questions underlining this work are: what is the absolute upper thermal limit tolerated by the host shrimp and, assuming that temperatures above 20°C are optimal for the epibionts (Wirsen et al., 1993), are exposures to such temperatures stressful for their hosts? To address these

questions, we undertook several experiments with live shrimps maintained in video-equipped pressurized aquaria. Our goal was first to determine the upper thermal tolerance (CT_{max}) of the shrimp by following behavior and survival upon severe heat shock (45°C peak). Second, we investigated some characteristics of the stress response (heat-shock protein accumulation) of *R. exoculata* during a 'mild' heat shock (25°C peak) that would presumably be biologically and environmentally relevant. In parallel, reference experiments (survival, behavior and oxygen consumption) were conducted at 15°C.

Materials and methods

Specimen collection

Rimicaris exoculata (Williams and Rona, 1986) specimens were collected during the 'ATOS' cruise (N/O *Atalante*, ROV *Victor 6000*; June 2001) at the Rainbow vent site (36°14.0′ N; Mid-Atlantic Ridge; 2300 m depth). Animals were sampled with a suction device operated with the submersible's hydraulic arm and stored inside insulated Perspex cylinders until transferred to the ship. Upon recovery, the temperature of the water inside the cylinders ranged from 10°C to 15°C.

Pressurized incubator IPOCAMP™

The stainless steel IPOCAMP (Incubateur Pressurisé pour l'Observation et la Culture d'Animaux Marins Profonds) pressure vessel (PV) has a volume of approximately 19 liters, as previously described (Shillito et al., 2001). The general design of the pressure circuit is inspired from flow-through pressure systems utilized by Quetin and Childress (1980), with flow rates that may exceed 20 liters h^{-1} at 230×10^5 Pa working pressure. Pressure oscillations due to pump strokes (100 r.p.m.) are less than 1×10^5 Pa at working pressure. The temperature of the flowing seawater (filtered at 0.4 μm) is measured constantly, at pressure, in the inlet and outlet lines (±1°C). A more accurate temperature measurement (±0.1°C) is achieved inside the pressure vessel through two Pt-100 probes (see Fig. 1). Temperature regulation is powered by a regulation unit (Huber CC 240) that circulates ethylene glycol around the seawater inlet line and through steel jackets that surround the PV. Finally, IPOCAMP allows video observations of the re-pressurized organisms by combining an endoscope (Fort, Dourdan, France) to a CCD camera (JVC, TK-C1380; Fig. 1). The resulting view is further displayed on a TV monitor (JVC) and recorded (Sony SVO-9500 MDP videotape recorder).

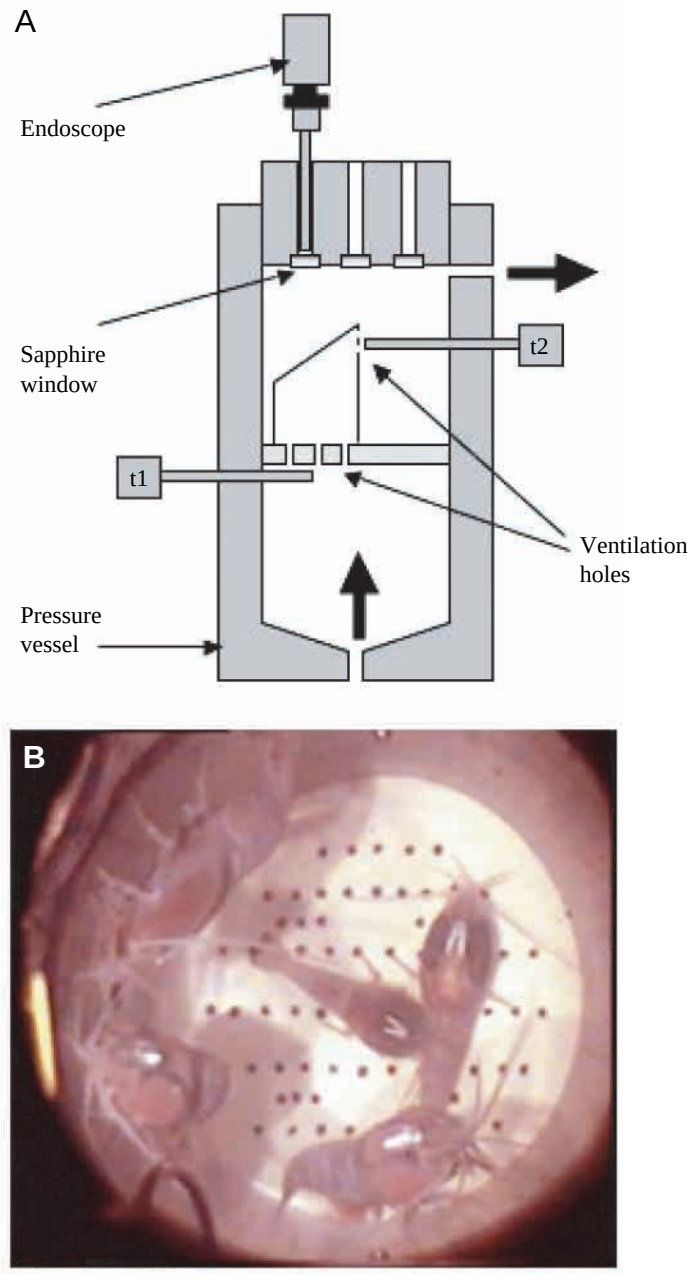

Fig. 1. Experimental set-up in the IPOCAMP (Incubateur Pressurisé pour l'Observation et la Culture d'Animaux Marins Profonds) pressure vessel for heat-exposure experiments. (A) The pressure vessel contains three cages (only one is represented here). Each cage is a PVC cylinder that is closed at the top with an inclined transparent Plexiglas lid (about 9 cm diameter; 15 cm at the highest point). Ventilation holes are located at the top and bottom of each cage, with Pt-100 probes that are positioned in the water flow, allowing direct reading of the upstream (t1) and downstream (t2) temperatures. Connections in the lid of the pressure vessel are terminated by sapphire windows. By inserting an endoscope into the appropriate connection, the content of one of the cages may be observed. Illumination is achieved through the other connections by means of optical fiber light-guides. Large arrows indicate the inlet and outlet of circulating seawater, which is forced through the cage ventilation holes. The internal diameter of the pressure vessel is 20 cm, while its height is approximately 60 cm. (B) Video-view of a nylon-mesh cage containing five *Rimicaris exoculata* specimens, maintained in the IPOCAMP at *in situ* pressure (230×10^5 Pa) and a temperature of 15°C. The white V-shaped ocular plates ('eyes') of the shrimps are clearly visible for two individuals that are resting on the bottom of the cage. Three other specimens (on the left of the image) are resting on the vertical wall at the top of the cage. Diameter of cage bottom = 9 cm, approximate length of shrimps = 3–6 cm.

In vivo *experiments*

Once on board, *R. exoculata* specimens were transferred to a 14°C cold-room. Most shrimps survived the collection trauma, and the individuals that swam apparently normally were sorted out to be re-pressurized for *in vivo* experiments. Only adult specimens were chosen, and these specimens were placed in PVC cages inside the pressure vessel at an initial seawater temperature of 15°C, as previously described by Shillito et al., (2001). Re-pressurization at 230×10^5 Pa was achieved in approximately 2 min. In all experiments, less than 2 h intervened between the time the samples began decompression (submersible ascent) and the moment they were re-pressurized.

Four experiments at *in situ* pressure (230×10^5 Pa; 20 liters h^{-1} flow rate) were performed on a total of 60 shrimps. For each experiment involving behavioral observations (all but Expt. 1), 15 shrimps were placed in three cages (five individuals per cage; Fig. 1B).

Expt 1 (respirometry experiments A and B, at reference temperature)

The shrimps were placed for 3 h (Expt 1A) or 4 h (Expt 1B) at 15°C in order to evaluate their oxygen consumption. These animals were not analyzed for their behavior. Because these experiments were performed in closed containers (see Oxygen-level measurements), the oxygen level decreased as a function of time, which limited the experiment duration to 4 h.

Expt 2 (reference experiment)

The shrimps were maintained at 15°C over 24 h. This 'control' experiment aimed to study behavior and survival in order to verify whether the shrimps recovered from the collection trauma. These shrimps also served as a control for immunochemical analysis (see below).

Expt 3 (lethal heat shock)

After 5 h at 15°C, the shrimps were heat-exposed, as previously described for the vent polychaete *Hesiolyra bergi* (Shillito et al., 2001), until the temperature reached 45°C, followed by cooling to 15°C. Maximum heating and cooling rates were 0.52°C min^{-1} and –1.05°C min^{-1}, respectively. The total duration of the experiment was 8 h.

Expt 4 (mild heat shock)

From the results of Expt 3, we tried a milder heat-exposure experiment to a temperature of 25°C. The heating phase started 21 h 45 min after the re-pressurisation, and the cooling phase to 15°C took place approximately 1 h later. Maximum heating and cooling rates were 0.35°C min and –0.29°C min^{-1}, respectively. The total duration of the experiment was 24 h, as for Expt 1 (control).

All the shrimps experimented on during Expt 2 and Expt 4 were frozen in liquid nitrogen on board until further analyses at the lab.

Video analysis of in vivo *experiments*

For all the *in vivo* experiments, survival of the re-pressurized shrimps was determined during the last minutes of the experiments by identifying each individual and witnessing its

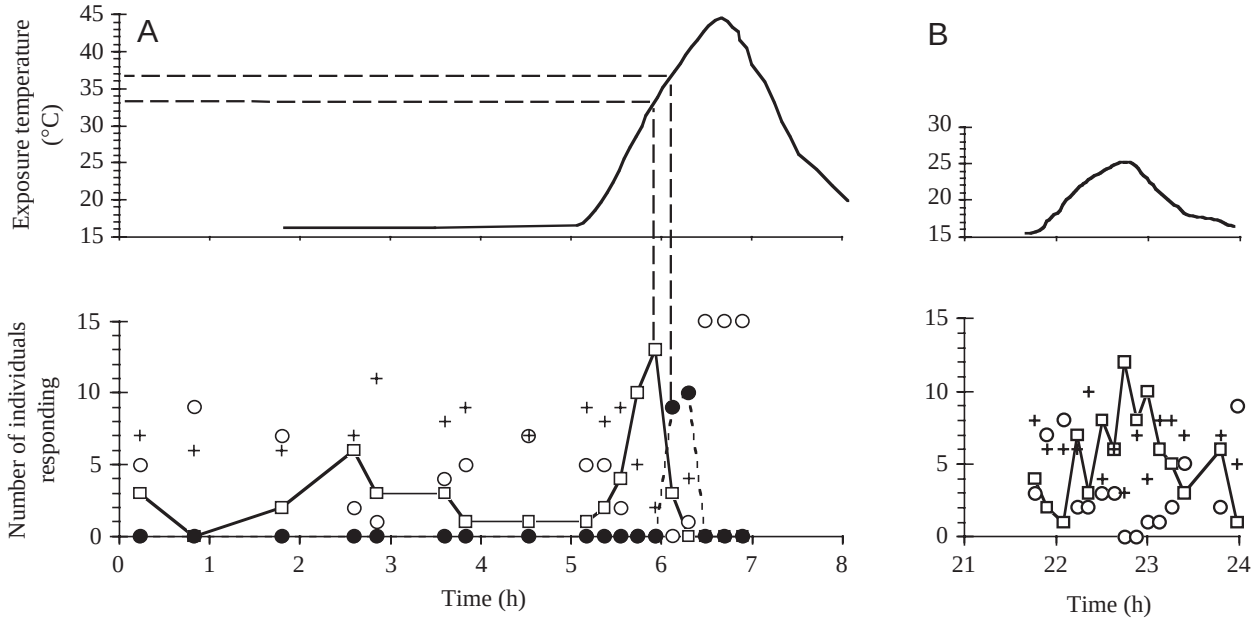

Fig. 2. Video analyses of *Rimicaris exoculata* behavior during *in vivo* experiments. Behavior during 30 s sequences, as a function of time since re-pressurization: ○, 'motionless'; +, 'moving'; □, 'active walking or swimming'; ●, 'spasms' (see Materials and methods). **(**A,B**)** Behavior (lower graphs) related to temperature (upper graphs, mean temperature between t1 and t2 probes; see Fig. 1A) during two heat-exposure experiments [Expts 3 **(**A**)** and 4 **(**B**)**; 15 individuals per experiment, split into three groups of five individuals]. For each observation time, the maximal error regarding the corresponding temperature is approximately ±2°C (A) and ±1.7°C (B).

movements. Additionally, survival could also be confirmed at atmospheric pressure after the experiments.

For the heat-exposure experiments (Expts 3 and 4), observations were recorded throughout the experiment.

The endoscope was moved successively from the first to the third cage (4 min observation period for each cage) about once every hour at 15°C and then continuously rotated during heat shock (each cage was observed for 4 min before moving to the next). The resulting behavioral data for 15 shrimps were pooled from the last 30 s in the first cage, the middle 30 s in the second cage, and the first 30 s in the third cage (see also Fig. 2 legend). Within each period of observation, the shrimps were individually classified into four categories:

(C1) 'motionless': no movement detected at normal tape-reading speed; this category was also applied when an individual's movement seemed to be the result of neighboring shrimp 'pushing', with no apparent reaction of the individual.

(C2) 'moving': any kind of detectable movement, at normal tape-reading speed, except that of category 3 and 4 (below): pereiopod or pleopod movements, scaphognathite beating, antennal lateral sweeping on dorsal side, or cleaning of the mouth parts by rubbing them along each other.

(C3) 'active walking or swimming': when the shrimp moved along a distance exceeding its own length in less than 30 s.

(C4) 'spasms': spasmodic motions (vibrations of the abdomen) without any coordinated movement; the temperature at which the animals were first observed to move in a jerky uncoordinated way was defined as the critical thermal maximum (CT_{max}; Wehner et al., 1992; Gehring and Wehner, 1995; Cuculescu et al., 1998).

All the categories are exclusive; i.e. one individual can only be assigned once among these four categories.

For each observation point of 15 individuals during heating periods (successive sequences on the three cages), we determined the error limits for the corresponding temperature. Maximum heating rates were 0.52°C min^{-1} (Fig. 2A) and 0.35°C min^{-1} (Fig. 2B) for Expts 3 and 4, respectively. Considering the maximum heating rates and the duration of each video survey (5 min, from the last 30 s of the first sequence, through the middle 4 min of the second sequence, to the first 30 s of the third sequence), a resulting maximum variation of 2.6°C (Expt 3) and 1.7°C (Expt 4) in the temperature measurements was obtained. Moreover, during heating, a maximum difference of 1.7°C was recorded between the two temperature probes placed at the top and the bottom of the cage. For each temperature point, the maximal error is thus approximately ±2°C (Expt 3) and ±1.7°C (Expt 4).

Oxygen-level measurements

Shrimps (15 individuals; Expt 1) were individually stored in soft polyethylene containers filled with 210 ml of surface seawater, which were sealed before pressurisation. Another 210 ml container without any animals was also pressurized for use as a control. After either 3 h or 4 h (Expt 1A or 1B, respectively), these containers were recovered and the oxygen concentration was estimated using a Clark-type microelectrode (DK; Unisense, Aarhus, Denmark) with an estimated precision of ±3%. These measurements were calibrated with air-equilibrated surface seawater (100% O_2) and deoxygenated surface seawater by addition of sodium sulphide (0% O_2). The 100% O_2 solution was standardised with the Winkler method (S.D. of the method = 2%; 95% confidence interval for N=1 is ±4%; Aminot and Chaussepied, 1983). The O_2 uptake rates were checked against the pressurized control to preclude possible uptake from bacteria in the seawater. No oxygen consumption was registered in the controls.

The shrimps were dried at 80°C on board the ship (for 48 h). They were then further dried at the lab at 80°C (for 5 days) and weighed (0.1 mg precision). Among the 15 shrimps treated, only the individuals for which the final oxygen concentration in the containers did not drop below 50% of the initial concentration were kept for the results interpretation (seven individuals). Final O_2 content was thus definitely above the oxygen level at which the shrimp O_2 consumption may decline rapidly (Prosser, 1973).

Electrophoresis and immunochemical analysis (western blot) of hsp70

Samples of shrimp abdomens (from Expts 2 and 4) without their cuticle were ground in liquid nitrogen, and the powder was homogenised in 1.5 ml of extraction buffer [10 mmol l^{-1} Tris/HCl, pH 7.4:protease inhibitor cocktail (Sigma, St Quentin Fallavier, France) 1:3 (v/v)]. The homogenates were sonicated (2×10 s) using a cell homogeniser and centrifuged at 10 000 ***g*** for 10 min at 4°C. The pellet was discarded and the extracted proteins were quantified in the supernatant with the Bio-Rad Protein Assay (Bio-Rad, Marnes-la-Coquette, France) using bovine serum albumin (Sigma) as standard. Proteins of the total supernatant were separated by minigel sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS–PAGE; 10% acrylamide:0.3% bisacrylamide (w/v); 4 μg protein $well^{-1}$]. After running the electrophoresis (15 min at 15 mA gel^{-1}, 1 h at 20 mA gel^{-1}), the bands were stained with silver nitrate (Wray et al., 1981). Protein molecular mass standards from 29 kDa to 205 kDa (Sigma) were used to evaluate the apparent M_r of the separated bands.

For western blotting, the proteins were transferred from the SDS–PAGE gel (20 μg protein $well^{-1}$) to a nitrocellulose membrane by semi-dry blotting at 20 V for 1.5 h (Trans-Blot semi-dry cell; Bio-Rad). Membranes were blocked in milk/Tris-buffered saline pH 7.4 (TBS) (5% w/v) for 1 h and incubated overnight with a polyclonal antibody (anti-human hsp70; StressGen, Victoria, BC, Canada) at room temperature. Subsequently, a 10 min washing step with TBS pH 7.4 was repeated three times, and the membranes were incubated with a polyclonal secondary antibody coupled to peroxidase (StressGen) for 2 h at room temperature. After another 3×10 min washing step with TBS, the antibody complex was detected using the substrate Bromo-chloro-indolyl-phosphate/Nitro Blue Tetrazolium (BCIP/NBT) (StressGen) incubated at room temperature for 10 min. The membranes were further digitalized using a UMAX (Hsinshu, Taiwan) Power Look 3

scan at 600 d.p.i. resolution. Density profiles were obtained from the western blot membranes using NIH Image 1.6 software.

Results

Reference experiments: survival, behavior and oxygen uptake at 15°C

Rimicaris exoculata withstood the decompression events, either during the collection process or after the *in vivo* experiments. At atmospheric pressure, just after the submersible recovery, the shrimps (except for some individuals, which may have been damaged by the suction sampler) were alive and active.

At 230×10^5 Pa and 15°C (Expt 2), all the shrimps were alive after 24 h. They can be maintained alive in pressurized vessels (230×10^5 Pa; 15°C) for at least 48 h with a survival rate of 100% (preliminary experiment with 15 specimens, not described here). A behavioral survey throughout Expt 2 (16 observations of 30 s sequences) allowed the detection of a broad diversity of movements. Table 1 summarizes the behavioral response of the shrimps during the different periods of maintenance at 15°C of Expt 2 (24 h), Expt 3 (first 5 h) and Expt 4 (first 21 h), which represents a total of 33 observations. This survey of periods at 15°C allowed us to set the threshold values for considering significant behavior during the heat-shock experiments. Throughout the 24 h of Expt 2, the shrimps were frequently observed immobile (C1) or slightly moving (C2). Several movements of the different appendages were identified: pereiopod or pleopod movements, antennal lateral sweeping on the dorsal side, and cleaning of the mouth parts (particularly the maxillipeds) by rubbing them along each other. The shrimps rarely swam or walked actively (C3), and a maximum of seven individuals were observed actively swimming at the same time. According to this, the threshold value considered in the heat-shock experiments for activity above the reference level was seven individuals. During these 15°C periods, the shrimps never had spasms (C4). The branchiostegites (lateral sides of the cephalothorax) sometimes appeared transparent, which allowed us to see the scaphognathite (large flaps on the second maxillae) beating in the gill cavities when the animals were in side view or when they faced the endoscope (vertical position on the cage wall; see Fig. 1). The scaphognathite movements create a water flow on the gills, and the beating rate was 24–58 beats min^{-1} (at 15°C; N=12).

Table 1. *Behavioral analyses of the different periods of maintenance at 15°C*

Behavioral category	Mean ± S.D.	N_{min}	N_{max}
C1 ‘motionless’	5.8±2.7	1	13
C2 ‘moving’	6.5±1.9	2	11
C3 ‘active walking/swimming’	2.6±1.8	0	7
C4 ‘spasms’	–	0	0

A total of 33 observations was performed throughout the 24 h of Expt 2, the first 5 h of Expt 3 and the first 21 h of Expt 4. All values are assigned to one observation of 15 individuals. Mean ± S.D., mean number of individuals ± standard deviation, based on all 33 sequences; N_{min} and N_{max} represent the minimum and maximum number of individuals ever observed during one 30 s sequence. According to this table, more than seven individuals actively walking or swimming (C3) would be considered as significantly above the reference level.

In the respirometry experiment, the initial oxygen content in the flasks roughly corresponded to the oxygen saturation level in seawater at the temperature of the experiments (254±9 μmol l^{-1}; N=5). This level is close to the oxygen content of 240±9 μmol l^{-1} (N=4) that we determined for deep waters surrounding the hydrothermal vent fields. The oxygen consumption rates (R; expressed in $\mu gO_2\ h^{-1}$) were related to the shrimps mass (M; expressed in mg dry mass) following the equation $R=1.748M^{0.891}$ (r=0.832, N=7, P<0.05; Expt 1A,B; Fig. 3). The mass-specific oxygen consumption rates for *Rimicaris exoculata* were 0.837–1.094 mg $O_2\ g^{-1}$ dry mass h^{-1} (means ± S.D. = 0.979±0.101 mg $O_2\ g^{-1}$ dry mass h^{-1}).

Heat-exposure experiments

Temperature resistance

Shrimps submitted to the first heat-shock experiment (Expt 3; maximum temperature 45°C; Fig. 2A) were all dead after 8 h. Moreover, they had stopped moving during the seventh hour of the experiment, before the temperature had reached its 45°C maximum. Indeed, from this point onwards, the shrimps all remained motionless at the bottom of the cage, laid on their back or side, and finally assumed a curved body shape in a *post mortem* position. The influence of temperature was analyzed by observing the behavior of these shrimps over 18 video-sequences of 30 s throughout the experiment (four behavioral categories; see Materials and methods; Fig. 2A). During heat

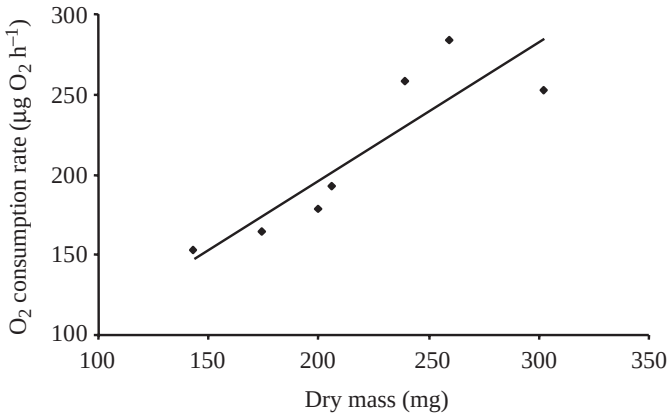

Fig. 3. Oxygen uptake in *Rimicaris exoculata* in relation to shrimp dry mass for seven individuals maintained at *in situ* pressure (3 h or 4 h; 15°C; 230×10^5 Pa; Expt 1; see Materials and methods). The oxygen consumption rates (R; expressed in $\mu g\ O_2\ h^{-1}$) correlate to dry mass (M; expressed in mg dry mass) of individuals following the equation: $R=1.748M^{0.891}$ (r=0.832, N=7, P<0.05).

exposure, an increase in shrimp activity (C3: active walking or swimming) above the reference level was observed, which started between 24±2°C and 28.5±2°C (see legend of Fig. 2; see Table 1 for reference behavior). The peak of this activity response corresponds to approximately 33°C (13 shrimps among the 15 were swimming or actively walking) and was followed by a fast decrease in activity until 45°C. This decrease was accompanied by an apparent loss of locomotory coordination, expressed as flicking of the abdomen (spasms) and rapid movements of the pleopods without any efficient displacement. All the animals were dead when the temperature reached 43±2°C.

Heat-shock response

During the second heat-exposure experiment (Expt 4; Fig. 2B), which was non-lethal, the maximum temperature was 25°C and all the shrimps survived after 24 h. The behavior during the first 21 h (15°C; 230×10^5 Pa) was similar to that of the reference experiment (Expt 2; see Table 1). The activity was relatively more intense during the heat exposure, as more than seven individuals were frequently observed swimming (C3) at the same time, with a maximum (12 individuals) at around 25°C. These shrimps were further analyzed for the detection of stress proteins.

The protein profiles of reference (Expt 2) and heat-shocked (Expt 4) *R. exoculata* abdomen samples are shown in Fig. 4 (only one profile is presented for each type of sample). In the heat-shocked individual, at least three proteins, with molecular masses of about 205 kDa, 90 kDa and 70 kDa, are overexpressed compared with the reference individual, for which these proteins display very low abundances.

The relative difference in the level of the hsp70 stress proteins between the groups of four reference (R) and four heat-shocked (HS) *R. exoculata* in response to a 25°C heat exposure (approximately 15 min at 25°C; see Expt 4 and Fig. 2B) was quantified by western blot and subsequent densitometry analysis using the NIH software (Fig. 5). hsp70 proteins were detected in both groups as two protein bands, one of them being more intense in the heat-shocked individuals [low molecular mass (LMM) band; Fig. 5A]. The intensity of the high molecular mass (HMM) protein band, which was revealed in all samples, is not significantly different between the R and HS samples (Mann–Whitney test; U=6.0, P=0.564). On the contrary, a significant increase of the LMM protein band intensity is observed on the western blot of the shocked animals (Fig. 5A,B; Mann–Whitney test, U=0.0, P=0.021).

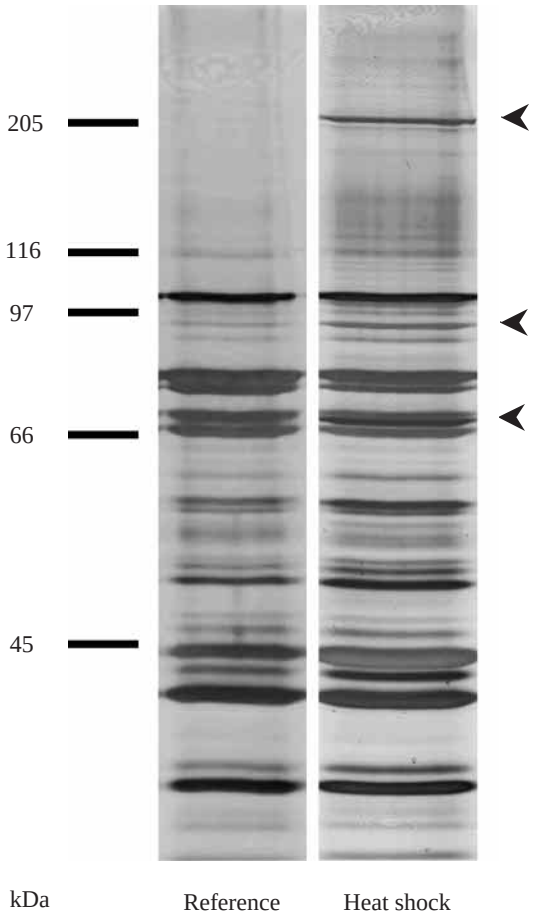

Fig. 4. Protein profile of heat-shocked and reference *Rimicaris exoculata* abdomen samples (4 μg total protein well^{-1}) as shown on a silver-stained 10% gel. The 'reference' lane shows individuals from Expt 2 (24 h; 15°C); the 'heat-shock' lane shows individuals from Expt 4 (24 h; 25°C heat shock; see Materials and methods). Arrowheads indicate bands of interest that are not apparent or are poorly represented in the reference samples and are visible in the heat-shocked samples at molecular masses of approximately 205 kDa, 90 kDa and 70 kDa.

Discussion

Reference experiments

Most *Rimicaris exoculata* survived the collection trauma, and the 100% survival rate of the individuals maintained at 15°C at *in situ* pressure over 24 h and 48 h, with no drastic change in their general behavior, indicates a relatively good physiological state. This conclusion is a pre-requisite to on-board *in vivo* experimentation on freshly collected deep-sea fauna. This is the first successful *in vivo* experiment performed at *in situ* pressure for a 2300 m depth Atlantic vent species. The respirometry experiments, performed at deep seawater oxygen levels (seawater surrounding the North Atlantic vents with an O_2 concentration of approximately 240 μmol l^{-1}; present study; Millero, 1996), confirm the good physiological state of the shrimps when repressurized. First, the correct fitting of individuals (P<0.05 for n=7) to a power law curve indicates a homogeneous physiological state. Second, the exponent value of this power law relation is 0.891, this value being very similar to the mean slope value of 0.85 expected for Crustacea (Bridges and Brand, 1980; Weymouth et al., 1944). Furthermore, it is quite similar to the values obtained at 15°C for other shrimps: 0.799 for the surf zone penaeid *Macropetasma africanus* (Cockcroft and Wooldridge, 1985) and 0.867–0.876 for the estuarine palaemonid *Palaemon pacificus* (Emmerson, 1985). Third, the oxygen consumption

rates of *R. exoculata* are very similar to those observed for *M. africanus* (R=1.806$M^{0.799}$; Cockcroft and Wooldridge, 1985) and for *P. pacificus* (R=1.378$M^{0.867}$ or R=1.383$M^{0.876}$; Emmerson, 1985). Third, the specific oxygen consumption rates for *R. exoculata*, using dry mass, fit perfectly those obtained for *M. africanus* (0.883–1.167 mg O_2 g^{-1} h^{-1}; recalculated from the data of Cockcroft and Wooldridge, 1985) and for *P. pacificus* (0.647–1.040 mg O_2 g^{-1} h^{-1}; recalculated from the data of Emmerson, 1985). Considered together, our data suggest that the metabolic rate of *R. exoculata* is, if not normal, at least far from reflecting that of moribund animals under the conditions of our reference experiment.

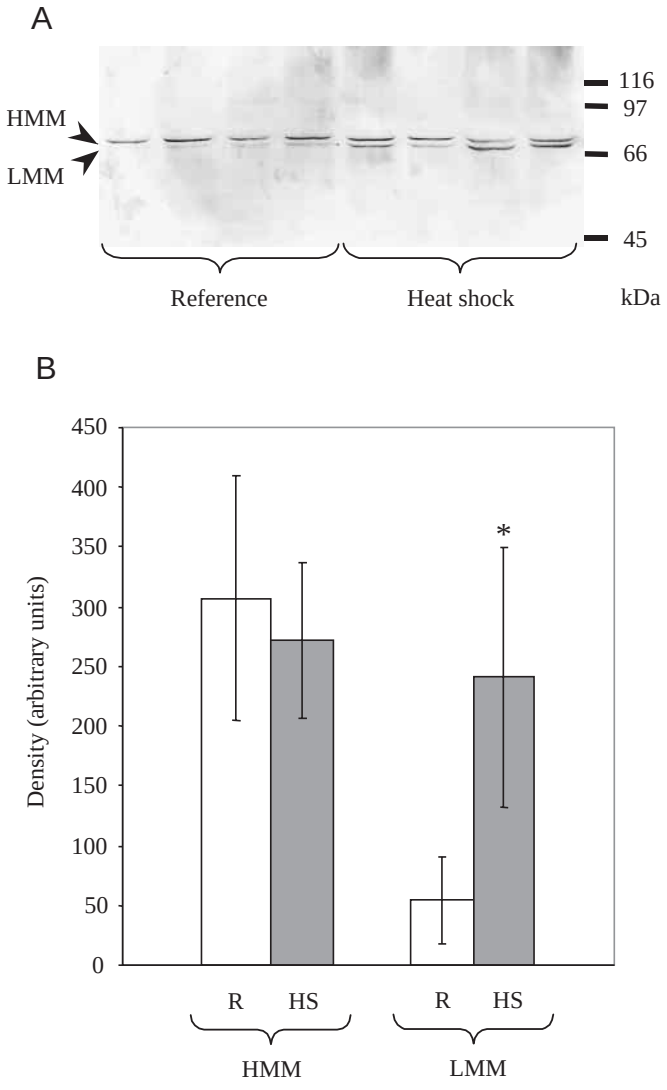

Fig. 5. **(**A**)** Western blot of *Rimicaris exoculata* abdomen samples for the detection of hsp70 proteins (anti-human hsp70). Each lane corresponds to a different individual. The 'reference' lanes show individuals from Expt 2 (24 h; 15°C); the 'heat-shock' lanes show individuals from Expt 4 (24 h; 25°C heat shock; see Materials and methods). Arrowheads indicate the hsp70 proteins, named band HMM (high molecular mass) and LMM (low molecular mass). (B) Comparison of hsp70 levels in abdomen samples as estimated by density measurements of the western blot protein bands (HMM and LMM). R = reference samples, HS = heat-shocked samples. Density profiles were obtained from the western blot membranes using NIH Image 1.6 software. The density of each band (expressed in arbitrary units) was calculated from the area of the corresponding peak on the profile. Each column represents the mean of band density for four individuals (± S.D.). The asterisk indicates a significant difference between treatments (Mann–Whitney test, U=0.0, P=0.021).

The reference experiments were performed in conditions comparable with those *in situ* for pressure (230×10^5 Pa) and oxygen level (240 μmol l^{-1}). Our 15°C reference temperature was chosen in agreement with the mean temperature values recorded so far in the shrimp microenvironment: 11°C (Geret et al., 2002), 13.2±5.5°C (Desbruyères et al., 2001) and 10–15°C (Segonzac et al., 1993). This reference temperature is significantly lower than the maxima suggested to be encountered in the shrimp habitat, where usual temperatures may reach, according to the various authors, 25°C (Desbruyères et al., 2000), 30°C (Van Dover et al., 1988) or 40°C (Gebruk et al., 1993).

Comparisons of experimental behavior of the shrimp with natural behavior should be made cautiously, since both environment and observation conditions differ radically. Moreover, whereas the individuals often live in dense swarms *in situ*, only five individuals were used in a single cage for pressure-vessel experiments. However, the various types of behavior observed experimentally resemble those occurring *in situ*, except for the lack of uncoordinated flicking of the abdomen (spasms). Most of the time, the shrimps were observed either motionless on the bottom or along the walls of the cage or making small movements of the appendages. In their natural environment, the shrimps may also remain motionless on the surface of the black smoker chimneys, moving only slightly in confined areas (Van Dover et al., 1988; Gebruk et al., 1993, 2000; Segonzac et al., 1993). They were occasionally observed actively swimming, when entrained by turbulent water, to move back towards the substrate.

Temperature resistance

In the 45°C peak heat-exposure experiment (Expt 3), none of the 15 shrimps survived. A significant increase in active crawling appeared in the 24–28.5°C temperature range, which, in view of the low activity at 15°C, can be inferred to be an escape response to avoid heat zones. A similar behavior occurs *in situ*, since the shrimps lay more or less motionless on chimney walls, where the measured temperature was 2°C, whereas they swam more actively as they came across warm water zones (Segonzac et al., 1993). However, the escape response observed for *R. exoculata* below 28.5°C is inconsistent with other *in situ* observations reporting a slight mobility of individuals in swarms where the temperature was reported to have reached 30°C (Gebruk et al., 1993, 2000). A more detailed description of the thermal characteristics of the *R. exoculata* microenvironment is necessary to understand the *in situ* shrimp behavior and, in particular, it is of interest to determine whether the shrimps are able to regulate their microhabitat temperature by inducing water flows with their locomotory appendages.

The critical thermal maximum (CT_{max}) is defined as the temperature at which the animal is no longer capable of proper locomotion and starts to move in a jerky uncoordinated way, i.e. the temperature at which the first signs of heat stress occur (Wehner et al., 1992; Gehring and Wehner, 1995; Cuculescu et al., 1998). CT_{max} for *R. exoculata* is evidenced by very characteristic spasmodic movements of the abdomen and is in the 33–37°C range (±2°C; see Fig. 2A). The shrimps were all dead when the rising temperatures reached 43±2°C, as they all sank to the bottom of the vessel and laid motionless on their backs or sides. *R. exoculata* may be compared with another vent crustacean species, the East Pacific Rise crab *Bythograea thermydron*, which survives a 1 h exposure at 35°C but succumbs after 1 h at 37.5°C (Mickel and Childress, 1982). The CT_{max} value for *R. exoculata* is lower than that of the vent polychaete *H. bergi* (41–46°C; East Pacific Rise), which lives on the chimney walls and is likely to encounter occasional high-temperature pulses (Shillito et al., 2001), and would also be very different from that proposed for the vent annelid *Alvinella pompejana* (East Pacific Rise), which, according to Cary et al., would lie above 60°C at least (1998). The upper thermal tolerance of *R. exoculata* is close to that of the Iceland hot spring beachflea *Orchestia gammarellus* (approximately 37°C; microhabitat temperatures ranging from 15°C to 25°C; Bjarnarstadir site; Morritt and Ingolfsson, 2000), which occupies similar harsh and unstable environments where temperatures can reach 42°C (thermal spring temperature). Finally, CT_{max} for *R. exoculata* is also comparable with that of the shore crab *Carcinus maenas* (approximately 32–34°C for 15°C-acclimated specimens; Cuculescu et al., 1998), which is regularly exposed to severe abiotic stresses and experiences very high and rapid changes in body temperature during the tidal cycles.

Stress response/stress protein induction

The heat-shock response is universal, as almost all organims studied to date are able to express heat-shock proteins (hsp; reviewed in Lindquist, 1986). Lindquist (1986) suggested that only some creatures living in the depths of the ocean may not have a heat-shock response, but even this was doubtful. This study constitutes the first analysis of the heat-shock response for a deep-sea vent organism living at 2300 m depth in a highly fluctuating thermal habitat that should cause frequent heat stress.

At least two proteins of the hsp70 family are present in both reference and heat-shocked animals. However, one of them (LMM band; see Fig. 5) was clearly more abundant in animals that were submitted to a heat shock, thus demonstrating its involvement in the heat-shock response. According to this, the presence of a less intense LMM band in reference animals could possibly indicate that 15°C is a temperature that approaches the hsp70 induction threshold. Alternatively, it may reflect a 'background' response to experimental stress (pressure variations upon recovery and conditioning in IPOCAMP, manipulation, etc.), since hsp70 expression can be triggered by many non-thermal stresses (Feder and Hofmann, 1999), including pressure variations (Welch et al., 1993). Finally, this low-intensity band may signal natural heat stress prior to sampling, even though the shrimps had been collected *in situ* at least 10 h before they were frozen for hsp detection. Indeed, it has been shown in the case of marine snails (Tomanek and Somero, 1999) that the presence of heat-inducible hsp70 was still detectable almost 50 h after an initial heat shock.

The HMM band was equally intense in both reference and heat-shocked animals (Fig. 5) and therefore appears to reflect a non-heat-inducible form of the hsp70 family. This protein could be either a constitutive form of the hsp70 chaperone [i.e. the so-called heat-shock cognates (hsc), which are expressed continuously in the organism] or could again be triggered by the experimental stress. None of the previous hypotheses can be favored but, because about the same level of this hsp70-like protein was found in all eight experimental animals, the HMM-band protein does not seem to be involved in the heat-stress response, whereas the LMM-band hsp70 form definitely is.

Under elevated thermal conditions, the hsp70 proteins function as 'molecular chaperones', preventing the aggregation and promoting the proper refolding of denatured proteins (reviewed in Parsell and Lindquist, 1993). In *R. exoculata*, the synthesis of an hsp70 heat-inducible form occurred following a heat exposure at 25°C (approximately 15 min at 25°C; Expt 4; Fig. 2B) and thus may reflect the emergence of cellular damage in the shrimps. The threshold induction temperature of the heat-shock response (i.e. the temperature at which heat-inducible hsp isoform synthesis is first observable) is thus lower than 25°C. The hsp70 enhanced synthesis threshold in *R. exoculata* would be in the same range of temperatures as for the 13°C-acclimated marine snail *Tegula brunnea* (24°C; Tomanek and Somero, 1999) or for various 10°C-acclimated teleost fishes (20–28°C range; Dietz and Somero, 1993). However, the hsp70 expression in *R. exoculata* occurred after a relatively brief heat exposure (15 min at 25°C) when compared with the heat exposure of the marine snail (2.5 h; Tomanek and Somero, 1999) and the teleost fishes (2 h; Dietz and Somero, 1993). As the induction of hsp reflects stress conditions for the organism (Parsell and Lindquist, 1993), the optimal habitat temperature range of *R. exoculata* may be below 25°C. This is quite low considering that the shrimps should approach the hydrothermal fluid to 'farm' their epibionts and are thought to tolerate *in situ* temperature spikes of at least 40°C (Gebruk et al., 1993). Furthermore, juveniles of *R. exoculata* have been observed in zones of diffuse vent fluid where the temperature is above 20°C (Gebruk et al., 2000), and adult shrimps are supposed to live at temperatures of up to 30°C (Van Dover et al., 1988; Gebruk et al., 1993). Several questions arise when considering the definition of the habitat temperature range of these shrimps. In such a highly fluctuating environment, the relevance of maximum temperature obtained from discrete measurement should still be considered cautiously. Furthermore, the shrimps can reach densities of 3000 individuals m^{-2} in the swarms (Gebruk et al., 2000), where they are packed side by side and often piled two

or more deep (up to 0.4 m thickness), which can interfere with the probing effort.

This study reports the first *in vivo* experiments in pressurized aquaria on a deep Mid-Atlantic Ridge vent organism. The heat-shock experiments with a protein induction assessment provide an indication of the thermal limits of this species, suggesting that the habitat temperature would be restricted to values lower than previously expected. The conclusion that can be drawn from our experiments is that *R. exoculata* does not tolerate exposure to temperatures in the 33–37°C range (CT_{max}) and succumbs to heat stress above this limit. Moreover, the shrimps synthesize heat-shock proteins when briefly exposed (15 min) to a temperature of 25°C. *R. exoculata* would thus be forced to live in a narrow thermal window in which the lower and upper limits are set by the epibionts 'farming' demands and heat stress, respectively, and would occasionally be exposed to high temperature peaks during very short periods. Considering the great difference in volume between the shrimps and their bacteria, they certainly undergo very different internal temperature patterns for the same environmental temperature conditions. The optimal thermal window or thermal regime allowing a successful epibiosis has still to be determined.

Further studies will help to pinpoint the threshold temperature for the induction of enhanced hsp70 expression in the shrimp. Since inducible hsp70 is thought to be deleterious when expressed under non-stress conditions (reviewed by Parsell and Lindquist, 1993; Krebs and Feder, 1997), the heat-shock response needs to be transient, which would imply for *R. exoculata* the ability to rapidly express and inactivate the hsp70. Future research will aim to determine whether adaptation to the fluctuating hydrothermal environment is reflected by particular kinetics of the hsp response in the vent shrimps.

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