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Thermal biology of the deep-sea vent annelid *Paralvinella grasslei*: *in vivo* studies

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SUMMARY

The annelid *Paralvinella grasslei* is a deep-sea vent endemic species that colonizes the wall of active chimneys. We report here the first data on its thermal biology based on *in vivo* experiments in pressurized aquaria. Our results demonstrate that *P. grasslei* survives a 30 min exposure at 30°C, and suggest that the upper thermal limit of this species is slightly above this temperature. The first signs of stress were noticed at 30°C, such as a significant increase in the animal's activity and the expression of HSP70 stress proteins. A preliminary investigation of the kinetics of stress protein expression surprisingly showed high levels of HSP70 proteins as late as 3.5 h after the heat shock. Finally, we provide here the first sequences for vent annelid *hsp70* (*P. grasslei*, *Hesiolyra bergi* and *Alvinella pompejana*). These constitute valuable tools for future studies on the thermal biology of these annelids.

Key words: Hydrothermal vents, heat shock proteins, stress response, heat stress, annelids, IPOCAMP.

INTRODUCTION

The deep-sea hydrothermal vent ecosystem is characterized by great spatial and temporal instability, especially in terms of temperature (Le Bris et al., 2005). As a consequence, the vent fauna have to deal with harsh and highly unstable thermal conditions. In the last few years, several studies have reported that some vent creatures, and in particular annelids belonging to the family Alvinellidae, feature exceptional thermal characteristics. This is the case for the emblematic polychaetous annelid *Alvinella pompejana*, believed to be one of Earth's most temperature-resistant metazoans, as suggested by the thermal stability of the molecules comprising its extracellular matrix (Gaill et al., 1993; Gaill et al., 1995). *In situ* recordings have suggested that *A. pompejana* could survive sustained temperatures of up to 60°C (Cary et al., 1998) and brief exposures beyond 100°C (Chevaldonné et al., 1992). Unfortunately, despite recent encouraging results (Shillito et al., 2004), the trauma associated with deep-sea collection has not yet allowed determination *in vivo* of the upper thermal limits of this species, an issue that remains controversial (Chevaldonné et al., 2000). Conversely, however, another member of the same family, *Paralvinella sulfincola*, which displays a similar association with hot fluid emissions (Juniper et al., 1992), survives throughout deep-sea collection, and was recently studied *in vivo* inside pressurized aquaria (Girguis and Lee, 2006). It was found to tolerate temperatures of 50–55°C for several hours, the highest temperature ever found for a marine metazoan. This worm species apparently preferred temperatures between 40 and 50°C. Beyond establishing the upper thermal limits of these peculiar congeners by using exposure at close to lethal temperatures, another way to explore the thermal biology of these animals is to seek the first signs of thermal stress, through 'mild' heat shocks, i.e. thermal exposures that are possibly stressful, but are not of long enough duration to be lethal. This approach may provide complementary data regarding the capacity of vent fauna to get close to vent emissions. This ability could provide a selective advantage in interspecies competition (Tunnicliffe, 1991), which is particularly relevant in this thermally oscillating habitat, where a temporary access to hotter areas may provide a refuge from predators and/or access to specific nutritional sources.

In this preliminary study, we firstly followed the behaviour and survival of the vent-endemic alvinellid worm *Paralvinella grasslei* during a 30 min heat shock at ~30°C in a video-equipped pressurized aquarium. Secondly, we investigated its stress response measured by heat shock protein accumulation after the heat shock. When organisms are exposed to a non-lethal thermal stress, the expression of a highly conserved set of polypeptides termed heat shock proteins (HSPs) is initiated (Feder and Hofmann, 1999). These proteins play an essential role in the repair or destruction of damaged proteins (Parsell and Lindquist, 1993). In most organisms studied, the most prominent proteins induced by heat stress are HSP70 proteins (so called because their molecular mass is approximately 70 kDa) (Feder and Hofmann, 1999).

P. grasslei is one of the most abundant polychaete species found at the East Pacific Rise (EPR) vents. It occurs both within *A. Pompejana* colonies, on the wall of active chimneys, and within *Riftia pachyptila* tube aggregations (Desbruyères et al., 2006). Therefore, this species may undergo temperature conditions as low as ~10°C [in *Riftia pachyptila* tubeworm clumps (Sarradin et al., 1998)], while it may also be exposed to temperatures well above 30°C [among *A. pompejana* colonies (Le Bris et al., 2005)]. Given the relative mobility of this species (Chevaldonné et al., 2000), could this rather wide thermal distribution reflect frequent displacements between different thermal environments, as has been suggested for the vent shrimp

Rimicaris exoculata (Ravaux et al., 2003), rather than a static distribution of fixed sub-groups, each adapted to different thermal regimes? By using video observations during *in vivo* heat exposure experiments at *in situ* pressure, and postshock detection of stress proteins, we aimed to demonstrate both the feasibility and scientific contribution of such shipboard physiological ecology studies. Finally, by presenting stress protein sequences of members of the Alvinellidae, this work provides valuable investigation tools for future studies on the thermal biology of this family of annelids.

MATERIALS AND METHODS

Specimen collection and experiments

Paralvinella grasslei (Desbruyères and Laubier 1982) specimens were collected during two cruises: 'HOPE' (R/V Atalante, ROV Victor6000, April 1999) and 'PHARE' (R/V Atalante, Nautile submersible, June 2002) along the EPR at the 13°N hydrothermal vent field at about 2600 m depth. Animals were sampled with a suction device operated by the submersible's hydraulic arm, and stored inside insulated Perspex cylinders until transferred to the ship. Most annelids survived the collection trauma, and only live adult specimens (4–7 cm in length) were placed in PVC cages inside the IPOCAMP pressurized incubator for *in vivo* experiments (Fig. 1) (for details, see Ravaux et al., 2003). In all experiments, less than 2 h passed between the time the samples began decompression (submersible ascent) and the moment they were re-pressurized. After experimentation, live worms for further analyses at the lab were immediately frozen in liquid nitrogen.

Nine experiments were performed at *in situ* pressure (26 MPa; 20 l h^{-1} flow rate) using a total of 111 *P. grasslei* specimens (Table 1). Five of these experiments (two reference and three heat shock experiments) were video monitored either at a constant 15°C temperature or throughout a heat shock.

Respirometry experiment (Expt 1)

Five worms were placed at 15°C for 6 h in order to estimate their oxygen consumption. This experiment aimed at evaluating the physiological state of the annelids after the collection process. These animals could not be observed, as this experiment was performed in individual closed containers.

Reference experiments (Expts 2 and 3)

Five and six *P. grasslei* were maintained at 15°C for 9 h (Expt 2) and 6 h (Expt 3), respectively. The aim of these experiments was to study behaviour and survival in order to determine whether the worms recovered from the collection trauma.

Heat shock experiments (Expts 4–6; see Table 1)

We performed three non-lethal heat shock experiments at 30°C with a similar temperature profile (Fig. 1B) but with different recovery periods. After being maintained at 15°C for 8 h, the worms were exposed to an abrupt heat shock. The temperature of the in-flowing seawater was increased in less than 5 min by immersing the inlet high-pressure tube in a 30°C regulated water bath while directing this flow into the experimental cages (see Fig. 1A). Animals were maintained for at least 30 min, followed by a rapid cooling to the original 15°C (within about 5 min). Animals were taken out of the chambers and frozen immediately (Expt 4a), or frozen 1.5 h (Expt 5a) or 3.5 h (Expt 6a) after the heat shock for further investigations on heat shock proteins. For each heating experiment, a reference experiment of the same total duration was conducted simultaneously,

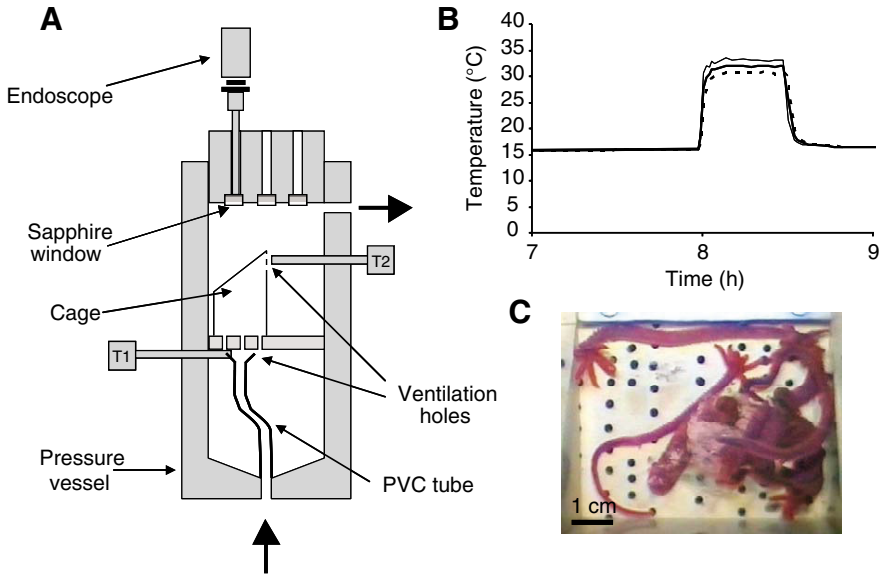

Fig. 1. Experimental set-up for *in vivo* experiments at *in situ* pressure (26 MPa) (modified from Ravaux et al., 2003). (A) The pressure vessel IPOCAMP (Incubateur Pressurisé pour l'Observation et la Culture d'Animaux Marins Profonds; internal diameter 20 cm, height 60 cm) contains two PVC cages (length 6 cm, width 5 cm, height 6 cm) closed at the top with a transparent polyethylene lid. Three sapphire windows in the pressure vessel lid allow the insertion of an endoscope and two optical-fibre light-guides, for the observation of the inside of the cages. Large arrows indicate the inlet and outlet of circulating seawater, which is forced through the cage ventilation holes *via* a PVC tube. Two Pt100 probes are positioned in the water flow indicating the upstream (T1) and the downstream (T2) temperature. (B) Temperature profiles for a typical heat shock (HS) experiment (solid line, T1 probe; broken line, T2 probe; bold line, mean temperature obtained from T1 and T2 probes). The animals were maintained at 15°C and subsequently exposed to a sharp heat shock at 30–33°C. After rapid cooling to 15°C, the animals were recovered at different times after the heat shock (see Materials and methods for more details). (C) Video view of a cage containing eight *P. grasslei* specimens, maintained at a temperature of *ca.* 30°C during a HS experiment. The approximate length of *P. grasslei* specimens was 7 cm.

Table 1. *In vivo* experiments performed at *in situ* pressure (26 MPa)

Experiment	Type of experiment	No. of individuals	Maximum temperature (°C)	Total duration of experiment (h)	Survival (%)
1	Reference respirometry	5	15	6	–
2	Reference survival and behaviour	5	15	9	100
3	Reference survival and behaviour	6	15	6	83
4a	HS response and behaviour with no recovery	20	31.7	8.5	70
4b	Reference associated with Expt 4a	30	15	8.5	–
5a	HS response and behaviour with 1.5 h of recovery	14	32.3	8	84
5b	Reference associated with Expt 5a	11	15	8	–
6a	HS response and behaviour with 3.5 h of recovery	8	31.4	12	100
6b	Reference associated with Expt 6a	12	15	12	–

For each heat shock (HS) experiment (a), a related reference experiment (b) was performed at 15°C. In all HS experiments, the animals were heat shocked at 30°C (see Fig. 1). However, the duration of the recovery period was different for each experiment (see Materials and methods). The maximum temperature reached for each experiment corresponds to the average obtained from the T1 and T2 probe values (see Fig. 1 for details of the experimental set-up). The survival rate was determined in the last 5 min of video-monitored experiments by identifying each individual and witnessing its branchial tentacle movements.

at a constant temperature of 15°C and at *in situ* pressure, using a second IPOCAMP pressure vessel (Expts 4b, 5b and 6b). All of the heating experiments were video monitored, in order to detect any type of behavioural response to thermal stress.

Oxygen level measurements (Expt 1)

Worms were individually sealed in soft polyethylene seawater containers (60, 125 or 150 ml) in order to evaluate their oxygen consumption. Another seawater container without any animals was pressurized as a control. After 6 h, all containers were recovered and oxygen levels determined by the Winkler method (s.d. of the method was 2%; 95% confidence interval for N=1 was ±4%) (Aminot and Chaussepied, 1983). The O_2 uptake rates were compared with the control to preclude possible uptake of oxygen by bacteria in the seawater. The worms were then dried at 80°C (48 h) and weighed (0.1 mg precision).

Determination of survival and video analysis of *in vivo* experiments

For all *in vivo* experiments with a video survey, survival of each individual re-pressurized worm was determined by witnessing its movements during the last 5 min of the experiments. Survival of each specimen was confirmed after the experiments at atmospheric pressure by identifying any kind of movement of the animal.

The pressure vessel IPOCAMP allows video observation of the animals by combining an endoscope (Fort, Dourdan, France) with a CCD camera (JVC, TK-C1380; Carrières sur Seine, France). For each video-monitored experiment we observed the behaviour of the worms during reference periods (15°C) and heating periods. These observations were aimed at characterizing specific behaviour during the heating periods. Compared with previous behavioural studies on other vent organisms (Shillito et al., 2001; Ravaux et al., 2003; Shillito et al., 2006), whole-body identification of each specimen was very difficult for *P. grasslei* because worms tended to aggregate and enlace themselves (Fig. 1C). For this reason, we focused on movements of branchial tentacles (beating, emergence–retraction movement, etc.) for determining survival.

Electrophoresis and immunodetection of HSP70

Samples of the posterior part of the worm body (heat shocked or not) were ground up in liquid nitrogen, and the powder was homogenized in 1 ml of extraction buffer [50 mmol l^{-1} Tris HCl, pH 7.4; protease inhibitor cocktail (Sigma, St Quentin Fallavier, France) 1:3 v/v]. The homogenates were centrifuged at 10 000 ***g*** for 10 min at 4°C, and the extracted proteins were quantified in the supernatant with a Bio-Rad protein assay (Bio-Rad, Marnes-la-Coquette, France) using bovine serum albumin (Sigma) as a standard.

For Western blotting, 20 µg of total protein was diluted in loading buffer [0.1% Tris HCL 0.5 mol l^{-1} pH 6.8, 0.1% glycerol, 0.2% SDS (10%), 0.05% β-mercaptoethanol, Bromophenol Blue ~0.001%] and separarated by minigel SDS-PAGE (10% acrylamide:0.3% bisacrylamide w/v). The proteins were transferred from the SDS-PAGE gel onto a nitrocellulose membrane using a Mini Trans-Blot Cell (Bio-Rad; 300 mA for 45 min) using transfer buffer (25 mmol l^{-1} Tris, 192 mmol l^{-1} glycine, 20% isopropanol, pH 8.3).

Dot blot assays were performed in a 48-well plate format using a Bio-Dot microfiltration apparatus (Bio-Rad). Typically, 30 µg of total protein from crude extract were suspended in 200 µl of Tris-buffered saline pH 7.4 (TBS; 50 mmol l^{-1} NaCl, 150 mmol l^{-1} Tris) and absorbed onto nitrocellulose membrane by gravity flow. The Bio-Dot was then washed with 200 µl of TBS per well, applying a constant vacuum flow.

Nitrocellulose membranes from the Dot blot and Western blot assays were then treated similarly for HSP70 detection, following a protocol described previously (Ravaux et al., 2003). Density profiles were obtained using a customized plug-in based on ImageJ software (Abramoff et al., 2004) that performed an automated background subtraction on the membrane's image before computing the integrated density of each band.

cDNA amplification and rapid amplification of the 3′ and 5′ cDNA ends (RACE), and cloning and sequencing of *hsp70*

Total RNA was extracted from the posterior part of the worm body, and reverse transcribed to cDNA as previously described (Ravaux et al., 2007).

The *hsp70* sequences were amplified by PCR amplification, using the primers HSP1, HSP2, HSP3 and HSP4 (Ravaux et al., 2007) or the primers 5′Primer and 3′Primer, designed from multiple alignments of homologous sequences (see Table 2 for primer sequences). The PCR amplifications were performed following a previously published protocol (Ravaux et al., 2007). The 5′ and 3′ ends of *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 Pag5, Pag6, Pag9 and Pag10 for *P. grasslei hsp70* form 1, and Heb1 and Heb3 for *Hesiolyra bergi hsp70* (see Table 2).

The PCR products were purified using a Geneclean kit (Q-Biogene, Illkirch-Grassenstaden, France), and subcloned into the pBluescript KS plasmid vector. The recombined vector was integrated into competent DH5α bacterial cells. Positive colonies

Table 2. Nucleotide sequences of primers used in PCR

Primer	Direction	Sequence (5′–3′)
HSP1	F	AAGGTGGARATCATCATCGCCAAYGAYCARGG
HSP2	F	TAYGTNGCVTTCACNGACAC
HSP3	R	AGGTTGTTGTCCTTGGTCATYGC
HSP4	R	TAGAARTCRATRCCYTCGAASAGACAGTC
5′Primer	F	ATGGCVAAGGCAMGYGCTGTSGGTAT
3′Primer	R	TTASTCRACTTCTCRATGGTGGG
Pag5	F	TCCGAGTGATTAACGAGCCAACAGC
Pag6	R	CCAGCCGTCTCCAGACCTAAACATA
Pag9	R	TCAATCGTCAATCACACCATCTAA
Pag10	F	ACACTCGGCCGTTAAAGATCTACT
Heb1	F	AACAGGAAACAAGCGAGCAGTCAGAC
Heb3	R	AGGTCTGGGTCTGCTTTGTGGGAAT
UnivP	F/R	CTAATACGACTCACTATAGGGCAAGCAGTGGT-ATCAACGCAGAGT
Nested	F/R	AAGCAGTGGTATCAACGCAGAGT

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

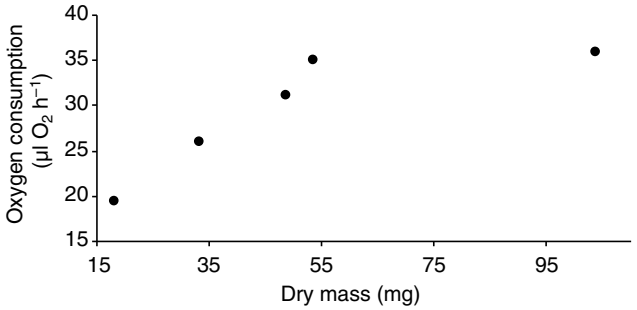

Fig. 2. Oxygen consumption (R; µl O_2 h^{-1}) as a function of dry mass (DM; mg) for five *Paralvinella grasslei* maintained at *in situ* pressure (26 MPa, 15°C, 6 h; Expt 1). R correlates with DM of individuals following the equation: $R=7.07DM^{0.37}$ (r=0.94, N=5, P<0.01).

were identified by white/blue selection, and the clones were further screened through *Pst*I/*Hin*dIII (Fermentas, Saint-Rémy-lès-Chevreuse, France) digestion of plasmid DNA. The sequencing was carried out by Genome Express (Meylan, France).

Sequence analyses

The sequences were analysed with the TGICL program (http://compbio.dfci.harvard.edu/tgi/software/) to find overlapping regions, and assembled into contigs. These sequences were further analysed using the Expasy Proteomics Server tools (http://www.expasy.org/).

The nucleotide sequences of the cDNA encoding *Paralvinella grasslei hsp70* form 1 and *hsp70* form 2 were deposited in GenBank under the accession numbers EF580992 and EF580993, respectively. We also deposited the sequence for *Hesiolyra bergi hsp70* under the accession number EF580994**.** The cDNA sequence obtained for *Alvinella pompejana* was partial and thus not deposited.

A molecular comparison was carried out between these sequences and those of other annelids: *Platynereis dumerii* (accession number ABB29585) and *Hirudo* sp. Since *Hirudo* sequences are not yet publicly available, we screened a molluscan HSP70 sequence (*Mytilus galloprovincialis*, AAW52766) against the *Hirudo* EST database (with the kind permission of Professor M. Salzet, ESA CNRS 8017, Université des Sciences et Technologies de Lille). The 10 best hits were then assembled using TGICL software, yielding one contig, which we used as the *Hirudo* HSP70 sequence in this paper.

This sequence dataset was automatically aligned by ClustalW and ambiguously aligned regions were removed, leading to 228 amino acid positions. Phylogenetic relationships were then reconstructed by maximum likelihood methods [PHYML (Guindon et al., 2003)] with the following model: JTT substitution matrix, estimated proportion of invariable sites and gamma-distributed substitution rates. The robustness of the topology was assessed by 100 bootstrap replicates in PHYML. Other reconstruction methods, such as parsimony or neighbour joining with the same evolutionary model, produced the same topology (data not shown).

RESULTS

Reference experiments

Survival and behaviour at 15°C

Most of the *Paralvinella grasslei* specimens appeared to withstand the decompression events, either during the collection process or after the *in vivo* experiments (except for some individuals, which may have been damaged by the suction sampler). At atmospheric pressure, just after the submersible recovery, the worms were alive but appeared motionless. However, following re-pressurization, it only took a few minutes before movements were observed.

Table 1 summarizes the survival determined at the end of each video-monitored experiment. At 26 MPa and 15°C, almost all animals were alive after 6 h or 9 h (Expts 2 and 3, respectively; mean survival ± s.d., 91.5±12%). Visual observations throughout these experiments showed a relatively low activity of the worms at 15°C. Most of the time, they were enlaced at the bottom of the cage, agitating their branchial tentacles slowly. Occasionally, they would crawl at the bottom of the cage.

Respirometry

In the respirometry experiment (Expt 1, Fig. 2), the oxygen consumption rates (R; expressed in µl O_2 h^{-1}) correlated significantly with the worms' dry mass (DM; expressed in mg) following the equation: $R=7.07DM^{0.37}$ (r=0.94, N=5, P<0.01), even though one specimen appeared to have a low oxygen consumption rate compared with its mass (DM=104 mg; R=35.9 µl O_2 h^{-1}). The mass-specific oxygen consumption rate for *Paralvinella grasslei* ranged from 347 to 1076 µl O_2 g^{-1} DM h^{-1} (mean ± s.d., 699±264 µl O_2 g^{-1} DM h^{-1}). At the end of the experiment, the oxygen concentration in the control was 246 µmol O_2, which is very similar to the initial oxygen concentration of about 250 µmol O_2.

Heat shock experiments

Survival and behavioural response

Most of the *Paralvinella grasslei* survived a 30 min heat shock at 30°C (Expts 4a, 5a and 6a; mean survival ± s.d., 85±15%; Table 1). Whilst the worms' activity was low at 15°C, a significant increase in activity was observed with increasing temperature, and throughout the heat shock. Moreover, some particular behaviours were detected at 30°C, e.g. worms were seen crawling actively around the cage (Fig. 1C), and in some cases the worms lifted the anterior part of their bodies above the substratum. In addition, we observed some *P. grasslei* quickly retracting their tentacles and then unfolding them progressively. This emergence–retraction movement of the worms' tentacles was very rarely observed at 15°C. More generally, with increasing temperature, animals tended to move towards the cage sidewalls. This crawling response was followed by a decrease in activity during the cooling process, with a progressive return to the reference behaviour.

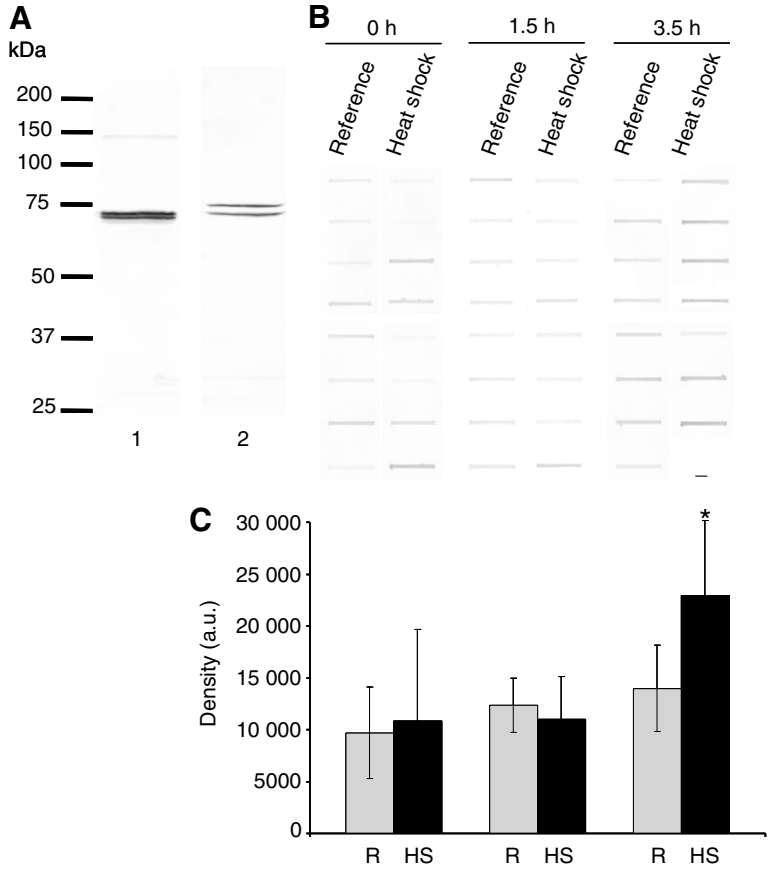

Fig. 3. (A) Western blot profiles obtained for the detection of HSP70 proteins in *P. grasslei* using the anti-rat polyclonal antibody (lane 1) or the anti-chicken monoclonal antibody (lane 2). Several bands with a molecular mass between 70 and 75 kDa were detected for both antibodies. A weak band around 150 kDa was also observed with the polyclonal antibody. (B) Dot blot detection of HSP70 proteins in *P. grasslei* using the anti-rat polyclonal HSP70 antibody. Each band corresponds to a different individual. The first two columns represent reference (R) and heat-shocked (HS) individuals frozen immediately after the shock (Expts 4a and 4b; 0 h). The two central columns show R and HS specimens that were maintained for 1.5 h at 15°C after the shock (Expts 5a and 5b, 1.5 h). Finally, the two last columns show R and HS animals maintained for 3.5 h at 15°C after the heat shock (Expts 6a and 6b, 3.5 h). (C) Dot blot signal intensity comparison of HSP70 levels for R (grey columns) and HS (black columns) *P. grasslei*. The density of each band (expressed in arbitrary units, a.u.) was calculated using a plug-in based on ImageJ software. Each column represents the mean of the band density (±s.d.) for the eight R or HS individuals from the corresponding column above (except for Expt 6a where only seven *P. grasslei* were used; the dash shows the empty well). The asterisk indicates a significant difference between treatments (Mann–Whitney test; U=7, P=0.007).

Heat shock response

Heat-shocked (HS; Expts 4a, 5a and 6a) and reference *P. grasslei* (R; Expts 4b, 5b and 6b) were further analysed for the detection of HSP70 stress proteins (Fig. 3). Two primary HSP70 antibodies were selected (anti-rat polyclonal and anti-chicken monoclonal). Their specificity was preliminarily checked by Western blot of *P. grasslei* body samples (Fig. 3A; only one profile is presented for each antibody) before considering the Dot blot approach. Several immunoreactive bands corresponding to the molecular mass of HSP70 proteins (~70–75 kDa) were detected with both antibodies. In addition, one high molecular mass band (~150 kDa), with a constant low density, was also observed with the polyclonal antibody. These results confirmed that the chosen antibodies detect HSP70 proteins in *P. grasslei*.

Dot blot detection and comparison of HSP70 signal intensity, using the polyclonal (Fig. 3B,C) and the monoclonal (data not shown) antibodies, were carried out on a total of 47 tested specimens. The relative difference in the level of HSP70 proteins between groups of R and HS specimens of *P. grasslei* in response to a 30°C heat shock followed by 0 h (Expts 4a and 4b), 1.5 h (Expts 5a and 5b) and 3.5 h (Expts 6a and 6b) of recovery at 15°C was quantified by densitometry analysis using a plug-in based on ImageJ software (Fig. 3C). HSP70 proteins were detected in all samples and, in spite of important variability between specimens, a significant increase in HSP70 expression following the heat shock was observed. This increase was detected, only when using the polyclonal antibody, in HS individuals that were maintained for 3.5 h at 15°C after the shock (Fig. 3B,C; Mann–Whitney test, U=7, P=0.007). The specimens recovered immediately or 1.5 h after the shock showed similar HSP70 levels in R and HS treatments either with the polyclonal (Fig. 3B,C) or with the monoclonal antibody (data not shown).

Annelid *hsp70* sequence analyses

To continue our investigations of the response to heat stress, we identified cDNAs for *hsp70* in *P. grasslei*. We also isolated and sequenced the cDNA for *hsp70* in the closely related vent annelids *Hesiolyra bergi* (full-length cDNA) and *Alvinella pompejana* (partial cDNA sequence). A comparison of vent annelid sequences was carried out with other annelid *hsp70* sequences (*Platynereis dumerii* accession number ABB29585 and *Hirudo* sp. assembled from *Hirudo* EST database, provided by Professor M. Salzet).

cDNA sequences for vent annelid *hsp70*

Two full-length cDNAs were obtained from *Paralvinella grasslei*, which were arbitrarily named form 1 and form 2.

The cDNA of *P. grasslei hsp70* form 1 (GenBank accession number EF580992) is 2207 bp in length, including a 1947 bp coding region and a 3′ UTR of 260 bp with a polyadenylation signal sequence (AATAAA at position 2162). The G–C content of the 1947 bp ORF is 47.8%. This ORF encodes a 648 amino acid protein with a predicted molecular mass of 71.65 kDa and a theoretical isoelectric point of 5.74.

The cDNA sequence of *P. grasslei hsp70* form 2 (GenBank accession number EF580993) is 2234 bp in length. A single reading frame of 1961 bp is followed by a 273 bp-long 3′ UTR, which contains the consensus polyadenylation signal AATAAA located at position 2192. The G–C content of the 1961 bp ORF is 49.4%. This ORF encodes a 653 amino acid protein (Fig. 4) with a calculated molecular mass of 71.38 kDa and a theoretical isoelectric point of 5.23. The cDNA we sequenced from *H. bergi* belongs to the HSP70 family. This cDNA is 2330 bp in length, including a 3′ UTR of 359 bp with the polyadenylation signal sequence at position 2286.

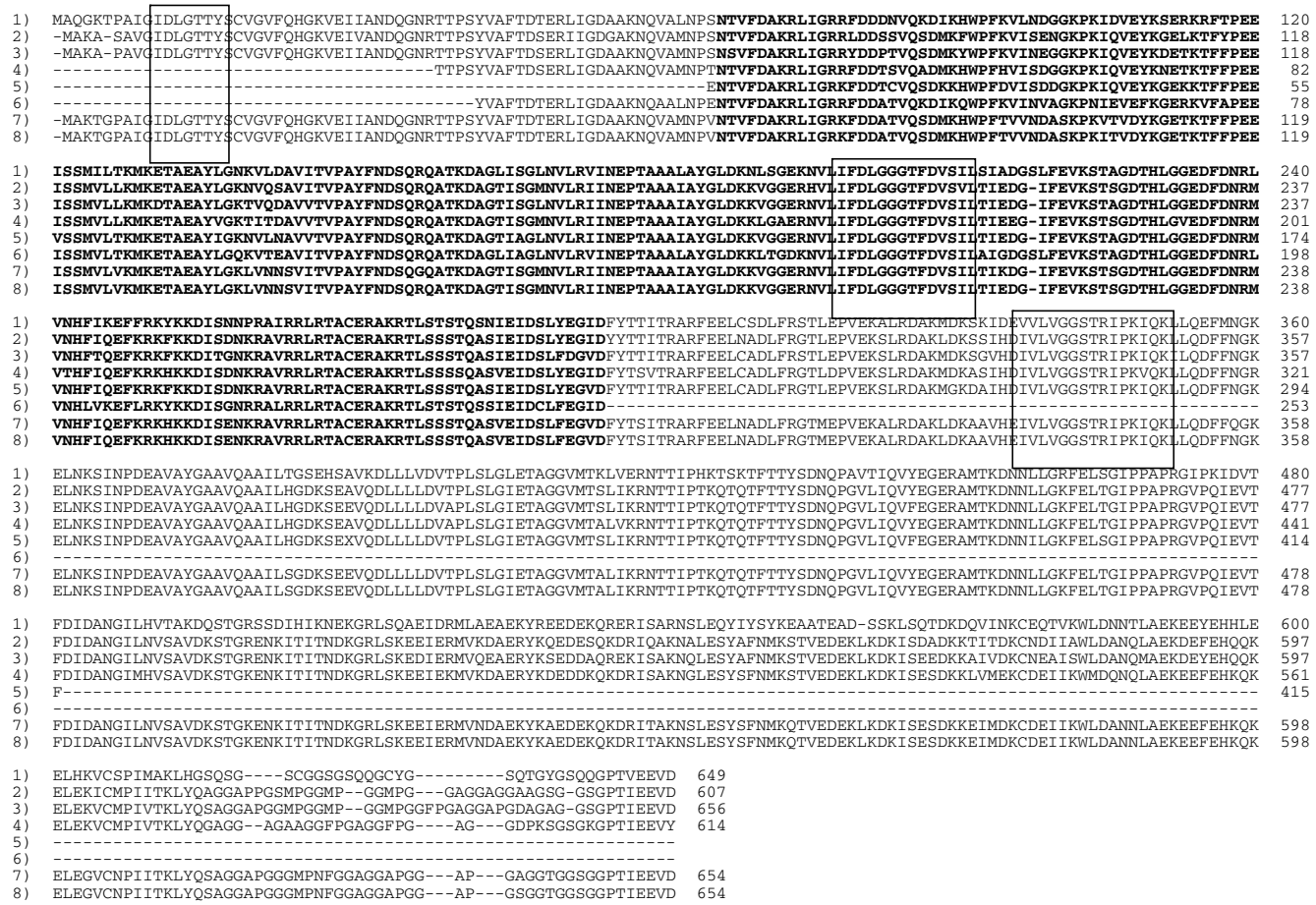

Fig. 4. Comparison of the HSP70 sequences used for phylogenetic analyses. 1, *Paralvinella grasslei* form 1; 2, *Paralvinella grasslei* form 2; 3, *Hesiolyra bergi*; 4, *Hirudo* sp.; 5, *Platynereis dumerii*; 6, *Alvinella pompejana*; 7, *Mytilus galloprovincialis*; 8, *Mytilus galloprovincialis*. The residues corresponding to the three HSP70 family signatures are boxed. The residues used for building the tree are indicated in bold (228 amino acid positions). The sequences used for rooting the tree are *Mytilus galloprovincialis* HSP70 (accession no. AAW52766) and HSC70 (accession no. CAH04109).

The 1971 bp ORF encodes a polypeptide of 656 amino acids with a predicted molecular mass of 71.64 kDa and a theoretical isoelectric point of 5.16. This sequence was deposited in GenBank under the accession number EF580994.

A partial cDNA sequence for *hsp70* was obtained from *Alvinella pompejana* using the degenerate primers HSP1, HSP2, HSP3 and HSP4. It is 762 nucleotides in length, which corresponds to a 253 amino acid peptide.

Comparison with other annelid HSP70 proteins

Several general eukaryotic HSP70 family motifs were identified in the amino acid sequences (see alignment in Fig. 4): three signatures [IDLGTTYS, amino acids 11–18; IFDLGGGTFDVS(I/V)L, amino acids 199–212; (V/I)VLVGGSTRIPK(I/V)Q, amino acids 337–350], and a putative ATP–GTP binding site (AEAYLG; amino acids 133–140). Two additional specific motifs were found: the non-organellar consensus motif (RARFEEL; amino acids 302–308) and the cytoplasmic HSP70 carboxyterminal region (GPT(I/V)EEVD).

The two sequences from *P. grasslei* were clearly different, as confirmed by their position in the tree presented in Fig. 5. This tree was built by a comparison of *P. grasslei hsp70* sequences with homologous annelid sequences, and did not aim to reconstruct the *hsp70* phylogeny amongst annelids. Indeed, the dataset available for annelid *hsp70* is weak, leading to low bootstrap values, except for one node that is highly supported (bootstrap percentage, 100) which corresponds to the group including *P. grasslei* form 1 and *A. pompejana* sequences. These two sequences are thus closely related whereas *P. grasslei* form 2 sequence unambiguously corresponds to a different form of HSP70 protein.

DISCUSSION

Reference experiments

Given the motionless appearance of freshly collected specimens, it is obvious that the collection process has deleterious consequences. Therefore, a pre-requisite to on-board *in vivo* experimentation is to ensure that re-pressurized control animals are healthy. The survival, oxygen consumption and behaviour at 15°C and 26 MPa were used as indicators of the physiological state of the *P. grasslei* specimens. The reference pressure corresponds to the *in situ* pressure encountered by the animals at 2600 m depth, and the reference temperature was chosen according to the mean temperature values recorded in the microenvironment of the worms, i.e. 12–20°C (Desbruyères and Laubier, 1982; Chevaldonné and Jollivet, 1993; Gaill and Hunt, 1991) (reviewed in Lutz and Kennish, 1993). This temperature is assumed to be tolerated by the animals since it is significantly lower than the maxima suggested to be encountered in the *P. grasslei* habitat, i.e. up to 40°C in *Alvinella pompejana* colonies (Le Bris et al., 2005).

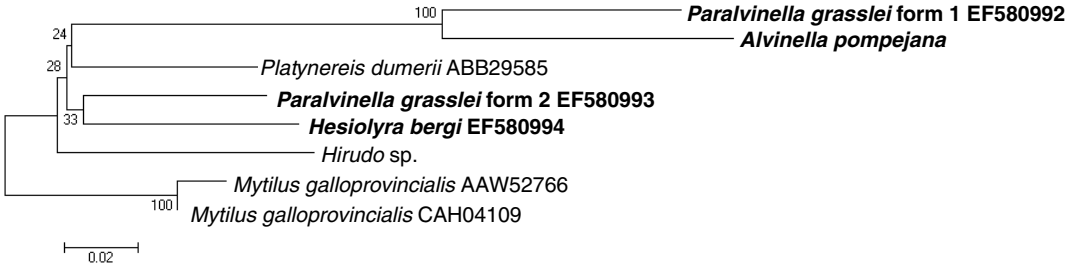

Fig. 5. Relationships amongst annelid HSP70 proteins. The tree was reconstructed by maximum likelihood methods [PHYML (Guindon et al., 2003)] under a JTT+invariant+gamma model (see Materials and methods). The accession number of the amino acid sequences is provided after each species name. The values indicated on the branches correspond to bootstrap percentages. According to the tree, the sequence *Paralvinella grasslei* form 1 is clearly related to the *Alvinella pompejana* sequence, while the *Paralvinella grasslei* form 2 sequence is unambiguously distinct from this group.

The almost immediate activity of the worms after re-pressurization and the high survival rate (91.5±12%) observed for individuals maintained at 15°C at *in situ* pressure for up to 9 h indicate a relatively good physiological state of the animals.

The oxygen consumption results reflect a metabolic rate of rather active animals for four *P. grasslei* out of the five specimens tested (Fig. 2). Only one worm showed a size-specific low oxygen uptake level, probably reflecting a trauma induced by the collection process. The oxygen uptake rates of the four *P. grasslei* specimens (654–1076 $\mu l\,O_2\,g^{-1}\,DM\,h^{-1}$) are very similar to those obtained for another deep-sea vent polychaete, *Hesiolyra bergi* [629–1133 $\mu l\,O_2\,g^{-1}\,DM\,h^{-1}$ (Shillito et al., 2001)] under the same experimental conditions. When compared with coastal annelids, the mean oxygen consumption rate for *P. grasslei* (787 $\mu l\,O_2\,g^{-1}\,DM\,h^{-1}$) is higher than the mean rate obtained at the same temperature for *Arenicola marina* [394 $\mu l\,O_2\,g^{-1}\,DM\,h^{-1}$; recalculated from the data of Toulmond (Toulmond, 1975)] or *Nereis diversicolor* [~380 $\mu l\,O_2\,g^{-1}\,DM\,h^{-1}$ (Ivlena and Popenkina, 1968)], and similar to that of *Nephtys hombergi* during its maximal swimming activity [~1000 $\mu l\,O_2\,g^{-1}\,DM\,h^{-1}$ (Newell and Norcroft, 1967)]. Since *P. grasslei* did not move actively at 15°C, our results would be rather high, possibly reflecting stress-induced hyperventilation, or caused by high oxygen levels in our experiments (surface seawater with an O_2 concentration of *ca.* 250 $\mu mol\,l^{-1}$ compared with the usual O_2 concentration in Pacific deep-sea water of *ca.* 130 $\mu mol\,l^{-1}$ (Johnson et al., 1986; Johnson et al., 1988; Millero, 1996) or even lower near deep-sea vents [*ca.* 0–100 $\mu mol\,l^{-1}$ (Desbruyères et al., 1998; McCollom, 2000)].

Comparisons of the experimental behaviour of the worms with their natural behaviour should be made cautiously, since both environment and observation conditions differ radically. For example, the space available in the cage is limited and devoid of refuges, in comparison with their natural environment where a lot of anfractuosities are accessible on the chimney surface. Moreover, the density of *Paralvinella grasslei* in the cages (equivalent to 1300–3300 individuals m^{-1}) is higher than the density described *in situ* on the chimney wall (200–800 individuals m^{-1}) (Chevaldonné and Jollivet, 1993). Another example is the temperature, which is constant in our experiments whereas it sharply fluctuates *in situ* (Le Bris et al., 2005). Nevertheless, the various types of behaviour observed experimentally resemble those occurring in their natural habitat. Most of the time, the worms are not very active, staying inside anfractuosities of the chimney surface and moving their branchial tentacles slowly. *P. grasslei* specimens can also move on the surface of the chimney and are often seen around *Alvinella pompejana* tubes (Chevaldonné et al., 2000).

Considered together, our data suggest that the metabolic rate of *P. grasslei* is, if not normal, at least far from reflecting that of moribund animals under the conditions of our reference experiments.

Temperature resistance and behavioural response to heat

Paralvinella grasslei can survive up to a 30 min heat shock at 30°C, as demonstrated by the high survival rate determined at the end of the three heating experiments (85±15%, Table 1). The survival rate of heat-shocked animals is comparable to that of reference animals. This observation shows that the critical thermal maximum (CT_{max}) of *P. grasslei*, defined as the temperature at which the worm is no longer capable of proper locomotion (Wehner et al., 1992; Ghering and Wehner, 1995; Cuculescu et al., 1998), is above this temperature.

Even if the 30°C shocks were not lethal for *P. grasslei*, signs of heat stress were clearly identified. The rise of temperature was accompanied by an increase in activity of the worms, with frequent displacements and a greater amplitude of beating of the branchial tentacles. The emergence–retraction movement of the branchial tentacles, which was very rare at 15°C, was frequently observed during periods of increasing temperature. This behaviour has also been described *in situ* (Chevaldonné et al., 1993) and Chevaldonné and colleagues proposed that alvinellids use it for thermoregulation (Chevaldonné et al., 1991). At 30°C, a peak of activity was observed and specific behaviours clearly appeared. The worms were seen actively crawling around the cage and sometimes lifting their bodies above the substratum. A similar behaviour was previously described for *Hesiolyra bergi*, which actively crawled and swam when the temperature reach 33.5°C, i.e. only a few degrees before reaching its CT_{max} at ~38°C (Shillito et al., 2001). In view of the low activity at 15°C, such behaviours can be inferred as an escape response to avoid heat zones, and therefore suggest thermal discomfort of the worms at around 30°C. When compared with other *Paralvinella* species living in a similar habitat, this is quite consistent with the observations on *Paralvinella palmiformis*, which avoids temperatures above 35°C, but rather lower than for *Paralvinella sulfincola*, which seems to be unaffected by temperatures in the 40–50°C range (Girguis and Lee, 2006). *P. grasslei* would thus avoid areas at temperatures above 30°C, where it might be exposed to excessive thermal stress.

Stress protein response

We detected HSP70 proteins in both reference and heat-shocked animals in all of our experiments (Fig. 3). The signal detected in reference animals was not significantly different between experiments and may correspond to the presence of the constitutive form since our antibodies are able to detect both forms. Alternatively,

it may also reflect a 'background' response to experimental stress, like pressure variation upon recovery and conditioning in IPOCAMP, since HSP70 expression can be triggered by many non-thermal stresses (Feder and Hofmann, 1999), including pressure variations (Welch et al., 1993; Kaarniranta et al., 2000; Elo et al., 2005). A great interindividual variability was observed for the HSP70 signal in each experiment, and especially for heat-shocked animals, which may reveal a difference in sensitivity to heat between specimens of *P. grasslei* (Fig. 3C). In spite of this variability, a significant increase of HSP70 level was detected in the heat-shocked specimens maintained for 3.5 h at 15°C after the shock, when compared with reference animals (see Fig. 3B). This increase was detected only when using the polyclonal antibody, which may be explained by the fact that the polyclonal antibody seems to detect more HSP70 isoforms than the monoclonal antibody (see preliminary Western blot, Fig. 3A).

A 30 min heat exposure at 30°C may thus be sufficient to trigger a heat shock response in *P. grasslei*. This suggests that the HSP70 enhanced synthesis threshold in *P. grasslei* may be lower than 30°C. This threshold would be in the same range as for the hydrothermal shrimp *Rimicaris exoculata* [25°C (Ravaux et al., 2003)] or the 13°C-acclimated marine snail *Tegula funebralis* [27°C (Tomanek and Somero, 1999)]. This temperature would appear to be quite low in view of the temperature that *P. grasslei* is supposed to experience in its habitat [up to 60°C among *Alvinella pompejana* tubes (Desbruyères et al., 1985; Chevaldonné et al., 1992; Le Bris et al., 2003; Le Bris et al., 2005)]. However, in such a highly fluctuating environment, the relevance of a maximum temperature obtained from a discrete measurement should still be considered cautiously.

This study is the first attempt to characterize the kinetics of the stress response in a vent animal. This is nevertheless not a classical kinetic study because, in order to avoid decompression events when opening the aquarium for the withdrawal of individuals, several independent experiments were performed to follow the heat shock response for various times of recovery. Our results showed a significant increase in the expression of HSP70 occurring 3.5 h after the heat shock. For comparison, the synthesis of HSP70 in the marine intertidal snail *Tegula brunnea* is induced from 2 to 14 h after a 30°C shock, and from 1 to 3 h in its congener *Tegula funebralis*, whose body temperature frequently exceeds 30°C during emersion (Tomanek and Somero, 2000). Although the heat exposure was shorter for *P. grasslei* (30 min *versus* 2.5 h for the marine snails), the kinetics of the response may be comparable to that observed for the intertidal species. In view of the frequent sharp spikes that the worms can encounter in their natural environment [up to 40°C within a few minutes (Cary et al., 1998; Di Meo-Savoie et al., 2004)], it is surprising to detect so late a heat shock response. However, further studies are required to determine the time at which the level of HSP70 returns to a reference level.

Relationship amongst annelid HSP70 proteins

Two *hsp70* sequences were identified from *P. grasslei*, which clearly correspond to two distinct forms of HSP70. When compared with homologous vent annelid sequences, the *P. grasslei hsp70* form 1 is closely related to the *Alvinella pompejana* sequence, whereas the *P. grasslei hsp70* form 2 is rather closer to the *Hesiolyra bergi* sequence. Several structural characteristics were proposed to differentiate *hsc70* and *hsp70,* like the presence/absence of introns, respectively (Gunther and Walter, 1994; Boutet et al., 2003; Liu et al., 2004), or the occurrence of the motif GGMP in the C-terminal region for HSC70 (Prapapanich et al., 1996; Liu et al., 2004). However, according to Leignel and colleagues, since these criteria are specific for each organism, gene expression studies still seem to be the only way to distinguish *hsp70* and *hsc70* (Leignel et al., 2007). Futures studies on *P. grasslei* thermal biology should aim to determine the regulation of expression of these two HSP70 proteins (constitutive *versus* inducible) in response to heat shock.

Conclusion

The heat shock experiments performed on *P. grasslei* suggest that the CT_{max} of this species is above 30°C. However, the significant increase in both the worms' activity and the HSP70 level upon a 30°C exposure gives evidence of thermal stress at this temperature. *P. grasslei* specimens may not encounter such severe heat shock in their natural habitat, but more probably brief spikes at temperatures above 30°C. Since *P. grasslei* would not exhibit a high tolerance to elevated temperature, the behavioural response, like escape or thermoregulatory movements (emergence–retraction of the branchial tentacles), may be sufficient to prevent exposure to deleterious temperatures.

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